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Abstracts

Oral Presentations

The Aquaporin Family of Membrane Water Channels

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Water is the major component of all life forms, so the entry and exit of water from cells may be a fundamental process of life. Physiologists have long recognized that plasma membranes of certain tissues must have water-selective pores to explain their high degree of water permeability. Attempts to identify molecular water channels by logical approaches proved unsuccessful, and discovery of AQP1 water channel protein was due to serendipity. Studies of AQP1 expressed in *X. laevis* oocytes demonstrated that this protein is a water-selective pore. Analysis of pure AQP1 reconstituted into membranes demonstrated lack of permeation by other solutes including protons. Sequence analysis predicted a protein structure which resembles an hourglass, and membrane crystallographic studies are now revealing the 3D structure at near-atomic levels of resolution. The presence of AQP1 has been documented in many tissues by immunohistochemistry and immunoelectron microscopy, including proximal tubules and descending thin limbs of kidney, aqueous humor secreting epithelia of eye, and capillary endothelia. Humans with mutations in *AQP1* were identified by the absence of the Co blood group antigen, and these individuals have a subclinical defect in renal concentration. Failure to identify AQP1 in renal collecting duct and other known water-permeable tissues predicted the existence of other aquaporins. Humans with defects in these other water channel proteins are being identified. AQP2 is expressed in principal cells of renal collecting duct, where vasopressin leads to redistribution of intracellular vesicles to the cell surface. Humans with mutations in *AQP2* suffer from nephrogenic diabetes insipidus. Rodent models of several clinical states such as lithium-induced polyuria, congestive heart failure, and pregnancy were found to produce have altered AQP2 expression. AQP0 is expressed in lens fiber cells, and humans with mutations in *AQP0* suffer from congenital cataracts. Other water-selective homologs have been identified in astroglia, AQP4, and secretory glands, AQP5. Aquaglyceroporins are subset of homologs which are permeated by water and small solutes, AQP3, AQP7, and AQP9. Recently, AQP6 was found in intracellular vesicles of acid secreting intercalated cells in renal collecting duct. Interestingly, AQP6 is permeated by anions, and the protein is believed to participate in acid-base homeostasis. Aquaporins have been identified in diverse species including insects and microbes. Plants express numerous aquaporins, and fascinating phenotypes are being uncovered. Thus, the workers in many labs worldwide are producing truly molecular understandings of how water crosses cell membranes.

Membrane channels for water and small anionic solutes

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Aquaporins are membrane channels involved in osmoregulation in bacteria, plants and animals. This large channel protein family consists of two clusters, the AQPs and the GLPs which allow passage of water and small hydrophilic solutes like glycerol, respectively [1]. Electron crystallography has shown monomers of AQP1 of human red cells to consist of six membrane spanning alpha-helices confirming sequence based predictions [2]. Two conserved loops fold back into the membrane to form the highly specific channel. AQP1 has a diameter of about 4 Å which suffices to allow passage of water molecules [3]. The bacterial glycerol facilitator GlpF reveals a more pronounced density minimum within the monomer, indicating a larger channel [4]. Surface exposed loops are the least conserved regions, allowing differentiation of aquaporins. Atomic force microscopy was used to image the surface of AQP1, aquaporin Z, the water channel of Escherichia coli [5], AQP0, the major intrinsic protein of lens fibre cells [6], and AQP2, the vasopressin regulated water channel of the collecting ducts. Significant changes of the cytoplasmic surfaces produced by proteolytic cleavage allowed the sidedness of AQP0 and AqpZ to be determined. Projection maps suggest the membrane resident core of aquaporins to be structurally conserved, whereas variations in the surface topography visualized by AFM are compatible with significant sequence variation of the loop regions.

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The projection structure of the glycerol channel GlpF at 3.5 Å

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The first structural analysis of the glycerol diffusion facilitator protein (GlpF)[1] from *E. coli* is presented. GlpF belongs to the GLP sub-cluster of the aquaporin-family. It is one of the few known diffusion facilitators in the inner membrane of *E. coli.* Its biological role is to mediate the diffusion of glycerol into the cell where it is phosphorylated by the glycerol kinase (GlpK) to prevent back-diffusion out of the cell. Apart from transport of glycerol, diffusion of polyols and urea derivatives were reported[2], but none of these substrates are transported in a phosphorylated state. In contrast the transport property for water remains unclear. The recombinant His-tagged protein was overexpressed and purified[3]. Concentrations up to 10 mg/ml were obtained. The purified GlpF was stable in 3% OG for weeks at 4EC. Transmission electron microscopy of the purified single particles showed a tetrameric structure of the solubilized complex. Two-dimensional crystallization produced highly ordered crystals with up to 40 Fm diameter, diffracting electrons to 3.3 Å resolution. Projections from vitrified 2D-crystals at 4 Å resolution confirmed the tetrameric structure, revealing a unit cell comprising two tetramers in p4-symmetry. In projection, GlpF was overall similar to AQP1[4], but clearly showed differences in the arrangements of density maxima. The central density minimum within the GlpF monomer, presumably the location of the diffusion pore, appeared to be larger in GlpF than in AQP1.

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Novel fold of AQP1

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In our previous electron crystallographic studies on AQP1, we have determined the 3D density map of AQP1 at 6 Å[1] and also 4.5 Å[2] resolution. As shown by 4.5 Å analysis, our new analysis clearly showed a novel fold of the AQP1 monomer to consist of six highly tilted, membrane-spanning á-helices that form a right-handed bundle. The scaffold of the AQP1 molecule was formed by two helical bundle of 1-3 and 4-6. These two helical bundles are unusual in that the three helices form a roughly linear arrangement but not according to their position in the primary structure, i.e 1-2-3 and 4-5-6, but the first helix of each bundle is sandwiched between the other two helices of the bundle, i.e. 2-1-3 and 5-4-6. Two helices of each bundle, helices 1 and 2 of the first bundle and helices 4 and 5 of the second bundle, run almost parallel to each other, tilting roughly to the same direction in the membrane. However, the third helix of the first bundle, helix 3 is oriented perpendicular to the axis defined by the first two helices of the bundle, and the same is found for helix 6 in the second helical bundle. The short helix HB from the first AQP1 repeat is strongly interacting with transmembrane helix 6 from the second AQP1 repeat, while short helix HE from the second AQP1 repeat interacts with transmembrane helix 3 from the first AQP1 repeat. This cross-interaction leads to an intimate link of the two protein halves.

After helix 2 which crosses the membrane adjacent to helix 1, near the four-fold axis of the AQP1 tetramer, loop B immediately folds back into the membrane positioning its NPA motif in the middle between the two helical bundles 1-3 and 4-6, close to the center of the lipid bilayer. After the NPA motif, loop B forms the short á-helix HB, which runs close to helix 6 of the second AQP1 repeat and brings the polypeptide chain back to the cytoplasmic surface. The remainder of loop B forms the cytoplasmic connection to helix 3 which crosses the membrane adjacent to helix 1, on the opposite side from helix 2. Helix 3 is the last structural element of the first AQP1 repeat that terminates on the extracellular surface. The second AQP1 repeat adopts essentially exactly the same fold as the first and the two repeats are connected by loop C.

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Three-dimensional fold of the human AQP1 water channel determined at 4Å resolution by electron crystallography of 2-dimensional crystals embedded in ice

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Three-dimensional (3-D) density map of deglycosylated, human erythrocyte AQP1 determined at 4Å resolution in plane and ~7Å resolution perpendicular to the bilayer is presented. The map was calculated by analyzing images and electron diffraction patterns recorded from tilted (up to 60E), ice-embedded 2-D crystals of AQP1 in lipid bilayer membranes. This map significantly extends the findings related to the folding of the AQP1 polypeptide chain determined earlier by us at a lower 7Å by ~20Å resolution. The solvent-accessible volume within a monomer has a vestibular architecture with a narrow, ~6Å diameter constriction near the center of the bilayer where the location of the water-selective channel is postulated. The clearly resolved densities for the transmembrane helices display protrusions expected for bulky side chains. The density in the interior of the helix barrel (putative NPA box region) suggests clearer linkage to some of the helices and may harbor short stretches of á-helix. At the bilayer extremities, densities for some of the inter-helix loops are visible. Consistent with these observed inter-helix connections, possible models for the threading of the AQP1 polypeptide chain are presented. A preferred model is deduced that agrees with the putative locations of a group of aromatic residues in the amino-acid sequence and in the 3-D density map.

Topological reorientation of aquaporin-1 topology during maturation in the endoplasmic reticulum

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The topology of most eukaryotic polytopic membrane proteins is established cotranslationally in the endoplasmic reticulum (ER) through a series of coordinated translocation and membrane integration events. For the human aquaporin water channel AQP1, however, the initial four-spanning topology synthesized in the ER membrane differs from the mature six-spanning topology observed at the plasma membrane. To understand the relationship between these two topological isoforms we have examined the transmembrane (TM) topology of key internal peptide loops during and following AQP1 synthesis in Xenopus oocyte and reconstituted cell-free expression systems. AQP1 constructs were generated by engineering a c-myc epitope tag within peptide loops flanking the N- or C-terminus of TM3 at residues Pro77 and Thr120, respectively. Constructs were then were sequentially truncated after TM segments 3, 4, 5 and 6 and fused to a C-terminus translocation reporter to mimic sequential stages of AQP1 synthesis. Topology of reporters was determined in full length and truncated constructs by protease protection and antibody accessibility in right-side-out ER membranes. This analysis revealed that AQP1 maturation in the ER membrane involves a novel topological reorientation of three internal TM segments and two peptide loops. During synthesis of TM's 4-6, TM3 underwent a progressive 180E rotation in which its C-terminus flanking residues were translocated from the cytosol to the ER lumen, and N-terminus flanking residues underwent retrograde translocation from the ER lumen to the cytosol. These events were sufficient to convert TM3 from a type I to a type II topology and reposition TM2 and TM4 into transmembrane conformations consistent with the predicted six-spanning AQP1 topology. Reorientation of TM3 was first initiated following synthesis of TM4 (27% of nascent chains), and was completed only after all six TM segments were synthesized, suggesting a cooperative effect between TM helices in facilitating AQP1 folding. Interestingly, topological maturation of full length AQP1 was ~80% efficient in intact Xenopus oocytes and in rabbit reticulocyte lysate (RRL) supplemented with oocyte-derived ER membranes. However, AQP1 remained predominantly in its immature four-spanning orientation when expressed in RRL supplemented with canine pancreatic rough microsomes (28% maturation). These results demonstrate that AQP1 utilizes a multi-step folding pathway for assembly into the ER membrane and that initial protein topology established via cotranslational translocation events is dynamic and may be modified by subsequent steps of folding and/or maturation.

P 1

Volume flux across red cell Aqp1 and *E. coli* AqpZ water channel proteins reconstituted into planar lipid bilayers

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The high water selectivity of aquaporin channel proteins was questioned by reports describing ion conductance. Forskolin, for example was claimed to stimulate cation permeability in aquaporin1 water channels when expressed in oocytes. Other examples are provided by Aqp0 (major intrinsic protein of Lens) and nodulin 26 (symbiosome membrane protein of soybean nodules) which exhibited channel activity after partial purification and reconstitution into planar lipid bilayers, but not when expressed in *X. laevis* oocytes. To clarify these discrepancies, a defined system was developed to simultaneously measure water permeability and possible ion conductance. Solute concentration changes within the unstirred layer adjacent to planar membranes were exploited to assess the osmotically induced transmembrane water flow. From uniexponential concentration profiles measured with microelectrodes, the hydraulic conductivity of the bilayer was calculated [1] whereas the electrical conductivity was obtained from voltage clamp experiments carried out with the same bilayer. Fusion of vesicles containing Aqp1 or AqpZ to Muller-Rudin planar lipid bilayers did not result in a measurable increase of water or electrical conductivities. To achieve a substantial augmentation of the protein : lipid ratio, solvent free planar lipid bilayers were formed from proteoliposomes. Under these conditions a large increase in the transmembrane water flux was observed. However, protein reconstitution did not induce an additional ion flux. Water movement across the highly purified human red cell aquaporin 1 channel protein [2] was inhibited by mercurial compounds. The temperature dependence of water transport rate across planar bilayers containing highly purified E. *coli* water channel protein, AqpZ [3] corresponded to an activation energy of 4 kcal/mol. The well defined planar bilayer system will permit direct analyses of putative electrogenic properties of other aquaporin proteins.

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P 2

Secondary structure, oligomerization and phosphorylation of lentil seed aquaporins

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We have purified two TIP (Tonoplastic Intrinsic Protein) isoforms from the PSV membrane of *Lens culinaris* seeds. The amino-terminal sequence of the 25 kDa protein show 87 % identity with the â-TIP of *Arabidopsis thaliana* and 60 % identity with á-TIP protein of *Phaseolus vulgaris*. The amino-terminal sequence of the 26 kDa polypeptide was blocked. To overcome this problem, an internal sequence was determined after trypsinolysis. Its sequence also shared a high homology with both *Arabidopsis* â-TIP (73 %) and *Phaseolus* á-TIP (78 %). To identify the 25 kDa and 26 kDa bands more precisely, they were cleaved with CNBr. For each protein, five proteolytic fragments have been sequenced.

Cross-linking experiments were performed in order to show that close physical association between different TIP molecules occurs within PSV membranes. SDS-PAGE revealed that formation of high molecular weight TIP oligomers is correlated with a net decrease in TIP monomers when the incubation times increase. Oligomers crosslinked with DTSP were dissociated into monomers in the presence of â-mercaptoethanol. Our results indicates that each oligomer was made of both 25 and 26 kDa proteins. Liposomes were fused with TIP-enriched membranes in order to minimize intermolecular coupling. This delayed the formation of trimers and tetramers but not that of the dimers. This is consistent with the presence of a dimer in the PSV membrane.

Both proteins are phosphorylated by a 52 kDa magnesium-dependent calcium-regulated membrane-bound kinase (CDPK). Incorporation of ^{32}P was maximal at 30EC and pH 6.5. Increasing the $MgCl_2$ concentration from 2 to 10 mM shifted the pH response curve to lower pH by about 0.5 unit. Addition of W-7, a calmodulin antagonist also known as a CDPK inhibitor to the reaction mixture resulted in a 30 % loss in kinase activity. The apparent molecular weight of the kinase was determinated by use of an in-gel assay. Calf histone H1 was used as substrate. An apparent molecular weight of 52 kDa was estimated from electrophoresis.

TIP oligomers were purified in Genapol X-080 and reconstituted into asolectine liposomes upon removal of detergent with Bio-Beads. The structure of the reconstituted TIP oligomer was investigated by attenuated total reflection Fourier transform infrared spectroscopy while the structure of the detergent-solubilized TIP oligomer was investigated by circular dichroism. Combination of both circular dichroism and infared spectroscopy techniques allows a acurate determination of the secondary structure of TIP oligomer. Our results indicates that lentil seed TIP oligomer and AQP-1 have a very similar secondary structure.

P 3

Substitution of loops E or/and C-terminal transmembrane domains in aquaporin and glycerol facilitator: effect on oligomeric state in non denaturing detergents

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We previously observed that aquaporins and glycerol facilitators exhibit different oligomeric states when studied by sedimentation on density gradients following non denaturing detergent solubilisation [1] or by freeze fracture analysis [2]. In order to determine the domains of MIP proteins involved in oligomerisation, we constructed chimeras corresponding to the aquaporin AQPcic substituted in the loop E and/or the C terminal part (including half of the sixth transmembrane segment) by the equivalent domain of the glycerol channel GlpF (chimeras called AGA, AAG and AGG). The analogous chimeras of GlpF were also constructed (chimeras GGA, GAG, and GAA). Functional analysis following expression in xenopus oocyte revealed no significant water or glycerol permeability for chimeras. This lack of effect is likely due to mistargeting in the oocyte system. Following expression in yeast, sucrose gradient sedimentation velocity analysis revealed that AQPcic is tetrameric while GlpF is monomeric when solubilised by 2% n-octyl glucoside or by 1% Triton X-100. In both non denaturing detergents, the chimera AAG exists in a tetrameric form while others are in dimeric (GGA, AGG) or monomeric form (GAG, GAA). Our data bring new evidence that in the MIP family, aquaporins and GlpFs behave differently towards non denaturing detergents. We show that the loop E and the sixth transmembrane domain are crucial for oligomeric assembly of MIP proteins.

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P 4

Loop B of the Yeast MIP Channel, Fps1p, May Play a Role in the Determination of Glycerol Transport Direction

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Fps1p is the glycerol exporter from *Saccharomyces cerevisiae*. This atypical Major Intrinsic Protein family member is notable because neither of the family's signature NPA motifs is preserved in its channel-forming loops. We have therefore addressed the functional consequences of NPS and NLA in Fps1p's respective loops B and E. Fps1p and its homologue, *E. coli* GlpF, were compared by site-directed mutagenesis and expression in a yeast *fps1Ä* mutant: NPA motifs were 'restored' in Fps1p, while in GlpF, NPA was changed to NPS and/or NLA, and Fps1p's loops B and/or E were swapped onto GlpF. Rather than employing the commonly-used *Xenopus laevis* oocyte system, our mutants were assayed in whole yeast cells. This is because it has not been possible to functionally express Fps1p in oocytes as is the case for many mutant aquaporins, which are often mislocalised in this system. We analysed the glycerol transport characteristics of our mutants in both the efflux and uptake directions as well as observing growth phenotypes on plates. Our data indicate that the Ser residue of the NPS motif in loop B of Fps1p is important for the channel to transport glycerol in both directions. Moreover, introduction of this residue into GlpF suggests that it may be involved in the determination of transport direction. Unexpectedly, our data for the mutant in which NPS is changed to QPS also support the role of loop B in the determination of glycerol transport direction, since it would appear that the presence of the Asn residue is important for glycerol *efflux*. We therefore propose that the unique deviation from the family's signature NPA motif in loop B of Fps1p reflects its function as an export channel.

P 5

Physiological roles of aquaporin water channels in the kidney

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Under normal circumstances, all animal cells maintain osmotic equilibrium regardless of whether or not they express water channels, because of the instrinsic water permeability of the lipid bilayer. The aquaporin-independent water permeability of mammalian cells is sufficient to allow osmotic equilibration in relatively short time frames, typically 30-90 seconds. Thus, aquaporins are required for normal function only in circumstances requiring extraordinarily rapid water transport. In the kidney, three such circumstances can be recognized. 1) In the proximal tubule, aquaporin-1catalyzes near-isosmotic fluid absorption driven by active NaCl transport.

Mathematical modeling of the process indicates that aquaporin-1 is theoretically unnecessary for fluid absorption to occur at normal rates, but that aquaporin-1 expression reduces the passive paracellular backleak of NaCl which can diminish net NaCl reabsorption. 2) In the descending limb of Henle's loop, aquaporin-1 permits rapid osmotic equilibration of the luminal fluid with the surrounding interstitium despite relatively rapid luminal flow. The ability of the descending limb to maintain a state of transepithelial osmotic equilibration is crucial to the countercurrent multiplication process that concentrates the urine. 3) In the collecting ducts, aquaporin-2 and -3 provide a target for hormonal regulation of water transport by vasopressin. The regulation includes both short-term control of water permeability via vesicular trafficking of aquaporin-2 and long-term control of water permeability through changes in the abundance of the aquaporin-2 and aquaporin-3 water channels.

Cotransport proteins as water channels and water pumps

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Cotransporters of the symport type have two modes of water transport: they act as water channels where the water transport is driven by the transmembrane osmotic difference and they act as molecular water pumps where the water is cotransported along with the other substrates.

The passive water permeability (L_p) per cotransport protein appears to be lower than that of aquaporins. For the Na^+/glucose cotransporter (SGLT1) by two orders of magnitude. Interestingly, the L_p depends on the presence of the non-ionic substrates: In the Na^+/glutamate transporter (EAAT1) L_p *increased* by 50% in the presence of glutamate (MacAulay et al, in prep); in the SGLT1, L_p was *unaffected* by the presence of glucose; while the L_p of the H^+/lactate cotransporter (MCT1) was *abolished* in the presence of lactate.

In the water pump mode, water transport is coupled to the substrate fluxes in a strict stoichiometric relationship. Coupling ratios range from 500 water molecules transported for each turnover in the K^+/Cl^- and H^+/lactate transporters to 50 water molecules in the H^+/aminoacid transporter (AAP5) from plants. Values between 200 and 450 are found for a variety of Na^+ coupled transporters. The water flux is coupled to the translocation of the non-aqueous substrates by a mechanism within the protein. The coupling ratio is independent of external parameters such as ligand concentrations, membrane potentials, and osmotic gradients. It appears that the free energy stored in the transmembrane (electro)chemical gradient of one substrate can be used for transport of the other substrates; uphill water fluxes, for example, are energized by downhill fluxes of the non-aqueous substrates.

With the introduction of molecular water pumps, cellular water homeostasis can be considered as a balance between water pumps and water leaks. The leak is passive, driven by osmotic gradients via the cotransporter itself and, if present, aquaporins. The concept may, at least in part, explain water transport and its regulation in a variety of biological systems. In the human small intestine, for example, half the daily uptake of water (4 litres) could be accounted for by cotransport of water, the other half by osmosis in the Na^+/glucose cotransporter.

How to construct a water-tight membrane: the apical membrane of MDCK cells as role model

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Epithelial cells of the stomach, bladder and kidney collecting duct have evolved specialized apical membranes which exhibit extremely low permeabilities to water and other small molecules. These barrier membranes protect underlying tissue and maintain strikingly high chemical gradients for water, protons, urea and NH_3. However, the structural features that contribute to barrier function are currently unclear. The aim of this study was to reconstitute the apical membrane of a well-characterized cell culture model of a barrier epithelium – the MDCK Type 1 cell. We also wished to further validate our earlier findings that each leaflet in a phospholipid bilayer offers an independent resistance to permeation [1,2]. This hypothesis can be summarized by the following, where the resistance offered by each leaflet is equivalent to the reciprocal of the permeability:

$$1/P_{AB} = 1/P_A + 1/P_B \text{ (Eq. 1)}$$

where P_{AB} is the permeability of a bilayer composed of leaflets A and B; P_A is the permeability of leaflet A and P_B is the permeability of leaflet B. We have exploited this property in attempting to recreate the MDCK apical membrane – an asymmetric bilayer. These cells have been shown to have extremely low permeabilities to water and urea [3]. Accordingly, we constructed liposomes from lipids which mimic the *exofacial* leaflet and the *cytoplasmic* leaflet of MDCK cells, and then measured their permeabilities to water, non-electrolytes, protons and NH_3. Using published analyses of MDCK Type I membranes we made *exofacial* liposomes containing (in mol%), phosphatidylcholine (PC–8.6%), sphingomyelin (SM–18.8%), glycosphingolipids (GSLs–18.4%), and cholesterol (54.3%); and *cytoplasmic* liposomes containing phosphatidylethanolamine (43.4%), phosphatidylserine (20.3%) and cholesterol (36.3%). Permeabilities were measured using a stopped-flow fluorimeter. The osmotic permeability of *cytoplasmic* liposomes to water (P_f), solutes and NH_3 was from 18 – 90-fold higher than for the *exofacial* liposomes [$P_f(ex) = 2.4x10^{-4}$ cm/s, $P_f(cy) = 4.4x10^{-3}$ cm/s; $P_{glycerol}(ex) = 2.5x10^{-8}$ cm/s, $P_{glycerol}(cy) = 2.2x10^{-6}$ cm/s; $P_{NH3}(ex) = 0.13$ cm/s, $P_{NH3}(cy) = 7.93$ cm/s]. By adding leaflet permeabilities (Eq. 1) our liposome studies predict a P_f for an MDCK apical membrane of 4.6×10^{-4} cm/s, which compares favorably with published values ($2 - 10 \times 10^{-4}$ cm/s). To determine which lipids were contributing to the low permeability values observed for the *exofacial* leaflet, we selectively removed SM, GSLs and cholesterol, and replaced them with PC. Removal of SM or GSLs resulted in a 3 – 6-fold increase in membrane permeability, indicating that each of these sphingolipids was contributing to barrier function. When cholesterol was removed, permeabilities increased by 50 – 200-fold indicating a critical role in reducing membrane fluidity. We conclude: (1) that the barrier function of water-tight epithelia resides in the *exofacial* leaflet, (2) that our reconstituted leaflets successfully reproduce the permeability properties of the MDCK apical membrane, (3) that both sphingomyelin and glycosphingolipids are important in reducing membrane permeability but that there is an absolute requirement for cholesterol to mediate this effect through lipid/lipid interactions and (4) that each leaflet in a bilayer offers an independent resistance to permeation [Supported by NIH grant DK43955].

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P 6

Microperfused rat intrahepatic bile duct units – a novel functional approach to study water, solute and ion transport in biliary epithelia

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Ductal bile is the net result of water, ion, and solute transport across the intrahepatic biliary epithelia. Progress in understanding physiology of bile formation requires functional approaches that allow measurement of transepithelial water, ion and solute transport in intact bile ducts. We previously reported a technique to microperfuse the lumen of intrahepatic bile duct units (IBDUs) isolated from normal rat liver (Gastroenterology 116:A1236, 1999). Our AIM here was to study transepithelial water, solute and ion transport using the microperfused IBDUs. METHODS. IBDUs (100-150 ìm diameter, 1-1.5 mm length) were isolated from rat liver and perfused in vitro with isotonic (290 mOsm) Ringer-HCO_3 buffer (KRB). To study water transport, IBDUs were perfused with a membrane-impermeant fluorophore, fluorescein-sulfonate (FS), as a volume marker. IBDUs were bathed simultaneously in KRB of different osmolalities – [70 mOsm (hypotonic), 290 mOsm (isotonic) or 530 mOsm (hypertonic)]. To study ion transport, particularly HCO_3^-secretion, IBDUs were perfused with the pH sensitive dye, BCECF-dextran. IBDUs were epiilluminated to excite FS or BCECF fluorescence in a small spot (50-100 Fm) at the distal end of their lumen. Osmotic water permeability (Pf) was calculated from the FS concentration, perfusate flow rate, lumen and bath osmolalities, and IBDU geometry. HCO_3^- transport was estimated by conversion of BCECF fluorescence to pH values. To study solute transport, IBDUs were perfused with 10-160 mM of taurocholic acid (TCA). Bile acid uptake was calculated from the concentrations of TCA in the perfused and collected solutions, the IBDUs geometry, and the time of perfusion. RESULTS. In the absence of an osmotic gradient, water did not move across biliary epithelia. In contrast, when inward (secretory) or outward (absorptive) osmotic gradients were established across IBDUs, water movement from bath to lumen ($Pf = 4.8 \pm 0.8 \times 10^{-3}$ cm/sec) and from lumen to bath ($Pf = 1.0 \pm 0.1 \times 10^{-3}$ cm/sec) was observed. Luminal pH was increased after addition of forskolin to the bath indicating active HCO_3^- secretion. The perfused IBDUs absorbed TCA in a saturable, sodium dependent manner; in addition, TCA uptake was blocked by S0960, a specific inhibitor of the Na^+/bile acid cotransporter, ASBT, known to be expressed on the apical cholangiocyte membrane. CONCLUSION. The results provide functional evidence that intrahepatic bile ducts can both secrete and absorb water in response to osmotic gradients, can transport ions, such as HCO_3^-, and can actively absorb solutes such as bile acids. The microperfused IBDU is a useful experimental model to study water, ion and solute transport across biliary epithelia.

P 7

Water channel protein, aquaporin 3, in epithelial cells

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Aquaporin 3 (AQP3) is localized at basolateral membranes of renal collecting duct principal cells and seems to play an important role of water reabsorption. AQP3 is expressed in extrarenal organs, but few works have been done for its localization and its functional roles were poorly discussed. We raised anti-AQP3 antibody and examined the comprehensive and detailed immunolocalization of AQP3 in rat epithelial tissues and the developmental changes of AQP3 in the skin. We further examined the changes of expression pattern of AQP3 in Madin-Darby canine kidney (MDCK) cells upon hypertonic stimulus.

AQP3 localization in rat epithelial tissues

AQP3 was present in the epithelia of the urinary tract, digestive tract, respiratory tract, and the epidermis of the skin. In the transitional epithelia of the renal pelvis, ureter, urinary bladder, and urethra, AQP3 was localized at cell membranes of basal and intermediate layers but not in the large superficial cells. In the stratified epithelia of the skin and the upper digestive tract from the oral cavity to the forestomach (nonglandular portion), AQP3 was localized at cell membranes of the basal and intermediate layers but not in the surface layer. In the simple epithelia of the fundic stomach, distal colon, and rectum, AQP3 was localized at basolateral membranes of surface-covering epithelial cells. In the pseudostratified ciliated epithelia of the respiratory tract from the nasal cavity to the intrapulmonary bronchi, AQP3 was localized at cell membranes of basal cells and at basolateral membranes of some ciliated cells. In summary AQP3 was present in the surface-covering epithelial cells exposed to an environment of possible water loss. Fetus rats have a low risk for the water loss from the skin because they are surrounded by the amniotic fluid. Upon delivery, newborn rats suddenly face a risk of dehydration because of their land life. Examination of AQP3 expression in the epidermis development revealed that it commenced late in the fetal life toward the birth. We suggest that AQP3 could serve as a water channel to provide epithelial cells with water from the subepithelial side to protect them against dehydration.

AQP3 expression in MDCK cells

AQP3 expression in MDCK cells was increased by treatment with hypertonic media containing raffinose or NaCl. AQP3 was localized at basolateral plasma membranes in these cells. These results show that the AQP3 expression is controlled by the extracellular fluid osmolality.

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P 8

Neurotransmitters regulate the amount of AQP5 in the apical plasma membrane via $[Ca^{2+}]i$ in parotid acinar cells

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The AQP5 cDNA was isolated from rat submandibular gland by Raina et al [1]. Northern blot analysis revealed that AQP5 mRNA is present in submandibular, parotid, sublingual, and lacrimal glands, trachea, eye, and distal lung. The AQP5 protein is located in the apical plasma membrane (APM) of acinar cells and intracellular secretory canaliculi [2, 3]. However it remains to be established whether subcellular distribution of AQP5 in the cells changes by autonomic nerve stimulation. We investigated by immunoblot analysis with anti-AQP5 antibody whether translocation of AQP5 in the parotid glands was regulated by neurotransmitters [4, 5].

The treatment of rat parotid tissues with acetylcholine (ACh) or epinephrine (Epi) induces the translocation of AQP5 from the intracellular membranes (ICM) to the APM with a maximal at 15 sec or 1 min, respectively. But the prolongation of the treatment of it with ACh for more than 5 min resulted in the converse translocation from APM to ICM. ACh-induced increase in the amount of AQP5 in APM was inhibited by atropine and *p*-F-HHSiD. This effect of Epi was mimicked by phenylephrine, but not by clonidine, dobutamine, or salbutamol, and Epi-induced increase in the amount of AQP5 was inhibited by phentolamine, but not by propranolol. The calcium ionophore A-23187 alone stimulates also the translocation of AQP5 between APM and ICM in the presence of extracellular Ca^{2+}. The translocation of AQP5 to APM was profoundly decreased in the tissues treated with U-73122, dantrolene or TMB-8. Cytochalasin D and tubulozole-C also inhibited ACh- and Epi-induced translocation of AQP5 to APM.

These results indicated that ACh and Epi act at muscarinic M_3 and_á$_1$-adrenergic receptors, respectively, and induce translocation of AQP5 via $[Ca^{2+}]i$ elevation in response to IP_3 and ryanodine receptor activation in the cells. In addition, cytoskeleton elements are shown to be involved in the translocation of AQP5 to APM.

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P 9

Molecular mechanisms of water hemostasis in brain: Are AQP4 molecules subjected to regulation?

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Vasopressin has been shown to increase the water content of brain tissue in several experimental paradigms and to increase the water permeability of glial cells in vitro. Furthermore, the brain is endowed with an intrinsic vasopressin containing fiber system, the function of which is still largely unknown.

Little is known about the mechanisms underlying the modulation of water transport in the brain, or if such a modulation indeed occurs. Attempts to address this issue in the intact brain are complicated by the need to distinguish between vascular and parenchymal contributions to water homeostasis. Here we explore whether vasopressin regulates brain water redistribution, using a superfused cortical slice preparation where vascular changes are irrelevant. In this model, water redistribution is assessed by measuring the intrinsic optical signals (IOS) generated by changes in the extracellular space volume.

Based on these data we hypothesize that vasopressin might play a role in regulating activity-dependent water redistribution. Our results indicate that this is indeed the case, and that the effect of vasopressin is mediated through V1a receptors. To adress whether this effect is through a modulation of AQP4 water permeability we are now employing astrocyte cultures. We will be studying the effect of vasopressin on cell volume of astrocyte cultures under two different experimental conditions; cultures treated with AQP4 antisense and cultures subjected to AQP4 overexpression. The results of these experiments will give us an indication whether the vasopressin effect is a function of the AQP4 content of the cells.

P 10

Rapid gating of AQP6, an intracellular water channel with anion conductance

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Aquaporin water channel proteins (AQPs) provide the molecular pathways for water permeation of cell membranes. Mammalian aquaporins reside in plasma membranes and are constitutively active. No evidence indicates that aquaporins are rapidly and reversibly gated or permeated by ions. Here we show that AQP6 is distinct from other known aquaporin in localization and function. Using a polyclonal antibody to the C-terminus of AQP6, immunohistochemical and immunoelectron microscopic studies revealed that AQP6 is localized exclusively in intracellular membranes in renal epithelia including acid-secreting type-A intercalated cells in collecting duct. When expressed in *X. laevis* oocytes, AQP6 exhibits low basal water permeability (P_f). Surprisingly, when treated with the known water channel inhibitor, Hg^{++}, the P_f of AQP6 oocytes rapidly rises ~10 fold and is accompanied by ion conductance. AQP6 colocalizes with H^+-ATPase in intracellular vesicles of acid secreting intercalated cells in renal collecting duct. At pH < 5.5, water permeability and anion conductance are rapidly and reversibly activated in AQP6 oocytes. Site-directed mutation of lysine to glutamate at position 72 in the cytoplasmic mouth of the pore changes the cation/anion selectivity but leaves low pH activation intact, suggesting that ions pass through AQP6 itself rather than passing through associated channels. These studies demonstrate unprecedented biophysical properties by an aquaporin, indicating that AQP6 does not function as a simple conduit for trans-epithelial water absorption or secretion but participates in diverse physiological processes, including acid-base metabolism.

Upregulation of Aquaporin-2 (AQP-2) gene expression in pathological state of water retention in glucocorticoid-deficient (GD) rats

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We determined alteration in renal AQP-2 mRNA expression and vasopressin (AVP) V_2 receptor function in association with water retention in GD rats. After adrenalectomy, SD rats (250-280 g) were administered aldosterone (42 ng/100g/h) by osmotic minipumps (GD rats). As a control, both aldosterone (42 ng/100g/h) and dexamethasone (58 ng/100g/h) were administered in adrenalectomized rats. Two groups of rats were subjected to the studies on day 7-14 (after the operation). Serum Na level was 138 mEq/l in the GD rats, less than that of 150 mEq/l in the control ($p < 0.05$). The expressions of AQP-2 mRNA and protein in kidney of the GD rats were increased by 1.5- and 1.3-fold as compared to those in the control, respectively. An acute oral water load (30 ml/kg) was carried out. The percent excretion water-loaded was 38% in the GD rats, which was less than that of 139% in the control ($p < 0.05$). The minimal Uosm was as high as 379 mOsm/kg H_2O in the GD rats, but it was diluted to 138 mOsm/kg H_2O in the control. One h after the water load, the expression of AQP-2 mRNA and protein were totally reduced in the control, but they remained unchanged in the GD rats as the values were 1.7- and 1.3-fold greater than those of the control, respectively. This was in concert with the nonsuppressible levels of plasma AVP despite hypoosmolality. OPC-31260 (30 mg/kg), a V_2 receptor antagonist, totally reduced the expression of AQP-2 mRNA and protein and normalized water diuresis in the GD rats. There was no difference in [^{3}H]AVP receptor binding (Bmax : 8.5 vs. 7.3 pM/mg protein) and AVP V_2 receptor mRNA expression between the two groups of rats. These results indicate that augmented expression of AQP-2 participates in water retention, dependent on AVP, in glucocorticoid deficiency, which is not associated with receptor downregulation.

P 11

Prostaglandins and the dehydration-induced upregulation of AQP2 expression

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Following stimulation with vasopressin, aquaporin 2 (AQP2) is rapidly transferred from a store in intracellular vesicles to the apical plasma membrane of the collecting duct principal cells. This increases the water permeability of the collecting duct, allowing water to be reabsorbed from the tubular fluid, resulting in a concentrated urine. It is now clear that this acute response is modulated by changes in the total AQP2 available in the cells. The signals controlling this expression are currently under investigation. While one signal is vasopressin itself, it is now clear that there are other factors involved, since under certain conditions AQP2 expression can be altered independent of vasopressin activity. In particular, dehydration appears to provide a strong stimulus for AQP2 expression, while overhydration results in reduced AQP2 levels.

We have investigated the hypothesis that prostaglandins may act as a stimulus for the production of AQP2. Preliminary studies showed that treatment with non-steroidal anti-inflammatory drugs (NSAIDs) caused a decrease in AQP2 expression. These drugs inhibit prostaglandin production by inhibiting cyclooxygenase (COX), the rate-limiting enzyme in the synthetic pathway. There are constitutive (COX-1) and inducible (COX-2) forms of the enzyme, and both have been shown to be present in the renal inner medulla [1]. To investigate whether prostaglandins might be involved in thirsting-mediated AQP2 expression we have now investigated the effects of the non-isoform-specific NSAID ibuprofen and the COX-2 specific meloxicam, in water-loaded and dehydrated animals.

Rats (male Wistars, 200 - 250g) were kept in metabolic cages for a two day baseline period, plus two days during which half received the drug under test. To ensure equal nutrition and drug dosages, and to regulate water intake, 15g of food was mixed with a known amount of water (0, 20 or 50 ml/day). Drugs (meloxicam (0.25 mg/day), or ibuprofen (10 mg/day)) were included in the diet as required. After the first day all food was eaten. Animals had free access to salt blocks throughout. At the end of the experimental period rats were anaesthetised, the kidneys removed, and the inner medullae prepared for immunoblotting and densitometry.

As previously reported [2], ibuprofen caused decreased AQP2 expression (to 24 " 5% of control levels) in rats with free access to water, while when given in conjunction with dehydrationit prevented the increase in AQP2 seen in thirsted controls [3]. When ibuprofen was given in conjunction with a moderate water load (50 ml/day), AQP2 levels were 57 " 11% of control levels (100 " 12%, $n = 6$, $p < 0.05$). Thus some reduction in AQP2 expression persisted. In contrast, in water-loaded animals there was no significant difference in AQP2 levels between meloxicam-treated (89 " 9%) and control animals (100 " 9%, $n = 8$ in each group, n.s.), while in water deprived rats AQP2 expression in meloxicam-treated animals was only 31 " 3% ($n = 7$) of the level found in controls which had been similarly water-deprived (100 " 8%, $n = 8$, $p < 0.01$).

These results suggest that prostaglandin production, mediated by COX-2, is important in the dehydration-induced increase in AQP2 expression. In the baseline state or during water loading, prostaglandin production probably plays only a minor part in driving AQP2 synthesis.

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P 12

Regulation of renal aquaporins and sodium transporters during vasopressin-escape in the rat

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Clinically, the syndrome of inappropriate antidiuretic hormone (vasopressin) secretion (SIADH) is associated with water retention and hyponatremia. However, patients are able to "escape" the antidiuretic actions of vasopressin by increasing urine volume and decreasing osmolality. Recently, using our rat model of vasopressin escape, we have begun to understand some of the molecular mechanisms underlying the escape. For these studies, male, Sprague-Dawley rats were implanted with osmotic mini-pumps to administer dDAVP (1-deamino-[8-D-arginine]-vasopressin), a V2-selective vasopressin agonist, for the entire study. After 4 days, rats were divided into two groups. For 1-7 additional days, Control rats received dDAVP, dry diet, and ad libitum water while Water-Loaded rats received dDAVP and a high-water diet (liquid or gelled). A significant increase in urine volume and decrease in urine osmolality in the Water-Loaded rats was observed by the second day, indicating onset of vasopressin escape. Furthermore, Water-Loaded rats experienced an early natriuresis and developed a marked hyponatremia (plasma Na^+ range approximately 98-122 mmol/L). The onset of escape coincided temporally with a marked decrease in renal aquaporin-2 protein (measured by semiquantitative immunoblotting and immunohistochemistry) and mRNA (Northern blotting). Additional studies have shown a decrease in V2 vasopressin receptor mRNA and V2-receptor binding in this model. In contrast, there were no decreases in the renal expression of aquaporins 1, 3, or 4. In fact, aquaporin-3 abundance was significantly increased in the Water-Loaded rats. Immunocytochemical localization and differential centrifugation studies demonstrated that trafficking of aquaporin-2 to the plasma membrane remained intact during vasopressin escape. The relative abundances of critical renal sodium transporters were also affected in this model. The abundances of the thiazide-sensitive NaCl cotransporter (NCC), the á-subunit of the epithelial sodium channel (ENaC) and the 70-kDa band of the ã-subunit of ENaC were all significantly increased in the Water-Loaded rats. No changes were observed for the â-subunit of ENaC. Similar protein changes have recently been associated with elevated aldosterone levels in rats. However, plasma aldosterone levels were significantly suppressed in this model. Together, these results suggest that escape from vasopressin-induced antidiuresis is attributable, at least in part, to a vasopressin-independent decrease in aquaporin-2 water channel expression in the renal collecting duct. Furthermore, several distal sodium reabsorptive mechanisms are upregulated during vasopressin escape. Increased sodium absorption due to upregulation of the transporters may act to ameliorate the hyponatremia resulting from water loading.

Aquaporin physiology revealed by phenotype analysis of transgenic mice

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Phenotype analysis of transgenic mice deficient in specific aquaporin water channels has provided new insights into the role of aquaporins in organ physiology. In kidney, deletion of AQP1, AQP2 or AQP3 produced distinct forms of nephrogenic diabetes insipidus, whereas AQP4 deletion produced only a mild defect in maximal urinary concentrating ability. Deletion of AQP5 in salivary gland resulted in defective saliva production. Deletion of AQP1 or AQP5, the endothelial and epithelial water channels in lung, gave a 90% decrease in airspace-capillary water permeability. In brain, deletion of AQP4 conferred marked protection from brain swelling induced by acute water intoxication and ischemic stroke. The general paradigm that has emerged from these phenotype studies is that aquaporins facilitate rapid near-isosmolar transepithelial fluid absorption/secretion, as well as rapid vectorial water movement driven by osmotic gradients. However, we have found many examples in which the tissue-specific expression of an aquaporin is not associated with apparent phenotypic abnormalities, such as unimpaired gastric acid production and skeletal muscle function in AQP4 null mice, unimpaired lung fluid handling in AQP1/AQP5 null mice, and unimpaired tear production in mice lacking multiple aquaporins. The physiological data in the aquaporin transgenic mice suggests the utility of aquaporin blockers and aquaporin gene replacement in selected human diseases.

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Requirement of AQP1 for Isotonic Fluid Transport in the Renal Proximal Tubule

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To assess the role of AQP1 in fluid transport across renal proximal tubules, we compared net fluid absorption in wild type and AQP1 knockout mice using in vivo micropuncture techniques. Fractional fluid absorption along the proximal convoluted tubule fell from 48 " 2.5 % in wild type to 25.6 " 2.5 in AQP1 -/- mice. Transtubular osmotic gradients were determined by measuring plasma and tubular fluid osmolalities. Tubular osmolality was lower than plasma by 38.7 " 3.8 mosm/l in AQP1-/- mice, but by only 11.9 " 2.4 mosm/l in AQP1+/+ mice. Despite the highly significant reduction in fluid absorption tubular flow rates at the end of the proximal tubule and in the distal tubule were not different between AQP1 +/+ and AQP1 -/- mice. Constancy of tubular flow was due to a significant reduction in nephron filtration rate from 11.1 " 1.6 nl/min in AQP1 +/+ to 5.1 " 0.4 nl/min in AQP1 -/- mice. The fall in GFR was mediated through tubuloglomerular feedback since it was seen only when the macula densa region was exposed to ambient tubule flow. Absorption of fluid along the loop of Henle of superficial nephrons (proximal straight tubule, thin descending, and thick ascending limb) was not different between genotypes at normal flow rates, but was significantly lower in AQP1 -/- mice at elevated flow rates. We conclude: 1) AQP1 is required for isotonic fluid absorption in the proximal tubule since in the absence of the water channel absorbate becomes markedly hypertonic; 2) The increased transtubular osmotic driving force permits water absorption to proceed at a half normal rate despite an 80% reduction in osmotic water permeability; 3) A reduction in glomerular filtration rate perhaps in conjunction with upregulation of downstream salt transporters prevents catastrophic renal salt losses; 4) Fluid absorption along the loop of Henle is relatively independent of AQP1 and may use another water channel, perhaps AQP4.

Aquaporins in gastrointestinal and skin physiology

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Fluid transport is a major function of the gastrointestinal (GI) tract. In humans, more than 9 litres/day of fluid are absorbed or secreted across epithelia and endothelia in salivary gland, stomach, the hepatobiliary tract, pancreas, small intestine and colon. Seven aquaporins have been localized to various epithelia and endothelia in the GI system: AQP1 in the intrahepatic bile duct epithelium and intestinal lacteals, AQP4 in gastric parietal cells and colon surface epithelium, AQP3 in small intestine, AQP5 in salivary serous gland, AQP7 in small intestine, AQP8 in liver, pancreas, salivary gland, and colon, and AQP9 in hepatocytes. These sites of aquaporin expression suggest a role of AQPs in fluid transport and digestive/absorptive functions in the GI tract. In the skin, AQP3 is expressed in epidermal keratinocytes and sebaceous glands, suggesting a possible role in skin moisture, epidermal lipid metabolism, and wound healing.

Transgenic mice lacking aquaporins 1, 3, 4, and 5 were generated in our laboratory by homologous replacement gene targeting. Initial phenotype analysis suggested a role of these aquaporins in GI and skin functions. In AQP5 knockout mice, pilocarpine-stimulated saliva production was reduced by more than 60%. AQP1 knockout mice on a high fat diet acquired an oily appearance and growth retardation, developed steatorrhea, and were relatively hypotriglyceridemic. Fecal and duodenal lipase activities were three to ten-fold greater in [-/-] than wildtype mice on low and high fat diets. TLC analysis of fecal lipids indicated more free fatty acids in [-/-] than wildtype mice, suggesting an absorptive defect. AQP3 [-/-] are also hypotriglyceridemic. AQP4 knockout mice were used to test the hypothesis that AQP4 is involved in colon water transport, fecal dehydration, and stomach acid secretion. Transepithelial water permeability was significantly lower in [-/-] mice when measured in full-length colon and proximal colon. While there was no difference in water content of cecal stool from wildtype vs. AQP4 null, there was slightly higher water content in defecated stool from [-/-] mice. However colonic secretory function was not impaired in a model of theophylline-induced secretion. In stomach, AQP4 deletion did not affect gastric acid secretion stimulated by a variety of agonists, nor were minimal pH or serum gastrin levels affected. Capacitance measurements of skin water content indicated relatively dry skin in AQP3 null mice with defective adaptation to changing atmospheric moisture. TLC analysis of the epidermal lipid profile demonstrated two to three-fold lower triglyceride in AQP3 [-/-] vs. wildtype mice. Together these results provide evidence that aquaporins are important in GI tract and skin.

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Aquaporins, cerebral edema, and brain water balance

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Cerebral edema contributes significantly to morbidity and death associated with many common neurological disorders. However, current treatment options are limited to hyperosmolar agents, steroids,and surgical decompression - therapies introduced more than 50 years ago. We find that mice deficient in aquaporin-4 (AQP4), a glial membrane water channel, have much better survival than wild-type mice in a model of brain edema caused by acute water intoxication. Brain tissue water content and swelling of pericapillary astrocytic foot processes in AQP4 deficient mice were significantly reduced at 30 minutes. Similarly, in response to focal ischemic stroke produced by middle cerebral artery occlusion, AQP4 deficient mice had improved neurological outcome. Cerebral edema, as measured by percentage of hemispheric enlargement at 24 hours, was decreased by 35% in AQP4 deficient mice and infarct size was smaller. By diffusion-weighted magnetic resonance imaging, AQP4 deficient mice showed remarkably reduced brain swelling after transient middle cerebral artery occlusion followed by reperfusion. These results implicate a key role for AQP4 in modulating brain water transport, and suggest that AQP4 inhibition may provide a new therapeutic option for reducing brain edema in a wide variety of cerebral disorders including head trauma, tumor and infection. Recently, remarkable effects of AQP4 deletion have also been demonstrated in a model of spinal cord injury and in chemically-induced seizure thresholds. Finally, experiments involving CSF dynamics are in progress to establish the role of AQP1 in CSF fluid production.

AQP5 deficient (-/-) acinar cells have decreased membrane permeability and altered cell volume regulation

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Salivary secretion is stimulated by the autonomic nervous system through cholinergic activation of muscarinic receptors on the surface of acinar cells. Pilocarpine, a naturally occurring sialagogue, stimulates exocrine secretions by acting as a muscarinic receptor agonist. The resulting change in tonicity requires a rapid and regulated adjustment of cellular volume. The mechanisms for this regulatory circuit are currently unknown.

Examination of AQP5 protein expression in mouse submandibular glands by Western analysis shows a sex-specific difference in the amount of total membrane AQP5 (in plasma membranes and vescicles), with females expressing approximately 4-fold more AQP5 protein ($p < 0.00001$; $N = 6$) compared to males. IP administration of pilocarpine to mice and subsequent Western analysis of protein from parotid glands shows a 2-fold increase in AQP5 in total membrane fractions ($p < 0.016$; $N = 8$). Sub-fractionation of plasma membranes and vesicles from parotid glands showed no significant difference in AQP5 expression between fractions in the presence or absence of pilocarpine treatment ($p < 0.26$; $p < 0.69$; $N = 8$). However, sublingual glands showed an 8-fold increase ($p < 0.001$; $N = 8$) in the amount of AQP5 protein in plasma membranes although the amount of AQP5 protein in vesicle fractions, appeared to remain unchanged. These results show that AQP5 is regulated differently in the submandibular, sublingual and parotid glands.

We previously reported the cloning, genomic characterization, and expression patterns of murine Aqp5 [1]. We have generated AQP5 knockout mice in which exon 3 of AQP5 has been deleted and replaced with a neomycin resistance cassette. Homozygous knockout mice show no AQP5 mRNA or protein expression in tissues in which AQP5 is normally expressed, including the salivary glands. Cell volume determinations were used to estimate the intrinsic water permeability in parotid acinar cells isolated from AQP5 +/+ and -/- mice. Calculating the rate of hypertonic-stimulated cell shrinkage and hypotonic-induced cell swelling, and the associated regulatory volume decrease (RVD), we determined that water permeability is significantly ($p < 0.005$; N$16) less in acinar cells from AQP5 -/- vs. +/+ mice. The magnitude of the water permeability sensitive to mercury in +/+ acini was determined to be ~50% ($p < 0.0001$; N$35). This value is comparable to that which is inhibited by knocking out AQP5 expression (~65%). In contrast, mercury did not inhibit water movement in -/- acini, but unexpectedly enhanced the water permeability of -/- acinar cells by an unknown mechanism ($p < 0.0001$; N$36). These results demonstrate that AQP5 is regulated both by sex-specific and gland-specific mechanisms, and that this water channel is required for the proper regulation of acinar cell volume and membrane permeability.

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The role of aquaporins in cell-to-cell water flow in plant tissues

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Cell-to-cell water transport within the plant is necessary to permit water to enter and exit the transpiration stream, to support cell enlargement – a major aspect of plant growth –, to facilitate stomatal opening or organ movements, and to permit the circulation of water between the xylem and the phloem. Thus, water needs to be transported through living tissues continuously in a growing plant. It has been known for a long time that water transport through living tissues can be regulated, and aquaporins may be the proteins through which such regulation is effected. Plants contain 25 – 35 different expressed MIP genes and many of these encode aquaporins. Most of the MIPs appear to reside in the two membranes that delimit the cytoplasm: the plasma membrane (PIPs) and the vacuolar membrane or tonoplast (TIPs). Cell to cell water flow involves passage of the water molecules through both membranes. Measurements of Pf show that the tonoplast is nearly always very permeable to water (Pf of 500 micrometer per second) , whereas the plasma membrane is much less permeable, and in a population of cells there is great variability in this permeability (1 to 500 micrometer per second). This situation permits the plant to regulate transcellular water flow at the plasma membrane, while the high permeability of the tonoplast ensures that there will be a minimum of volume perturbations when osmotic strength changes suddenly. To determine the role of PIPs in membrane permeability we expressed an Arabidopsis PIP2 sequence in the sense and antisense orientations and screened plants for protein expression with antibodies that recognize the entire PIP2 family (F. Barrieu and M.J. Chrispeels, unpublished results). Protoplasts from the over-expressing plants have the same permeability as those from wild type plants, but protoplasts from the antisense plants have a much lower water permeability than those of the wild type plants. (R. Morillon and M. J. Chrispeels, unpublished results). These results will be discussed with respect to the role of the two groups of aquaporins in the permeability of the plasma membrane and the regulation of cell-to-cell water flow.

Physiological and genetic analysis of plasma membrane aquaporin functions in *Arabidopsis*

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The *pip* subfamily of plant MIP homologues encodes putative plasma membrane aquaporins and comprises 13 members in the model species *Arabidopsis thaliana*. The function of the PIP proteins at the cell and tissue levels and the significance of their diversity are still poorly understood. These questions were addressed by a combination of reverse genetics and cell physiological analyses.

In a first approach, we used *Arabidopis* suspension cells to investigate the regulatory mechanisms which target PIP aquaporins. To characterize water transport at the cell level, cell pressure probe measurements were set up in cooperation with the group of Prof. Ernst Steudle (University of Bayreuth, Germany). Using this technique, we found that the cell hydraulic conductivity (L_p) of *Arabidopsis* suspension cells can vary up to 8-fold depending on the measuring conditions. When cells were impaled in the presence of calcium in the pipette and in bathing solution, cell L_p was reduced 4-fold as compared to control values obtained in the absence of calcium. In contrast, a nearly 2-fold increase in L_p was observed when chloride ions were replaced by fluoride ions, the latter presumably acting as a protein phosphatase inhibitor. The mechanisms which underly these changes in L_p were investigated *in vitro*. Plasma membrane vesicles were isolated from *Arabidopsis* suspension cells by aqueous two phase partitioning and their water transport properties were characterized by stopped-flow spectrophotometry. Membrane vesicles isolated in standard conditions exhibited a reduced water permeability together with a lack of active water channels. In contrast, when prepared in the presence of fluoride and chelators of divalent cations, plasma membrane vesicles showed a 5-fold higher water permeability and active water channels. Furthermore, addition of calcium to these purified membrane vesicles reduced their water permeability down to the basal level of membranes isolated in standard conditions. The cellular and molecular mechanisms of this plasma membrane-delimited regulation of *Arabidopsis* water channels by calcium are under investigation.

In parallel to these studies, an undiscriminate analysis of plasma membrane aquaporin function was undertaken by isolating knock-out mutants of *pip* genes in *Arabidopsis*. For this, a library of plant lines mutagenized by insertion of *Agrobacterium tumefaciens* T-DNA was screened by PCR, using a combination of primers specific for *pip* genes and T-DNA border sequences, respectively. The isolation of six insertion mutants for genes both in the *pip1* and *pip2* subfamilies will be presented. The phenotype of these mutant lines is being investigated by characterizing their growth and development in normal and stress conditions. Parallel characterization of gene expression will help in understanding the function of the corresponding genes. A *pip* mutant showed a normal growth and development when grown in soil in standard conditions. However, the mutant showed an altered root growth, as compared to the wild type, when grown *in vitro* in the presence of NaCl. These preliminary observations need to be confirmed in a second, independently isolated mutant which is disrupted in the same *pip* gene. They suggest a role for aquaporins in the response of plants to salinity.

Post-translational regulation of aquaporins

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In the model plant *Arabidopsis thaliana*, 30 genes have been found that code for aquaporin homologues. Some of these genes code for highly abundant constitutively expressed proteins and some genes are known to be temporally and spatially regulated during development and in response to stress. At a given time, cells express several different aquaporins and it is probable that vacuolar and plasma membrane aquaporins acting in concert are responsible for cytosolic osmoregulation necessary for maintaining normal metabolic processes. Inhibition studies of aquaporins *in vivo* and antisense mutant studies suggest that, in addition to cytosolic osmoregulation, aquaporins are important for water homeostasis in plants. The water transport activity of two aquaporins is regulated at the protein level by phosphorylation and dephosphorylation. The post-translational regulation of one of these proteins will be discussed in detail.

Protoplasmic pH regulates an aquaporin water pathway in plant vacuoles

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Water is essential for plant growth and development. Therefore, the study of the mechanisms that regulate water permeability in plants is basic to understand plant responses under limiting factors like water stress or drought. The discovery of water channels (aquaporins) have requestioned the role of the cell to cell pathway in water transport and the progressive understanding of their possible regulatory properties is giving important clues to comprehend their ubiquitous presence [1]. In a previous work we focused our interest in understanding water movements in *Beta vulgaris* storage roots, and we were able to describe a transcellular water movement sensitive to mercurial compounds [2]. In this species a tonoplast intrinsic protein (TIP) has been described in the root parenchyma [3]. In general, it has been reported that water permeability at the tonoplast level is very high, which strongly supports the role of TIPs in water transfers across vacuole membranes. We therefore focused our goal in studying the properties of water pathway transfer in sugar beet storage root vacuoles and its possible regulation.

For this puropose, vacuoles were mechanically isolated from parenchyma of *Beta vulgaris L.* storage roots to study volume changes using videomicroscopy. After a hypertonic challenge employing a non permeant solute (PEG-3350), the vacuole volume stabilized in a new value, corresponding to a perfect osmometer response. When the vacuoles were exposed to anisosmotic conditions by modifying mannitol concentration and when urea partial and isosmotically replaced mannitol, a more complex response was observed, probably reflecting both water and solute movements. If vacuoles were pre-incubated in the presence of $HgCl_2$ (isosmotic conditions) and then a hyposmotic challenge was applied, a reduction in the equilibrium volume was observed. This reduction was concentration-dependent. When $HgCl_2$ concentration arrived to 300 ìM the volume change was completely abolished. The volume changes induced by a hyposmotic challenge were also strongly dependent on medium pH. Surprisingly, the response to medium hyposmolarity was completely abolished at pH 6.6, being this effect fully reversible. The pH-dependent volumetric change induced by a hyposmotic shock was a classical dose-response curve. This evidences are consistent with recently findings that the AQP3 transport of water and glycerol is gated by H^+ [4].

We concluded that mercury sensitive water pathway present in the studied are strongly regulated by the pH of the medium, showing that plants also probably developed proton regulation mechanisms of water pathways.

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The response of plants to salinity involve root water channels

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Irrigation with saline water occurs in many semiarid regions of the world where it decreases the growth and yields of crop plants. There is a general agreement that whole plant growth responses to salinity are multigenic and that a better knowledge of the underlying physiology is required, in order to understand why some plants are more resistant than others. It has been hypothesised that plant growth is initially inhibited by cellular responses to the osmotic effect of external salt, but in a later response, growth is inhibited by the toxic effect of excessive salt accumulation within the plant. In our experiments, we have centred our attention on the effects of NaCl on water channels and their role in the response of plants to salinity. Firstly, pepper plants were stressed with two isotonic solutions: NaCl and increased macronutrients of the Hoagland nutrient solution (HNS). Root hydraulic conductance, L_0, was always less in NaCl plants than in controls and HNS. In control and 6xHNS plants, $HgCl_2$ treatment (50 FM) caused a sharp decline in L_0, to values similar to those of NaCl-stressed roots, but L_0 was restored by treating with 5 mM DTT. However, in NaCl roots, only a slight effect of Hg^{2+} and DTT was observed. The results suggest that the putative reduction in water-channel function of NaCl-treated plants did not seem to be due to the osmotic effect. In other experiments, melon plants stressed with NaCl, or with NaCl following previous treatment with $CaCl_2$, were compared with controls and $CaCl_2$ treated plants. L_0 of NaCl-treated plants was markedly decreased when compared to control and $CaCl_2$-treated plants, but the decrease was smaller when NaCl was added to plants previously treated with $CaCl_2$. A similar effect was observed when the flux of calcium into the xylem and the Ca^{2+} concentration in the plasma membrane of the root cells were determined. The inhibition of L_0 and the osmotic water permeability (P_f) of root protoplasts by $HgCl_2$ was high for control, $CaCl_2$ and $NaCl + CaCl_2$ but only slight for NaCl. Therefore, the ameliorative effect of Ca^{2+} on NaCl stress could be related to water-channel function. Also, P_f measurements were carried out on protoplasts treated with NaCl plus okadaic acid. Okadaic acid produce an opening of aquaporins by inhibiting phosphatase activity. The effect of okadaic acid on P_f values after and before NaCl addition was similar, showing (with respect to control protoplasts) a smaller decrease of P_f than with NaCl alone. The results showed that the negative effect of NaCl on water channel activity was not due to a high ion concentration effect on channel pores or to the increase in osmotic pressure. We suggest that it was due to a direct action of NaCl on protein regulation. However, in other experiments with *Arabidopsis*, a reduction of mRNA expression of the aquaporin PIP1a after 1 d of exposure to NaCl, has been observed. Therefore, we hypothesise that there should be two different effects of NaCl on water channels, one at the protein level (gating regulation) and the other through gene expression.

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Regulation of water channel activity and stomatal conductance are coordinated events in honey locust (*Gleditsia triacanthos*)

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Since the discovery of aquaporins in plants and subsequent demonstration of their functionality *in vivo*, progress in elucidating the role of this interesting family of transmembrane proteins in plants has been limited. We have investigated the presence of water channel activity in honey locust (*Gleditsia triacanthos*), a leguminous tree species that exhibits a strong circadian rhythm in root water transport. The volume flux of water (Jv) through roots of 2-month-old honey locust seedlings was estimated by using applied hydrostatic pressure. Severed whole root systems were mounted in the lid of a pressure chamber with the cut stump protruding through a gasket. The chamber, filled with Hoagland solution, was pressurized to 0.34 MPa, and exudate was delivered from each root system to an electronic balance via an automated system. Jv was expressed on a total root surface area basis and plotted over time. A highly-defined endogenous circadian rhythm in Jv was observed, with maximal fluxes occurring from sunrise until noon and minimal fluxes occurring at sunset; the rhythm continued for up to 90 h. Midday root water flux was inhibited by more than 90% upon treatment with mercuric chloride, but complete recovery occurred upon subsequent treatment with mercaptoethanol. Interestingly, root hydraulic conductivity values measured at midday [1], coincident with the period of maximal flux, were 50% higher than at sunset. These data suggest that root membrane water channels are open during periods of high water flux and closed when water flux through the plant is reduced. When whole-plant transpiration and root water flux were studied simultaneously, the circadian pattern of water vapor loss from leaves closely resembled that of Jv, although the former was phase-shifted forward by approximately 4 hours. A model that relates root water channel activity and stomatal conductance in *Gleditsia triacanthos* is proposed.

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P 15

Passage of H_2O_2 through water channels: coupling with metabolism and effect of OH radicals

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A model is presented describing the permeation of hydrogen peroxide (H_2O_2) across a cell membrane and the implications of chemical degradation of H_2O_2 inside the cell due to the action of catalase. The model and its predictions have been verified in pressure probe experiments with internodal cells of *Chara corallina*. Series of osmotic pressure relaxation curves with different concentrations of H_2O_2 of up to 350 mol•m^{-3} are presented. A procedure is describted how to determine permeability (P_s) and reflection coefficients ($ó_s$) for H_2O_2 in the presence of the chemical reaction in the cell. Mean values were P_s = (3.6 " 1.0)•10^{-6} m•s^{-1} and $ó_s$ = (0.35 " 0.12) (" SD, N = 6 cells). Besides transport properties, coefficients for catalase reaction following a Michaelis-Menten type of kinetics were determined directly from the pressure relaxation curves. Mean values of the Michaelis constant (k_M) and maximum rate of decompositon (v_{max})) were k_M = (91 " 57) mol•m^{-3} and v_{max} = (45 " 36) nmol•(s•cell)$^{-1}$, respectively. The absolute value of P_s of H_2O_2 was high and about half of that of the diffusional permeability of water (P_d) measured with heavy water (HDO; Henzler and Steudle, 1995). This and the high value of $ó_s$ indicated that hydrogen peroxide – a molecule with chemical properties close to that of water - uses water channels to rapidly cross the cell membrane. Results suggest that some of the water channels in *Chara* (and, perhaps, in other species) serve as 'peroxoporins' rather than as 'aquaporins'.

Results also indicate that internodal cells of *Chara* could stand a short term treatment with fairly high concentrations of H_2O_2 up to 350 mol•m^{-3}. However, in the presence of agents that mediate the formation of hydroxyl radicals, even submillimolar concentrations of H_2O_2 resulted in a considerable decrease of the hydraulic conductivity of the plasma membrane. Upon treatment with hydroxyl radicals, cells showed anomalous osmosis during osmotic pressure relaxations with rapidly permeating test molecules. When radicals were removed from the medium effects were completely reversible. Since the aquaporin blocker $HgCl_2$ produced the same behaviour [1], this indicated that hydroxyl radicals affect the function of water channels. Effects of OH radicals were much more pronounced than those of $HgCl_2$ suggesting that OH radicals may reversibly affect a greater number of water channels than $HgCl_2$. It is shown that the use of OH radicals is a good tool for a functional analysis of water channels in plants.

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Expression, location and function of a plasma membrane aquaporin from Tobacco

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An aquaporin with a high similarity to the plasma membrane located PIP1 protein family was isolated from Tobacco and characterized with respect to gene expression, cellular and tissue location. The protein-function was analyzed in the heterologous *Xenopus*-Oocyte expression system and by physiological studies of transgenic tobacco plants expressing the respective antisense-RNA.

The described aquaporin is permeable for water and small solutes like glycerol and urea as confirmed by heterologous expression in oocytes. The aquaporin induced water permeability is not sensitive to heavy metals.

The corresponding gene is expressed in all parts of the plants, although to different extends. Results from a promoter analysis by transient transformation into Tobacco protoplasts suggested a gene-regulation by specific phytohormones.

The aquaporin protein could be identified by immuno-localization in plant tissues contributing to water transport from symplast to apoplast or *vice versa*. In addition, the specific localization pattern in leaf petioles indicated for a transcellular pathway of water flow.

Membrane dissection and western blotting revealed a localization in the plasma membrane. Electron microscopy as well as analysis of chloroplast membranes and antibody detection provided evidence for the occurrence of an aquaporin in specific membranes of the chloroplast. To our knowledge this is the first indication for an aquaporin in photosynthetic active cell-organelles.

In order to study the aquaporin function in plants an antisense construct was used to transform Tobacco. The resulting reduction of aquaporin RNA-levels was confirmed by quantitative RT-PCR and Northern analysis. Anti-aquaporin plants show an altered phenotype and differences in some physiological parameters like root or shoot water conductance. The studies strongly indicate for a function of aquaporins in plants.

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Ammonia and water permeation across the peribacteroid membrane of legumes

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Reduced nitrogen released by symbiotic N_2-fixing bacteria is an important nitrogen source in legumes [1]. In soybean root nodules nitrogen fixed by *Rhizobium* bacteria has to pass the peribacteroid membrane (PBM) to be assimilated in the plant cytoplasm. Whereas ammonium, as a charged molecule (NH_4^+), is supposed to enter the cytoplasm through specialised Ca^{2+}- and Mg^{2+}-regulated channels [2], ammonia (NH_3), as a small uncharged molecule, has been assumed to simply diffuse through the lipid phase of the membrane. Nodulin 26, a prevalent MIP in the PBM that is only expressed when the bacteria infect a root cell, is also a member of the aquaporin family. NOD26 confers increased water permeability [3], but is also permeable to small molecules like formamide and glycerol [3]. It has been suggested that the main function of NOD26 in the symbiosome membrane is that of osmoregulation [4], the feature of its' glycerol-permeability being less understood. If NH_3 uses the same pathway as other small solutes (H_2O, glycerol) then it could potentially permeate via NOD 26 and display features of those transports.

Ammonia permeability of the PBM from N_2-fixing soybean nodules was measured (8×10^{-5} m s^{-1}) using isolated PBM in a stopped-flow spectrofluorimeter. Ammonia (NH_3) uptake into PBM vesicles was inhibited by up to 42% by $HgCl_2$ (EC_{50} = 2.9 ìM, mercaptoethanol-reversible) and reduced by ATP-pre-incubation. The activation energy of NH_3 uptake (52 kJ mol^{-1}) increased (118 kJ mol^{-1}) with $HgCl_2$. Water transport was also $HgCl_2$-sensitive (EC_{50} = 52.6 ìM), but increased by ATP pre-incubation.

In conclusion NH_3-permeation is partially protein-mediated in native PBM-vesicles. The H_2O (osmotic) and NH_3 permeabilities were both inhibited by mercury and modulated by ATP pre-incubation. However, P_{NH3} was ten times more sensitive to mercury inhibition than P_{OS}, and P_{NH3} was reduced by ATP pre-incubation, whereas P_{OS} increased after ATP pre-incubation. A possibility to be discussed is whether NH_3, glycerol and H_2O permeate via different pathways through the NOD26-tetramer aggregates.

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Role of aquaporins during elongation growth of castor bean seedlings

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The functional identification of membrane intrinsic proteins (MIPs) as transmembrane water channels has immediately led to new models in plant water relations. Varying hydraulic permeabilities of different cell membranes are now attributed to the abundance and/or activity of aquaporins in these membranes, at least to a certain extent. However, direct measurements of aquaporin activity in plant cells is difficult and a direct genetic approach is time consuming, if available at all. Moreover, the MIP gene family seems to be larger in plants compared to animals, yeasts or bacteria (more than 30 MIP genes known to date with the majority shown to be expressed). Therefore even a knock-out plant will not necessarily produce a phenotype and thus make the detailed investigation of a specific aquaporin difficult. What is then the function of aquaporins when we can't see a effect?

Since it is so difficult to find out the physiological function of specific plant aquaporins, we decided to go the other way: to start from a defined physiological process and to look which aquaporins are functionally involved in this process. This approach should help us identify those aquaporins (and aquaporin genes) which then should be investigated in detail.

We decided to look at the hypocotyl elongation growth of castor bean (*Ricinus communis* L.) seedlings, whose function is to expose the first leaves of a plant emerging from the seeds towards the light (scotomorphogenesis). The major advantage of this tissue is that each cell of the hypocotyl originates from a cell division zone in the apical part of the hypocotyl directly below the cotyledons. These small cells are first filled-up with osmotically inactive starch granules. Within the cell elongation zone at the hypocotyl hook the starch is degraded to hexoses and additional assimilates (mainly sucrose) are supplied via the phloem; this increase in osmotic value together with cell wall loosening results in water uptake and thus in cell elongation and hypocotyl growth. Below that zone are fully expanded cells which will not further grow or take up nutrients or water besides what they need for basic processes (respiration etc.). Of experimental advantage is the size and germination time of the seedling and the accessibility of phloem and xylem sap.

Since hypocotyl cells originate from the cell division zone, go though the elongation zone to become fully elongated hypocotyl cells, the hypocotyl axis represents a time scale of cell differentiation. We are interested how the onset (elongating cells) and offset (elongated cells) of cell growth (water uptake) correlates with the expression rate of several aquaporin genes. The results indicate that the expression of certain aquaporin genes is indeed up-regulated in the elongation zone and down-regulated again when cells became fully elongated. In order to confirm that aquaporin gene expression really leads to different hydraulic conductivities of the membranes, we measured the water transport rates of vacuolar membranes isolated from different regions along the hypocotyl axis. Again, we found a transient increase in hydraulic conductivity of these membranes in the elongation zone.
Illumination stops hypocotyl cell elongation (photomorphogenesis) and, therefore, water uptake and could serve as an endogenous switch to shut-off water uptake into the cells of the elongation zone either directly or indirectly. MIP gene expression analysis showed that the amount of putative aquaporin mRNA is slightly reduced in the former elongation zone but strongly induced in the now rapidly growing apical part of the hypocotyl. This again indicates a direct involvement of aquaporin even in processes where the amount of water flow is quite low as it is the case in cell elongation which takes several hours to complete.

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EphB2 guides axons at the midline and is necessary for normal vestibular function

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Mice lacking the EphB2 receptor tyrosine kinase display a cell-autonomous, strain-specific circling behavior that is associated with vestibular phenotypes. In mutant embryos, the contralateral inner ear efferent growth cones exhibit inappropriate pathway selection at the midline, while in mutant adults, the endolymph-filled lumen of the semicircular canals is severely reduced. EphB2 is expressed in the endolymph-producing dark cells in the inner ear epithelium, and these cells show ultrastructural defects in the mutants. A molecular link to fluid regulation is provided by demonstrating that PDZ domain-containing proteins that bind the C termini of EphB2 and B-ephrins can also rcognize the cytoplasmic tails of anion exchangers and aquaporins. This suggests EphB2 may regulate ionic homeostasis and endolymph fluid productions through macromolecular associations with membrane channels that transport chloride, bicarbonate, and water.

Microbial MIP channels

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Most microorganisms studied so far, for instance by systematic genome sequencing, possess MIP channels. Commonly, bacteria have either none or up to four MIP channels, glycerol facilitators and/or water channels. Glycerol facilitators appear to mediate uptake of glycerol and related compounds for catabolism as carbon source and glycerol uptake is coupled to phosphorylation. The identification of water channels in bacteria was an unexpected finding, since the high permeability of the plasma membrane and the high surface to volume ration suggested that water flow would not require aquaporins. Two presentations in this session address the role water channels in bacteria.

Also fungal organisms have MIP channels. The yeast *Saccharomyces cerevisiae* has two putative highly similar water channels and two related glycerol facilitators. Expression analysis indicates that the two aquaporins, though highly similar, may have distinct physiological roles. Since most laboratory strains have inactivating mutations in both genes, the aquaporins are not essential for yeast. Two presentations will discuss the yeast aquaporins.

Fps1p is a well-characterised yeast glycerol facilitator. It does not appear to play a role in glycerol uptake but rather in export during osmoregulation. In fact, Fps1p is a regulated osmolyte exporter and its proper function is central to yeast adaptation to high as well as to low osmolarity. Further involvement of Fps1p in processes such as cell fusion during mating hints to the complex involvement of osmoregulation in cellular behaviour. Fps1p is used in our laboratory as a tool to manipulate glycerol accumulation and to study signalling events in yeast osmoadaptation. The function and regulation of Fps1p is subject of different presentations at this meeting. The physiological role of the second yeast glycerol facilitator, for which homologues exist in other fungi, is still unknown.

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Biological properties of the microbial *Escherichia coli* Aquaporin-Z water channel

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Transport of water across cell membranes is a fundamental process of life of all living organisms. However, the molecular pathway of the osmotic water movement across plasma membranes remained unknown until recognition of the aquaporin water channels, proteins belonging to the MIP family of transmembrane channels. A number of distinct aquaporins have been found in higher organisms where they have been showed to play essential physiological roles. Consistent with the fact that in their natural habitats microbial cells are often exposed to changes in environmental water activity, aquaporins were recently found also in microorganisms. The bacterial *Escherichia coli* Aquaporin-Z water channel (AqpZ) was the first known microbial aquaporin (1). In the intervening time, 27 additional aquaporins have been already identified and partially characterized both among eukaryotic and prokaryotic microorganisms; many more await yet to be discoved. Due to their structural and biological features as well as to their potential applications as model systems, a growing interest is characterizing microbial aquaporins. Considerable information is already available on the *E. coli* AqpZ. The *E. coli aqpZ* gene has a monocistronic nature, a structural feature shared with other known bacterial aquaporin genes. As showed by heterologous expression in *Xenopus laevis* oocytes (1) and functional reconstitution in proteoliposomes (Borgnia *et al.*, 1999), the AqpZ channel is selectively permeable to water by excluding small uncharged solutes such as glycerol, sorbitol and urea and lacking apparent ionic conductances. Borgnia *et al.* (1999) devised a homologous expression system to produce milligram quantities of the AqpZ protein which was then used to describe the strikingly stable homotetrameric organization of AqpZ in its native state. Electron crystallography and atomic force microscopy studies performed by Ringler *et al.* (1999) and Scheuring *et al.* (1999), respectively, confirmed the homotetrameric structure and demonstrated experimentally the six transmembrane domains topology of the AqpZ monomer, a structural feature shared with the high eukaryotics aquaporins. As usual, the two typical Asn-Pro-Ala (NPA) aquaporin signature motifs of AqpZ were located in loops B and E that have been shown to fold back into the membrane to form a highly specific channel (hourglass model). The *E. coli aqpZ* gene is maximally expressed during the exponential phase of growth and is osmotically regulated because its transcription rate is increased under steady-state hypoosmotic conditions and strikingly reduced in hyperoosmotic environments (2). Disruption of the *aqpZ* gene indicated a direct role for AqpZ in osmoregulation. AqpZ was showed to mediate the rapid osmotic fluxes of water across the cytoplasmic membrane triggered by sudden shifts of the environmental osmolality (3) and to participate in the adaptive response of *E. coli* to prolonged hypoosmotic stress (2). Requirement for AqpZ by rapidly growing cells was also observed. Interestingly, deletion of an *aqpZ*-like coding region (ORF10S) carried by the pathogenetic *Shigella sonnei* eliminated the form I antigen and conseguently the bacterial virulence (Houng and Venkatesan, 1998). It is anticipated that use of AqpZ and, more in general, of microbial aquaporins as model systems will continue to provide information useful for defining the biophysical, structural, biochemical, evolutionary and physiological features of aquaporins and other MIP family proteins.

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Study of fast water movements in bacteria by cryoelectron microscopy

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Movement of water into or out of cells is a fundamental process of life found throughout nature. In bacteria, the osmotic movement of water across the cytoplasmic membrane is one of the mechanisms triggered to maintain the cell turgor, a function essential for growth and survival. The first known prokaryotic aquaporin water channel gene, *aqpZ*, have been reported in *Escherichia coli* [1] and functionally characterized [2, 3]. Several *Mip* genes have been subsequently identified in other bacterial species. This indicates that, in spite of the high surface-to-volume ratio characterizing bacteria, the simple diffusion of water through the membrane lipids could not be always sufficient to preserve the turgor.

In order to functionally characterize new aquaporins in bacteria, we have studied water transport properties of bacterial MIPs, by cryoelectron microscopy, using *E. coli* as a heterologous expression system. Cryoelectron microscopy enables biological specimens to be imaged under physiological conditions and at different stage of a biological process [4]. Thus, this technique makes it possible to perform time-resolved studies. We have monitored the response to an osmotic shock of an AqpZ-deficient *E. coli* strain containing or not an exogenous *Mip* gene. Using this approach, new bacterial aquaporins have been functionally characterized in *E.coli*. These results provide new insight into the bacterial osmoregulation and define a suitable model to study the physiology of procaryotic water transport.

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Molecular Cloning and Characterization of AqpX a Water Channel from *Brucella abortus*

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The genus *Brucella* is formed by different species of Gram negative bacteria able to infect animals and humans. They have the ability to survive and multiply in macrophages. The objective of this work was to look for putative MIP proteins in *Brucella* in order to study their functionality and possible implication in the pathogenic mechanisms of these bacteria.

We used degenerate primers designed from the highly conserved NPA regions of the MIP proteins to amplify genomic DNA from the *Brucella abortus* strain 2308. Several amplification fragments were cloned and sequenced. In one of the fragments we found a gene, that was called *aqpX*, homologous to the *aqp*Z gene from *E. coli*. The *aqp*X from *B. abortus* contained a 687 base pair open reading frame encoding a polypeptide 28-38 % identical to known aquaporins. The AqpX protein has short NH_2 and COOH termini, as observed in *E. coli* AqpZ.

Expression of *B. abortus* AqpX after cRNA injection in *Xenopus* oocytes increased the osmotic water permeability of the oocytes, indicating that the protein facilitates water transport through the oocyte membrane. Transport experiments using ^{14}C -glycerol and complementation studies with *E. coli* mutants showed that AqpX is unable to transport glycerol.

We used allelic replacement to construct an *aqpX* null mutant from the *B. abortus* strain 2308. The mutant showed a marked decrease of survival in hypertonic medium. Macrophage infection assays using this mutant did not revealed any effect mon intracellular survival of *B. abortus.*

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Aquaporin water channels in *Saccharomyces cerevisiae*: function and expression studies

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Aquaporin water channels facilitate the transmembrane diffusion of water in many organisms. The genome of the yeast *Saccharomyces cerevisiae* contains two highly similar aquaporin genes, *AQY1* and *AQY2*. *AQY1* has been shown to encode a functional water channel but only in certain laboratory strains [1, 2]. The *AQY2* gene is interrupted by an 11-bp deletion in most of the laboratory strains with the exception of strains from the Ó1278b background, which also exhibit a functional Aqy1p [3]. However, although the *AQY2* gene from Ó1278b is highly homologous to functional aquaporins we did not observe Aqy2p-mediated water transport in *Xenopus* oocytes. A survey of 52 yeast strains revealed that all industrial and wild yeasts carry the allele encoding a functional Aqy1p while none of these strains appear to have a functional Aqy2p. We conclude that natural and industrial conditions provide selective pressure to maintain *AQY1* but apparently not *AQY2*. Although phenotypic analysis of aquaporin deletion mutants did not permit to assign any physiological role to aquaporins in yeast, first experiments on gene expression seems to implicate Aqy1p in sporulation events and Aqy2p in osmoregulation.

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Functional Analysis of a Second Aquaporin Homolog from *Saccharomyces cerevisiae*

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The *S. cerevisiae* genome database contains two open reading frames with homology to aquaporins. One of the open reading frames, *AQY1*, has been shown to be a functional aquaporin in some strains. *AQY2*, the second open reading frame, is disrupted by a stop codon in most strains. However, *AQY2* from strain Ó1278b is an intact open reading frame that encodes a polypeptide 87% identical to Aqy1p. When Ó1278b Aqy2p-expressing *Xenopus* oocytes were tested, the P_f of the oocytes was not different from control. Indirect immunofluorescence of Ó1278b Aqy2p-expressing oocytes revealed that the polypeptide does not traffic to the plasma membrane. *AQY2* was sequenced from other strains of yeast to search for different alleles of *AQY2*. Of twelve strains examined, only one strain contained an *AQY2* sequence that functioned as a water channel in oocytes. Despite the identification of a functional Aqy2p the physiological role in yeast remains unclear.

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Function and regulation of the yeast MIP glycerol export channel Fps1p

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Yeast cells encounter drastic and very rapid changes in osmolarity of the surrounding growth medium. The eukaryotic model organism *Saccharomyces cerevisiae* (baker's yeast) adapts to such changes by altering the intracellular concentration of the compatible solute glycerol. Control of the intracellular glycerol content is exerted at the level of production and retention. We have recently demonstrated that glycerol retention is mediated by the MIP glycerol export channel Fps1p.

The transport activity of Fps1p is rapidly regulated by altered osmolarity. Fps1p rapidly closes after an increase in external osmolarity, thereby enabling the cells to accumulate glycerol. Fps1p rapidly opens again upon a drop in osmolarity of the growth medium allowing glycerol export. Hence, Fps1p is a gated channel. Mutants lacking Fps1p are unable to rapidly release glycerol and are sensitive to a hypo-osmotic shock, illustrating the importance of Fps1p-mediated glycerol export after a decrease in external osmolarity.

While none of the known yeast osmosensing signalling pathways seems to be involved in controlling the opening and closing of Fps1p, a short domain within the N-terminal extension of Fps1p is crucial for channel closure. This regulatory domain is rich in proline residues and is located close to the first transmembrane domain (TMD1). Deletion of this sequence, or mutation of specific residues within the regulatory domain, results in a constitutively open channel. Strains expressing a constitutively open Fps1p channel permanently lose glycerol and grow slowly in high osmolarity medium, illustrating the importance of glycerol retention under these conditions. Furthermore, the precise position of the regulatory domain appears to be essential for channel closure, since moving it somewhat closer to or further away from TMD1 results in loss of channel regulation.

In addition to targeted mutagenesis within the regulatory domain, we have also isolated mutants that suppress the osmosensitivity of a strain expressing a constitutively open Fps1p channel. Some of these intragenic mutations are located on the predicted extracellular face of Fps1p and exert the suppression effect apparently by reducing the glycerol export capacity of the channel. Identification and analysis of more suppressors is underway.

By combining yeast genetics and biochemical analysis, we aim at understanding the molecular mechanisms of osmoregulated gating of this MIP member.

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Characterization of the first microbial mixed MIP : Gla of *Lactococcus lactis*

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No MIP protein has yet been functionally characterized in gram-positive bacteria [1]. We have studied glycerol and water transport properties of a *Lactococcus lactis* MIP, in two models : *Xenopus* oocytes and *E. coli*. This MIP is permeable to glycerol, like *E. coli* GlpF, and also mediates significantly water fluxes. This is the first characterization of a microbial MIP protein presenting a mixed function. Furthermore, using freeze-fracture electron microscopy [2], we demonstrate that *L. lactis* MIP expressed in *Xenopus* oocytes presents 3 oligomeric states. In addition our findings bring new evidences to demonstrate that, in MIP proteins, transport specificity is correlated to their oligomerization states in the membrane [3 and 4] and that a mixed function is the result of a dynamic equilibrium between monomers (glycerol transport), dimers, tetramers (water transport).

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P 23

Molecular cloning and functional expression of a water channel - aquaporin gene in *Plasmodium falciparum*

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Comparative Blast searches in databases of the Malaria Genome project of Plasmodium falciparum revealed an open reading frame of 774 bp which has considerable sequence similarity (32.1%) to a known glycerol-uptake facilitator protein from E. coli (GenBank acc. # P11244). Therefore, this Plasmodium gene likely codes for a member of the aquaporin – water channel family and has been preliminarily designated as PfAQP1. Aquaporins are characterized by a topology consisting of six transmembrane spans and a duplicated, extremely conserved NPA tripeptide motif which is central in the formation of the actual water pore. The predicted protein sequence of the Plasmodium gene has six putative transmembrane regions. However, the canonical, tandemly arranged NPA motifs of the aquaporins are changed to NLA and NPS, respectively. This is similar to a known glycerol pore from *Saccharomyces cerevisiae* which has an inverted arrangement, i.e. NPS and NLA at these conserved positions (1,2). We used specific primers and genomic DNA from Plasmodium as a template and obtained a 792 bp PCR product. The coding DNA sequence of 774 bp has an A/T content of 71% and was intronless as far as we could predict by visual inspection. It coded for a protein of 258 amino acids and was identical to the translated reading frame identified in the Malaria Genome Project. The DNA was cloned into pOG1, a vector designed for cRNA-transcription. Upon injection of this cRNA into oocytes from Xenopus laevis, water permeability was greatly increased and oocytes ruptured shortly after application of a hypoosmotic stress. Control injected oocytes (DEPC-water) remained unaffected by these conditions. Thus we functionally established the presence of a water channel protein in the malaria parasite Plasmodium falciparum. Currently, our studies focus on the functional consequences possibly caused by the highly unusal NLA/NPS motifs.

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The trafficking of native and mutant mammalian MIP proteins

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Several *in vivo* and *in vitro* studies on different mammalian aquaporins have revealed that their subcellular localization and regulation of trafficking is cell-type dependent and protein specific, implicating that targeting motifs are interpreted differently by distinct cell-types. Aquaporin-2, which is the best studied water channel, is essential for vasopressin (AVP)-regulated urine concentration. AQP2 is predominantly expressed in intracellular vesicles under basal conditions and is re-distributed to the apical membrane of collecting duct cells through activation of a cAMP signalling cascade initiated by binding of AVP to its V2-receptor. AQP2 trafficking has been reconsituted in several cultured cell models, and these have provided important information on trafficking pathways and targeting signals for this protein. For example, phosphorylation of AQP2 at S256 by protein kinase A is essential for its redistribution to the plasma membrane, but does not not seem to alter the single channel water permeability of AQP2. SNARE proteins, dynein, microtubules and an intact cytoskeleton may also be important for AQP2 trafficking, but confirmation of their functional involvement is lacking in most cases. Mutations in the AQP2 gene result in autosomal recessive and dominant nephrogenic diabetes insipidus (NDI), a disease in which the kidney is unable to concentrate urine in response to AVP. AQP2 missense mutants in recessive NDI are usually retained in the endoplasmic reticulum. An AQP2 mutant in dominant NDI, however, is retained in the Golgi complex. The ability of this latter mutant to heterotetramerize with wild-type AQP2, thereby impairing its further routing to the plasma membrane, provides an explanation for dominant NDI.

Regulation of aquaporin-2 water channel trafficking by phosphorylation: lessons from transfected epithelial cells

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The short term regulation of collecting duct water permeability by vasopressin is mainly a consequence of regulated trafficking of the aquaporin 2 (AQP2) water channel back and forth the apical membrane of collecting duct principal cells. Many progress in our understanding of the cellular biology of AQP2 recycling came from the establishment of cell colture models in which the regulated pathway of AQP2 insertion into the plasma membrane is retained. In cultured renal CD8 cells transfected with AQP2 vasopressin stimulation caused AQP2 redistribution from an intracellular site to the apical membrane increasing the water permeability coefficient (P_f) by approximately 6-fold [1,2]. The water channel AQP2 has a potential phosphorylation site (ser-256) for cAMP-dependent protein kinase (PKA). PKA-mediated phosphorylation of AQP2 has been proposed to be essential in regulating AQP2-containing vesicle exocytosis. Recent finding from our laboratory however, demonstrate that in CD8 treated with the phosphatase inhibitor okadaic acid (OA), phosphorylation of AQP2 is not required for the translocation of AQP2. OA induces a full vasopressin-like effect promoting AQP2 redistribution and 4-5 fold increase of the osmotic permeability coefficient P_f. Interestingly, OA treatment resulted in a slight (60%) increase in AQP2 phosphorylation which was abolished when this treatment was performed in the presence of 1ìM H89, *vs* the threefold increase induced by FK stimulation. The finding that OA increases water transport through AQP2 translocation independently from AQP2 phosphorylation, suggest that different intracellular pools of AQP2-bearing vesicles under different regulatory mechanisms might exist in renal collecting duct epithelial cells. This hypothesis was also sustained by the observation of the partial additive effect of OA and FK in increasing the P_f. Confocal laser microscopy analysis suggests that cytoskeletal elements might be involved in OA action. These finding suggest that the increased phosphorylation of unidentified protein/s distinct from AQP2 itself, is involved in the activation of AQP2 trafficking. *In vitro* phosphorylation experiments in CD8 cells revealed that, during AQP2 maturation in the Golgi apparatus, ser-256 is also a potential target for a Golgi-resident kinase, termed Golgi casein kinase (G-CK). AQP2, as well as a peptide reproducing the sequence around ser-256 is readily phosphorylated by purified G-CK. This suggests a potential role for G-CK in regulating constitutive AQP2 trafficking within the Golgi apparatus. Interestingly, an autosomal dominant form of inheritance NDI caused by a mutation of the Glu-258 in the AQP2, affects an aminoacid that is irrelevant to PKA targeting, while it is essential to make ser-256 a good target for G-CK. Previous studies have shown that when expressed in oocytes mutated AQP2 was retained in the Golgi. For this case of NDI in which the phenotype is caused by an impaired routing of AQP2, the possible involvement of the G-CK might give some hints. To clarify the physiological role of the G-CK in intact cells, wild type CD8 cells were transfected with E258K AQP2 mutant. Preliminary results demonstrate that mutated AQP2 was retained in a perinuclear region probably corresponding to the Golgi apparatus. We speculate that phosphorylation of AQP2 by G-CK might represent a regulatory signal for constitutive trafficking of AQP2 within the Golgi network, whereas phosphorylation of AQP2 by PKA controls the targeting to the apical membrane.

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cAMP-independent pathways of AQP2 membrane insertion: role of nitric oxide and cGMP in AQP2 trafficking

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It is known that cAMP-mediated phosphorylation of AQP2 is required for the accumulation of this water channel at the cell surface during vasopressin stimulation. However, the S256A residue on AQP2 is also a concensus site for protein kinase G (PKG) phosphorylation, and concensus sites for other kinases are also present within the cytoplasmic domains of AQP2. Thus, we sought to determine whether activation of other signal transduction pathways would lead to the cell surface insertion of AQP2. Three separate lines of investigation from our laboratory have together shown that the vasopressin-sensitive water channel AQP2 can traffic from intracellular vesicles to the plasma membrane of various epithelial cells in a cAMP and vasopressin-independent pathway.

1) LLC-PK1 epithelial cells stably transfected with AQP2 cDNA show an accumulation of AQP2 at the cell surface if the cells are first cooled to 20•C for 2 h, allowing AQP2 to accumulate in the trans-Golgi network (TGN), and then moved to 37•C for 10 mins. During the rewarming, "blocked" AQP2 is released from the TGN and rapidly appears at the cell surface. ELISA assays show that the intracellular cAMP levels remain at baseline levels during this time. Thus, the cell-surface appearance of AQP2 during this temperature-shift protocol is cAMP independent.

2) We next examined AQP2 in the rat vas deferens. This tissue has a high level of AQP2 on the apical plasma membrane of epithelial cells lining the duct lumen, even in Brattleboro rats that lack vasopressin, and few intracellular vesicles are present. Using FITC-dextran as an endocytotic marker, we showed that AQP2 is not extensively recycled in this tissue, and AQP2 was apically-located under all conditions tested. Microtubule disruption, which preferentially disrupts pathways involving rapidly-recycling membrane proteins (including AQP2 in the kidney), did not result in redistribution of AQP2 into intracellular vesicles, although an increased amount of basolateral membrane staining was observed 8 h after colchicine administration to rats. The results indicate that AQP2 is a constitutive, apical membrane protein in the vas deferens.

3) We have now shown that both nitric oxide (sodium nitroprusside - SNP) and L-arginine treatment cause extensive insertion of AQP2 into the plasma membrane both in collecting ducts *in vitro*, and in AQP2-transfected LLC-PK1 cells in culture. The magnitude of the response is quantitatively similar to that caused by vasopressin and/or forskolin. Both the SNP and the vasopressin effect are blocked by the kinase inhibitor, H-89, and neither SNP nor vasopressin stimulated the membrane insertion of AQP2 in cells expressing a phosphorylation-deficient S256A mutation of the AQP2 protein. In cultured LLC-PK1 cells, SNP caused a large increase in cytosolic cGMP levels, but cAMP remained at baseline levels. In the same transfected cells, cGMP alone stimulated AQP2 membrane insertion. An in vitro phosphorylation assay using a fusion protein comprised of GST and the entire C-terminal cytoplasmic tail of AQP2 showed that both PKA and PKG were capable of phosphorylating the AQP2 C-terminus. In cultured LLC-PK1 cells, atrial natriuretic peptide (ANP) caused a striking relocation of AQP2 from vesicles to the plasma membrane, indicating the presence of the full signaling cascade for ANP that results in PKG activation in this cell type. We conclude that AQP2 membrane insertion can occur in a cAMP-independent manner, and that membrane insertion of AQP2 can be mediated via the cGMP pathway. The role of other potential phosphorylation sites in AQP2 trafficking is under investigation in our laboratory.

Identification of protein kinase A anchoring proteins potentially involved in AQP2 translocation and the role of the small GTPases of the Rho family in the translocation

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The antidiuretic hormone arginine-vasopressin (AVP) regulates water reabsorption in inner medullary collecting duct (IMCD) principal cells by inducing the translocation of water channels (AQP2) from intracellular vesicles into the apical cell membranes. AVP initiates the activation of protein kinase A (PKA) by stimulation of the G_s/adenylyl cyclase system. Using a recently established primary culture model of rat IMCD cells [1] it was shown that the tethering of PKA by protein kinase A anchoring proteins (AKAPs [2]) is a prerequisite for the translocation of AQP2 [3]. In subcellular fractions from renal inner medulla and primary cultured IMCD cells enriched for AQP2-bearing vesicles various AKAPs were detected. One of the AKAPs identified by a combination of 2D electrophoresis, RII overlay technique and mass spectrometry was moesin of which the functional involvement in AQP2 translocation is currently being investigated. In a second approach cDNA expression library screening with PKA RII subunits led to the isolation of two clones which represent the rat homologues of AKAP18 and Ht31. Northern blot analysis demonstrated the presence of at least two different splice variants of each AKAP all with stronger expression in renal inner medulla, the main site of AQP2 expression, than in residual kidney tissue. An antiserum was raised against a peptide derived from an AKAP18 sequence which is common to all splice variants. Subsequent Western blot and immunofluorescence microscopic analysis of primary cultured IMCD cells with affinity-purified antibodies from this serum showed that AKAP18 variants are localized intracellularly and at the cell membrane. Immunofluorescence microscopic studies of sections of renal inner medulla demonstrated that these variants (as AQP2) are expressed mainly in cells lining the collecting ducts (most likely principal cells). Taken together these data indicate a possible involvement of AKAP18 in AQP2 translocation.

The involvement of the small GTPases of the Rho family (Rho, Rac and Cdc42) was investigated by incubation of primary IMCD cells with bacterial toxins. *C. difficile* toxin B inhibits all small G proteins of the Rho family while cytotoxic necrotizing factor I derived from pathogenic *E. coli* activates Rho, Rac and Cdc42. Both toxins - to varying degrees - induced translocation of AQP2 in the absence of AVP, which is still active after toxin treatment. These data indicate the functional involvement of Rho proteins in AQP2 translocation. They also point to the presence of different pools of vesicles which may depend on different signals for their release, one pool may dependent on activation of Rho proteins while another requires inactivation of these proteins. Identification of the responsible Rho family member is attempted by the use of dominant negative or constitutively active mutants of these GTPases.

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Complex Regulation of AQP5 Expression by Osmotic Stress

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Aquaporin-5 (AQP5) is a water channel protein expressed in the apical membrane of type I pneumocytes and submucosal glands of the respiratory tract, salivary and lacrimal gland epithelia, and corneal epithelium. Each of these sites may experience fluctuations in extracellular osmolarity. To date, investigation of cellular responses to osmotic stress has focused on regulation of organic osmolyte transporters. In this study, we demonstrate that AQP5 induction by hypertonic stress in a cultured mouse lung epithelial cell line (MLE-15) is mediated by extracellular signal-regulated kinase (ERK). Following incubation of cells with medium containing 200 mosM sorbitol, AQP5 protein increased by 8 h, with maximum induction of 4-5 fold at 24 h. This induction was dose-dependent and required the presence of relatively impermeable solutes (NaCl, sucrose, sorbitol). Two distinct agents which block ERK activation (U0126 and PD98059) blocked induction of AQP5 mRNA and protein in response to hypertonic stress. Exposure of cells to TPA activated ERK but did not induce AQP5, suggesting that other signaling steps are required. Additionally, incubating cells with a PKC inhibitor (Ro-31-8425) had no effect on either ERK activation by hypertonic stress or AQP5 induction, indicating that PKC is not involved in this signaling pathway. Studies in hyperosmolar rats demonstrated that AQP5 protein increased 2-7 fold in lung, lacrimal gland, and submandibular gland, suggesting this induction is not an isolated *in vitro* phenomenon. This is the first demonstration of osmotic induction of an extra-renal aquaporin as well as the first association between MAP kinase signaling and aquaporin expression. Dynamic regulation of AQP5 may prove integral to regulated secretion of water in the respiratory tract and may play a critical role in maintaining both lung fluid tonicity and alveolar cell volume. These studies were funded by the National Institutes of Health (PA, LK) and the Cystic Fibrosis Foundation (PA, LK).

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Vasopressin release, water channels, and vasopressin antagonism in cardiac failure, cirrhosis, and pregnancy

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Vasopressin (AVP) is released in response to both osmotic and nonosmotic stimuli. Nonosmotic-stimulated AVP release occurs in cardiac failure, cirrhosis, and pregnancy in response to alterations in arterial circulatory integrity. Cardiac failure in rats is associated with increased plasma AVP and hypothalmic AVP mRNA, and in humans, it is associated with cardiac failure. Plasma AVP concentrations are elevated when measured with a sensitive radioimmunoassay. Urinary concentrations of AVP-responsive aquaporin-2 water channels are also elevated in cardiac failure. V2 receptor antagonists correct the impaired solute-free water excretion seen in rats with low-output cardiac failure and reverse the upregulation of renal aquaporin-2 water channels. Orally active non-peptide-selective V2 receptor antagonists administered to patients with congestive cardiac failure decrease urinary concentrations of aquaporin-2, increase solute-free water clearance, and correct the hyponatremia. Cirrhosis of the liver results in splanchnic arterial vasodilation and increased vascular capacity, most likely secondary to increased nitric oxide production. This relative underfilling of the arterial circulation stimulates nonosmotic AVP release with resultant water retention. Aquaporin-2 gene expression is upregulated in the kidneys of rats with cirrhosis of the liver. AVP-2 receptor antagonists administered to animals with cirrhosis reverse the water retention. Human studies using orally active, non-peptide-selective V2 receptor antagonists in patients with cirrhosis are currently underway. Pregnancy is another state of nitric oxide-mediated arterial vasodilation that is associated with plasma AVP concentrations that are relatively high for the degree of hypoosmolality. Upregulation of the water channel aquaporin-2 in the renal papillae of pregnant rats has also been demonstrated, and this effect is reversed by administration of a V2 receptor antagonist.

Water transport in peritoneal dialysis

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In peritoneal dialysis (PD) 2 litres of a sterile dialysis fluid (essentially a lactated Ringer's solution containing glucose as osmotic agent) is repeatedly exchanged with the peritoneal cavity via a chronically implanted abdominal catheter. Exchanging 8-10 litres of fluid per day will result in adequate removal of uremic toxins and water. Water removal occurs by glucose induced crystalloid osmosis. The exchanges across the peritoneal membrane can be described according to the "three-pore model" of peritoneal transport. The major exchange pathway for small solutes and water is represented by the clefts between endothelial cells, commonly denoted "small pores", having a functional radius of 40-50 Å. Macromolecules mainly reach the peritoneal cavity via large pores of radius 250-300 Å. In addition, we postulated the presence of transcellular, "water-only", pores, to account for the marked sieving of small solutes (such as Na) occurring during glucose induced osmosis. Assuming that only 2 % of the total peritoneal ultrafiltration coefficient (L_pS) is accounted for by such transcellular pores, nearly one-half of the water flow can be calculated to occur through these pathways during peritoneal dialysis when hypertonic (glucose) solutions are used.

Aquaporin-1 (AQP-1) is likely candidate for the "transcellular", water-only pathway in PD. In collaboration with Søren Nielsen it was possible to immunolocalise AQP-1 to the capillary endothelium of the microvessels in the peritoneal membrane. To further evaluate the role of AQP-1, we exposed the peritoneal cavity of anaesthetised Wistar rats ($\sim$300 g) *in vivo* to increasing concentrations (0.1-1 mmol/l) of $HgCl_2$ for 10 minutes and then performed hypertonic dwells (3.86 % glucose solutions). In these experiments, however, we obtained a peritonitis-like reaction for $HgCl_2$ conc. exceeding 0.3 mmol/L, with marked increases in both small-solute and protein transfer across the peritoneum. Because of that, we had to introduce a modification of the protocol, including a very short-term and "gentle" pre-fixation of the peritoneal cavity using 10 mL of 1 % glutaraldehyde (in phosphate buffer) over 1.5 minutes. After the pre-fixation, one group of animals was exposed to 1 mmol/L $HgCl_2$ for 10 minutes, while another group was exposed to buffer only, without being exposed to $HgCl_2$. When pre-fixed rats were exposed to hypertonic dialysis fluid the sieving of Na was almost completely intact. However, when pre-fixed rats had been exposed to $HgCl_2$, the sieving of Na was more or less completely abolished. Furthermore, water transport, although slightly reduced by the pre-fixation alone, was reduced to nearly one-half by the combined pre-fixation and $HgCl_2$ treatment.

These results have recently been corroborated, as Verkman and his associates have been able to produce a transgenic knockout mouse, deficient in AQP-1. When performing PD-like dwells in these mice using hypertonic sucrose (+300 mmol/L) in saline, water transport during the first hour was reduced to one-half (to one-third) of that of normal mice. These data demonstrate the importance of AQP-1 in PD.

In long-term PD (lasting for more than 4 years), more than 30 % of the patients develop ultrafiltration failure (UF volume for 3.86 % glucose less than 400 mL in 4 hours), which is nearly always related to an increased transfer of small solutes (glucose), most likely due to an increased microvascular "surface area" developing over time. However, the expression of AQP-1 has *not* been shown to decrease during a long-term PD.

Accumulating evidence thus seems to support the three-pore model of peritoneal transport, which predicts that approximately one-half of the total peritoneal osmotic water transport occurs through water-only pathways in PD. We thus conclude that AQP-1 plays a major role in osmotic water removal during PD.

Aquaporin-3(AQP3) and Aquaporin-4(AQP4) protein expression in developing Aquaporin-1(AQP1) knockout mouse kidney

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Recent studies demonstrated impaired urinary concentrating ability in AQP1 knockout mice. We reported that AQP1 gene deletion is associated with an upregulation of AQP3 and a downregulation of AQP4 (JASN 1999:10:14A). It is known that newborn rodents are not capable of producing a concentrated urine and has a ability to concentrate urine during first 3 weeks after birth. The purpose of this study was to establish that compensatory changes of AQP3 and AQP4 are occur in neonatal mice with the lake of AQP1. Kidneys from 3-, 7-, and 15-day-old AQP1(+/+) and AQP1(-/-) mouse pups were preserved by in vivo perfusion with PLP and processed for immunohistochemistry using a preembedding peroxidase method with rabbit polyclonal antibodies to AQP3 and AQP4. In 3-, 7- and 15-day-old AQP(+/+) pups, both AQP3 and AQP4 were expressed on the basolateral plasma membrane of principal and IMCD cells throughout the collecting duct, with more AQP3 in the proximal segments of collecting duct and more AQP4 in IMCD. Distinct AQP4 immunostaining was observed in the S3 segment of proximal tubules from 15-day-old pups; however, the intensity of staining was less than in adults. In 3- and 7-day-old AQP(-/-) pups, no differences of mophology and immunoreactivity for AQP3 and AQP4 were observed. In 15-day-old AQP(-/-) pups, there was an increase in AQP3 immunostaining in principal cells of collecting duct. However, AQP4 immunostaining was slightly decreased in both S3 segment of proximal tubule and collecting duct, and there was a decrease in cell height and intensity of immunolabeling of the lateral cell membrane in IMCD cells. In summary, in the deveoping AQP(-/-) pups no distinct changes of AQP3 and AQP4 immunoreactivity was observed until 15 days after birth. These observations suggest that compensatory changes of AQP3 and AQP4 in AQP1 knockout mice appear around the time of having an concentrating ability of urine.

Perinatal increase in rat skin AQP 3 expression parallels maturation of skin barrier function and is influenced by antenatal corticosteroid treatment

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Background: Extremely preterm newborn infants have a high transepidermal water loss (TEWL) carrying a risk for hyperosmolar dehydration. We have speculated that AQP's might be involved in the process of water movement through the skin in the perinatal period. Antenatal corticosteroid treatment (ANS) is widely used in obstetric practice, has been shown to have effects in different tissues and has been shown to regulate AQP expression.

Aim: To investigate the perinatal development of skin AQP expression in relation to skin barrier function and to study the effect of ANS on AQP expression and skin barrier function.

Methods: Immunohistochemistry identified two AQP isoforms in rat skin (AQP1 and 3). AQP mRNA expression was investigated in embryonic (E) and postnatal (P) skin, using semi-quantitative RT-PCR with beta-action as internal control. Skin barrier function was measured as TEWL from the skin of rats of different ages. In a second series in preterm rats (E18), we studied AQP expression and TEWL after betamethasone treatment compared with controls.

Results: AQP expression and TEWL in relation to age: AQP1 mRNA was highest before birth (E18, E19 and P0) and then decreased slightly from P1 to adult. AQP3 decreased from E18 to P0 and then increased 3-fold from P0 through P1 and P12 to adult. Immunohistochemistry revealed AQP1 expression in dermal capillaries and AQP3 in basal cells of the epidermis.

TEWL was highest in E18 and then decreased dramatically with increasing gestational age (E20) and further with postnatal age (P4), the values at E18 being 10-fold higher than at P4 ($p < 0.001$).

Effect of ANS: AQP1 mRNA was not changed by ANS. However, ANS resulted in almost a 3-fold increase in AQP3 mRNA, similar to what was seen with spontaneous age-related maturation of the skin. TEWL was 30% lower after ANS than in control rats ($p < 0.001$).

Conclusion: In the perinatal period, there is an increase in skin AQP3 expression with increasing age. The increase parallels the maturation of epidermal barrier function. Antenatal corticosteroid treatment increases AQP3 expression and improves skin barrier function.

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Deficiency of membrane localization of aquaporin 5 in lacrimal glands of Sjögren's syndrome patients and model mice

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Purpose : Sjögren's syndrome (SS) is characterized by lymphocyte infiltration and apoptotic cell death, which leads to the defective tearing. The present study was undertaken to determine whether membrane presentation of aquaporin 5 (AQP5), a lacrimal gland (LG) water channel is related to the defective tear secretion in SS patients and SS-liked model mice. **Methods** : Lacrimal samples were obtained from SS, non-SS and Mikulicz's disease (MD) patients. The models were female MRL/*lpr*, MRL/*gld* and NOD mice at 5, 10 and 15 weeks old. The *lpr* and *gld* are mutations in Fas and Fas ligand. Control mice were female BALB/c and ICR mice at 5, 10 and 15 weeks old. LGs were immunostained with anti-AQP5 or anti-Na^+/K^+ ATPase and anti-Na^+ channel antibodies. **Results** : In human controls, AQP5 expression was localized in apical membrane of acinar and duct cells of LG. In SS patients which is lack of both basic and reflex tearing, LG showed severe lymphocyte infiltrations. Immunoreactivity of AQP5 was weak in apical membrane, and diffused in all cytoplasm. In contrast, membrane localization of AQP5 was observed in non-SS and MD patient which are maintained the reflex tearing, although LG of MD patients showed massive lymphocyte infiltrations. On the other hand, significant difference in immunolabeling of Na^+/K^+ ATPase and Na^+ channel were not observed in all patients. In the model mice, lymphocyte infiltrations in LG were progressed with age. In MRL/*lpr* and MRL/*gld* mice, there is no difference in immunolabeling of AQP5 throughout the observation period. These results are similar to that observed in non-SS and MD. In NOD mice, immunoreactivity of AQP5 was a tendency to disappear at the age of 15 weeks old. This results was similar to that observed in SS. The mean basic tear secretion in NOD mice was significantly decreased by the aging. These results suggested that the lack of membrane localization of AQP5 is related to the defective tear secretion in SS patients and NOD mice.

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Oxytocin: one of the factors for regulating AQP2 localization and urinary AQP2 excretion

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Oxytocin has been suggested as an antidiuretic hormone with some controversies. Aquaporin-2(AQP2), an essential water channel for urinary concentration, is mainly located in the apical plasma membrane of collecting duct epithelial cells, but there has been some evidences of a moderate amount of basolateral localization of AQP2 at least in the inner medullary collecting duct(IMCD). We hypothesized that oxytocin may play some role in vasopressin-independent urinary concentrating mechanisms by regulating AQP2 expression in the kidney.

First, we tested acute effect of exogenous oxytocin on AQP2 localization in the kidney using male Sprague-Dawley rats. Immunocytochemistry and semiquantitative immunoblot analysis were carried out using polyclonal peptide-derived antibodies to AQP2 after a single intraperitoneal injection of 10 units of oxytocin. We found that AQP2 localization was shifted from diffuse cytoplasmic predominance in control rats to both apical and basolateral membranes in oxytocin-treated rats along the connecting tubule and collecting duct. Semiquantitative immunoblotting from whole kidneys revealed a higher band density ratio of plasma membrane-rich fraction over cytoplasmic vesicle-rich fraction in oxytocin-treated rats than in controls. Next, we examined the effect of V_2 antagonist pretreatment on the oxytocin-induced redistribution of AQP2 protein in rat kidney. In V_2 antagonist pre-treated (20 mg/200 g BW/d for 3 days) rats, AQP2 labeling of basolateral plasma membrane was vanished and translocated to cytoplasm along the collecting duct. However, the apical staining of AQP2 was not affected by V_2 antagonist pre-treatment. Finally, we tested whether exogenous oxytocin affects urinary concentration in human and accompany changes in urinary AQP2 excretion. Intravenous oxytocin was administered at a rate of 20 mU/min over 2.5 hours in 10 healthy male volunteers(NC), 7 patients with central diabetes insipidus(CDI) and 3 patients with nephrogenic diabetes insipidus(NDI). Urine volume and free-water clearance decreased significantly with oxytocin administration in NC and CDI but not in NDI. Urinary AQP2 excretion, measured by Western blot analysis, increased significantly with oxytocin administration in NC and CDI but not in NDI.

In conclusion, localization of AQP2 protein is affected by short-term oxytocin administration in both rat and human. We are left with the question about physiologic significance of basolaterally targeted AQP2 water channel along the connecting tubule and collecting duct. Further studies are required to elucidate intracellular pathways from oxytocin binding to AQP2 targeting in the kidney.

Dysregulation of renal aquaporins in postobstructive polyuria

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Urinary tract obstruction is a common disorder seen in both children and adults, which, depending on the location and the degree of obstruction, may cause serious deterioration of renal function, including inability to produce a concentrated urine. Aquaporin-2 (AQP2) is the vasopressin regulated water channel of the kidney collecting duct, which has been shown to be critically involved in numerous pathophysiological conditions associated with nephrogenic diabetes insipidus. Release of bilateral ureteral obstruction (BUO) results in polyuria and impairment of urinary concentrating capacity. After release of unilateral ureteral obstruction (UUO) GFR is reduced and the fractional excretion of both sodium and water is increased. Consequently, both BUO and UUO is characterized by a markedly impairment in the ability of the postobstructed kidney to concentrate urine, which is resistant to vasopressin treatment. To examine whether this urinary concentrating defect was related to changes the expression of several renal aquaporins, we used a rat model in which both ureters were reversibly obstructed. After obstruction for 24 hours, AQP2 expression was reduced to 26"8%, supporting the view that diuresis per se is not the cause of the decrease in AQP2 levels. Following release of the obstruction, there is a marked polyuria, which initially is primarily osmotic. Immunoblotting demonstrated a significant downregulation in protein expression of all 3 AQP´s 5 hours after release of BUO: AQP2 (0.32"11 vs 100"4%), AQP3 (14"5% vs.100"14%), and AQP1 (7"2% vs.100"22%). Forty-eight hours after release all 3 AQP´s remained significantly downregulated: AQP2 (0.13"5 vs 100"1%), AQP3 (10"5% vs.100"1%), and AQP1 (24"5% vs.100"10%) concurrent with a persistant polyuria (104"10 vs. 30"3 ìl/min/kg, $p<0.05$), and a marked reduction in free water clearance (-64"11 vs -181"8 ìl/min/kg, $p<0.05$) suggesting a defect in the water reabsorptive capacity at the collecting duct level. The polyuria persists for several days after the plasma biochemistry has normalised associated with an increase in solute-free water clearance together with a persistant reduction in AQP2 expression until at 15 days after release obstruction (44"10% of control levels). Urinary concentrating capacity remained impaired until 30 days after release evidenced as an inability to increase urine osmolality in response to thirst (1773"218 vs. 2880" 81 mosm/kg) consistent with impairment of collecting duct water reabsorption. In parallel, AQP1 was persistently downregulated at 3 days (39 " 9%), 15 days (57 " 8%) and 30 days (59 " 5%) after release of BUO, suggesting that AQP1 may also play an important role for the postobstructive polyuria. Thus, the present study suggests that dysregulation of aquaporins located to the collecting duct and to the proximal tubule may important for the impaired urinary concentrating capacity following ureteral obstruction.

Studies of AQP3 oligomerization by sucrose gradients and cryofracture

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The proteins of the MIP family are membrane proteins that facilitate water (aquaporin) and/or solute (aquaglyceroporin/glycerol facilitator) transport across biological membranes. In order to determine the basis of the selectivity difference observed between these MIP proteins, we attempted to analyze the oligomerization of AQP3 in membranes. AQP3 expressed in native or heterologous membranes was solubilized in non-denaturing detergents and analyzed in sucrose gradients [1]. The comparison between the AQP1 and AQP3 apparent sedimentation coefficients was performed on human red blood cells solubilized in non-denaturing detergents (Triton X100, n-octyl â-D-glucopyranoside and n-lauroyl sarcosine). We confirmed the tetrameric form of AQP1 and we demonstrated that its quaternary structure is not altered by these detergents. On the contrary, AQP3 is present in much slighter gradient fractions that could not account for a tetrameric form but rather a monomeric and a dimeric one. These results suggest two possibilities: (1) AQP3 is present in monomeric and dimeric forms in membranes, (2) AQP3 resides as a tetramer in membranes, but is very sensitive to non-denaturing detergents. Freeze fracture electron microscopy was also used to compare the oligomeric structure of AQP1 and AQP3 expressed in plasma membrane of Xenopus oocyte according to Zampighi *et al.* [2]. We showed an increased intramembrane particle (IMP) density in the P-face of AQP1 and AQP3 expressing oocyte: 723 " 165 and 406 " 76 IMPs/Fm^2, respectively, versus 181 " 40 IMPs/Fm^2 for control oocyte, representing the count of 8000 to 10000 IMPs in each condition. The IMP size repartition in each case corresponded to a Gaussian curve with the mean at 8.4 " 1.6 nm (n = 597) for the control oocytes, 8.1 " 1.5 nm (n = 786) and 7.1 " 1.4 nm (n = 752) for oocyte expressing AQP1 and AQP3 respectively. The size difference between AQP1 and AQP3, as also observed in CHO cells [3], cannot be explained by the presence of AQP3 in a dimeric or monomeric form in the oocyte membrane. However, this result cannot exclude a different quaternary structure in native tissues (red blood cell or collecting duct principal cells). AQP3 structure studies, on reconstituted proteoliposomes, or on crystals by crystallography, would be necessary to determine whether different interactions between monomers (tetrameric hypothesis) can explain the weak selectivity of AQP3 compared to that of AQP1.

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P 29

Missense mutations in the human *MIP* gene, encoding the major intrinsic protein of the lens, underlie autosomal dominant "polymorphic" and lamellar cataracts on 12q

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Background:

Water and solute homeostasis are thought to be critical to the maintenance of optical clarity in the crystalline lens of the eye. Recently, the gene encoding the "major intrinsic protein of the lens" (MIP), the most abundant protein in the mature lens fibre cell membrane has been localised to 12q14 and its protein product shown to be a member of the aquaporin family of water channels (AQP0).

Materials and Methods:

Linkage analysis (positional-candidate approach) by PCR-based microsatellite marker genotyping was used to identify the disease locus in two families with autosomal dominant congenital cataract. Mutations within the *MIP* gene were detected by direct sequencing and confirmed by restriction fragment length analysis.

Results:

We obtained maximally positive two-point lod scores at marker D12S1676 in both family A ($Z_{max} = 3.91$ at $\_ = 0$) and B ($Z_{max} = 2.11$ at $\_ = 0$) and defined the disease interval as D12S375-D12S1064, which spans the *MIP* gene locus on 12q. In family A, sequence analysis of the *MIP* gene revealed a missense mutation in affected individuals; the C_G transition results in a threonine-to-arginine substitution at codon 138 (T138R). Restriction fragment length analysis (gain of an *AocI* restriction site) confirmed co-segregation of the mutation with the disease. In family B, affected individuals were found to have an A_G transversion that results in the substitution of glycine for glutamic acid at codon 134. Restriction fragment length analysis (loss of a *BglII* restriction site) confirmed co-segregation of the mutation with the disease.

Discussion:

Our data constitutes the first evidence implicating the major intrinsic protein of the lens, MIP (or aquaporin-0, AQP0), in human cataract. Both mutations arise in highly conserved residues within the putative fourth transmembrane domain of the protein and are thought to be critical for water transport. These studies provide the first confirmation that *MIP* plays a vital role in the maintenance of transparency of the human lens and further evidence of the important physiological role of aquaporins.

P 30

Identification and localization of aquaporins expressed in human salivary glands

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Aquaporins are expressed in a variety of epithelia, most of which have well-defined functions in fluid transport. Previous studies have shown that AQP1 [1], AQP4 [2], and AQP5 [3] are expressed in rat salivary tissue. The purpose of the present study was to identify and localize the aquaporins expressed in human salivary glands. Total RNA was extracted from human major glands (parotid, submandibular and sublingual), minor glands (labial) and from surrounding tissues. Expression of AQP mRNA was assessed by semiquantitative RT-PCR using primers specifically designed for the human aquaporin 1, 3, 4 and 5 sequences. First PCR products were further amplified with nested primers and the products sequenced. The cleaned first PCR products were also used as ^{32}P-labelled cDNA probes in a Northern analysis of whole gland total RNA. Expression levels of aquaporins in the various human glands were quantified by densitometry and normalized to GAPDH expression. Following fixation and cryosectioning, aquaporins 1, 3, 4 and 5 were localized in human labial glands by immunohistochemistry using affinity-purified primary antibodies and peroxidase-linked secondary antibodies. Both the semiquantitative RT-PCR data and the Northern analyses showed that all four aquaporins are expressed at detectable levels in human salivary glands. The sequenced RT-PCR products were 100% identical to the expected nucleotide sequences. Expression levels for AQP3 and AQP5 were noticeably lower, and AQP4 significantly higher, in the sublingual glands compared with the other major glands. The labial glands showed expression levels similar to the parotid and submandibular glands apart from a markedly lower level of AQP4 expression. Immunohistochemistry of the labial glands showed that AQP1 was localized to endothelial and myoepithelial cells, AQP3 was present in the basolateral membranes, and AQP5 in the luminal and canalicular membranes, of the mucous acinar cells. AQP4 was not detected. These findings suggest the first time that aquaporins may participate in the transepithelial movement of fluid in human salivary glands. This work was supported by the Wellcome Trust, BBSRC and Hungarian Ministry of Education.

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P 31

The Expression of Aquaporin Homologues in Fish

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One of the major differences between terrestrial and aquatic vertebrates is that the latter group have a potentially greater difficulty in maintaining osmotic homeostasis because they are surrounded by an external environment which is almost always in osmotic dis-equilibrium with their body fluids. In comparison to terrestrial animal species, aquatic organisms such as teleost fish may well possess a distinct set of mechanisms in order to adapt and survive the osmotic challenges posed by the seawater (SW: hyper-osmotic) or freshwater (FW: hypo-osmotic) environments. However, to date, little is known about how these mechanisms manifest themselves at the molecular level. In order to begin an evaluation of which proteins may be involved in osmoregulation in both the freshwater and marine environments, investigations have been initiated using the euryhaline teleost the European eel (*anguilla anguilla*) which naturally inhabits both environments.

From an osmoregulatory perspective the key organs concerned with exchanging water and ions with the external environment are the gills, intestine and kidneys. In the gills, osmotic water permeabilities have been measured previously and were found to be considerably higher in FW fish. The intestine of marine eels possess a mechanism to absorb both water and ions in large quantities, but water can be absorbed against the osmotic gradient and the extent of this ability is correlated to the level of hyper-osmolality of the external environment to which the fish is adapted. The kidneys of teleost fish do not possess a mammalian-like urinary concentrating mechanism, but the production of dilute urine is significantly enhanced in FW eels.

The purpose of the molecular investigations undertaken in these studies has been to examine the role that fish aquaporin water channel homologues play in the physiological processes mentioned above. As there was no published evidence for the presence of aquaporins in fish, initial studies focused on the cloning and identification of aquaporin homologues using degenerate RT-PCR. The first aquaporin homologue identified shared highest levels of nucleotide and amino acid homology with AQP 3 and subsequent Northern blotting showed that it is expressed as a 2.4 kb mRNA in the eye, oesophagus, intestine and gill with a minor amount of a 7 kb mRNA species also present. Quantitative studies revealed that the major site of expression was in the gill of FW eels, where levels decreased in some eels to 3% of FW values 3 weeks after transfer of fish to SW. Further studies revealed that the down-regulation of mRNA coding for this AQP3 homologue in SW occurred rapidly with a half-time of around 10 hours. The levels of mRNA expression in the intestine were relatively low in both FW or SW fish. Consequently, the existence of further intestinal aquaporins was investigated and a homologue of AQP 1 was identified. This homologue had a wider tissue distribution than that of the AQP 3 homologue and expression of a 1.4 kb mRNA was found in brain, eye, heart, pancreas, oesophagus, stomach, and intestine, with much lower levels in skeletal muscle, gill and kidney. A minor 3.1 kb mRNA component was also present in some tissues. Quantitative studies revealed that the level of mRNA expression in the intestine increased 10-25x following the transfer of FW eels to SW. Messenger RNA abundance was also significantly decreased to 28% of FW values in the kidneys of SW adapted fish. As the kidney is a major site of mammalian aquaporin expression and with only a small amount of AQP 1 homologue mRNA expression present in this tissue, a search was recently undertaken for further eel kidney aquaporin homologues. These studies have resulted in the discovery of a third aquaporin which is a duplicate isoform of the AQP 1 homologue. Further expression studies on this isoform are currently underway.

The significance of the results of these aquaporin expression studies will be discussed in relation to the osmoregulatory strategies adopted by both freshwater and seawater acclimated fish.

P 32

Cloning and functional expression of MIPfun, a Major Intrinsic Protein homolog from killifish (*Fundulus heteroclitus*) lens

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The Major Intrinsic Protein (MIP) of lens fiber cells is a member of the aquaporin (AQP) water channel family. The protein is expressed at very high levels in lens fiber cells, but its physiological function is presently unknown. We have cloned a full-length cDNA encoding a MIP from killifish (*Fundulus heteroclitus*) lens by homology to known AQP:s. The predicted protein (263 amino acids; GenBank accession number AF191906) shows 77% identity to amphibian MIPs, 70% identity to mammalian MIPs and 46% identity to mammalian AQP1. The protein-encoding sequence was subcloned into an oocyte expression vector flanked by the 5' and 3' *Xenopus* beta-globin untranslated regions, and in vitro transcribed cRNA was injected into *Xenopus* oocytes (2.5 ng/oocyte). Expression of MIPfun caused a 10-50 -fold increased in the water permeability of the oocyte, which is comparable to that seen with AQP1 but at least an order of magnitude larger than seen with mammalian MIP. MIPfun is similar to mammalian MIP in that the water permeability was not sensitive to block by mercurials, and no glycerol permeability was detected.

P 33

Phosphorylation of Ser256 is essential and seems sufficient for re-distribution of AQP2 from vesicles to the apical plasma membrane in MDCK cells

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Aquaporin-2 (AQP2) is the vasopressin-regulated water channel in the collecting duct cells of the kidney. Without stimulation, AQP2 is localized in subapical storage vesicles in these cells. Upon binding of vasopressin to its V2-receptor, intracellular cAMP levels increase, resulting in phosphorylation of Ser256 of AQP2 (p-AQP2) and presumably other proteins, by protein kinase A (PKA). Consequently, vesicles containing AQP2 fuse with the apical plasma membrane, thereby redistributing AQP2 from vesicles to this membrane. In a previous study we have shown that heterologously expressed in MDCK cells, wild-type AQP2 (wt-AQP2) shuttling to the apical membrane upon stimulation by vasopressin/forskolin is similar to that in collecting duct cells.

To determine whether phosphorylation of AQP2 at Ser256 is necessary and sufficient for trafficking to the apical membrane, MDCK cells were stably transfected with AQP2 expression constructs encoding an AQP2 mutant in which Ser256 was replaced by alanine (AQP2-S256A) or by aspartic acid (AQP2-S256D). It has often been shown that a protein in which a phosphorylatable Ser is changed into an Ala acts as a constitutively-unphosphorylated protein, whereas changing it into Asp, a constitutively-phosphorylated protein is mimicked. This also seems to be the case for AQP2, because in LLC-PK cells, AQP2-S256A was found in vesicles [1]. In addition, expressed in oocytes, AQP2-S256A was localized intracellularly, whereas AQP2-S256D was only localized at the plasma membrane [2].

Of selected MDCK clones, confocal Laser Scanning Microscopy (CLSM) revealed that, with or without stimulation by forskolin, AQP2-S256A was also localized intracellularly in these cells. Co-localization studies with Giantin revealed that AQP2-S256A was not localized in the Golgi complex. Subsequent electron microscopic (EM) analysis revealed that AQP2-S256A was localized in intracellular vesicles. In contrast, CLSM and EM analysis revealed that, with or without stimulation, AQP2-S256D was localized within the apical plasma membrane.

The subcellular localizations of AQP2-S256A and AQP2-S256D, clearly indicate that they mimick unphosphorylated and phosphorylated AQP2, respectively. The intracellular localization of AQP2-S256A shows that in MDCK cells, phosphorylation of AQP2 is essential for redistribution to the apical membrane. In addition, the plasma membrane localization of AQP2-S256D suggests that phosphorylation of just AQP2 at S256 is also sufficient for its redistribution to the apical membrane. However, future studies have to reveal whether contribution of other proteins in the redistribution process can be excluded. Previously, it has been speculated that phosphorylation of Ser256 by a Golgi casein kinase and possibly subsequent de-phosphorylation is important for trafficking out of the Golgi complex [3]. Our results clearly show that, if this process is important for AQP2 routing, it is not essential for AQP2 routing in MDCK cells.

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P 34

Renal aquaporin expression in aging rat: effect of food restriction

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In rodents, aging is associated with polyuria and decreased urinary osmolality. The mechanisms underlying this reduced renal concentrating ability were investigated in 10, 30 mo-old female WAG/Rij rats fed ad libitum and in 30 mo-old rats that were food restricted by 30% for 20 months. The survival rate of food restricted animals was similar to that of rats fed ad libitum. Urinary volume and osmolality were 3.7 " 0.4 ml/24h and 2389 " 112 mosm/kg H_2O in adult rats and 8.3 " 1.7 ml/24h and 1339 " 157 mosm/kg H_2O in 30 mo-old rats. Interestingly, chronic food restriction prevented polyuria (3.0 " 0.4 ml/24h and 1960 " 154 mosm/kg in 30 mo-old restricted rats). Vasopressin V2 receptor mRNA, quantified by RT-PCR in the whole papilla or in dissected collecting tubules, did not significantly differ with age but ^{3}H-vasopressin binding sites were reduced in membrane papilla of 30 mo-old (51.7 " 49 fmol/mg protein) as compared to the 10 mo-old rats (81.9 " 9.4 fmol/mg proteins). This decrease in V2 receptor density did not affect cAMP content of the papilla (1.51 " 0.10 pmol/mg in 10 mo-old animals and 1.47 " 0.21 pmol/mg in 30 mo-old animals). On the other hand, papillary osmolalities (1084 " 103 mosm/kg H_2O in 10 mo-old animals), were significantly reduced in senescent animals (645 " 49 and 678 " 35 mosm/kg H_2O in rats fed ad libitum and food restricted respectively). Aquaporin-1, -2, -3 and -4 (AQP1 to AQP4) expression was quantified by Western blot analysis in the different regions of the kidney. The expression of AQP1 and AQP4 in cortex and medulla was mostly unchanged in all groups. In contrast, AQP2 and AQP3 expression was down regulated by 80% and 50%, respectively, in inner medulla of senescent animals fed ad libitum, and to a lesser extent in outer medulla, but not in renal cortex. Similarly, AQP2 expression was markedly decreased in the inner medulla of food restricted animals, although to a slightly lesser extent, whereas AQP3 expression remained stable in food restricted 30 mo-old rats. Indirect immunofluorescence and gold labeling electron microscopy of kidney sections showed a similar distribution of AQP1,3,4 in all groups. AQP2 staining was markedly reduced in the inner medullary collecting duct of all 30 mo-old animals. In addition, AQP2 was improperly distributed throughout principal cell cytoplasm in 30 mo-old animals fed ad libitum. In contrast, AQP2 was properly localized to the apical region of principal cells in food restricted 30 mo-old animals like in 10 mo-old rats. These results demonstrate that aging is associated to a marked decrease in inner medullary AQP2 expression, which is correlated to a decrease in papillary osmolality and independent from vasopressin-mediated intracellular cAMP accumulation. The polyuria observed only in 30 mo-old rats fed ad libitum, is associated to an intracellular redistribution of AQP2, which was not observed in food restricted animals. These observations suggest that AQP2 expression and AQP2 recruitment at the apical plasma membrane are regulated by two independent mechanisms in this model.

P 35

Functional expression of AQP4 in a human gastric cell line

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HGT-1 is a human cell line sharing a number of physiological features with gastric parietal cells [1,2]. HGT-1 cell monolayers were able to secrete H^+ when stimulated with histamine (ΔpHe 0.46"0.05). Omeprazole treatment inhibited (by about 60%) the histamine-induced apical acidification. pHi measurements using the fluorescent pH-sensitive dye 2',7'-bis-carboxyethyl-5(6)-carboxyfluorescein (BCECF) demonstrated the expression of a functional omeprazole-sensitive H^+/K^+-pump. A monoclonal antibody directed against the alpha subunit of the H^+/K^+-ATPase immunoprecipitated a protein of 95 kDa from HGT-1 cells and human stomach which corresponds to the expected molecular size of the native protein. HGT-1 cells resulted also positive for the anion exchanger AE2 specifically expressed in gastric parietal cells. In addition, in HGT-1 cells, we identified a histamine and pHi sensitive Na^+/H^+ exchanger which might correspond to the functional expression of the NHE4 isoform which has been detected gastric epithelial cells as well as in primary cultured parietal cells. HGT-1 cells were stably transfected with the cDNA coding for rat AQP4. Western blot analysis using specific AQP4 antibodies revealed the expression of a 30 kDa in transfected cells (clone 10C1) not detected in wild type HGT-1 cells. Indirect immunofluorescence studies showed that AQP4 was confined to the basolateral membrane of 10C1 cells as in native parietal cells. Functional studies employing the phase contrast techniques showed that in 10C1 cells, the time constant (_) of cell swelling in response to changes in perfusate osmolality was more than sevenfold lower and temperature-insensitive, when compared to wild type HGT-1 cells consistent with the expression of a functional water channel. By freeze-fracture electron microscopy, numerous orthogonal arrays of particles (OAPs), typical of the presence of AQP4, were observed in basolateral plasma membranes of AQP4-transfected cells, but were totally absent from wild type cells. Whether histamine stimulation of these cells is accopanied by a reorganization of AQP4 OAPs is under investigation.

AQP4-transfected 10C1 cells therefore might represent an interesting cell culture model for studying the regulatory mechanisms of water transport driven by acid secretion.

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P 36

Effect of expressing MIP-MOD and Nodulin-26 on the NH_3 permeability of *Xenopus* oocytes

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Recently we demonstrated that expressing AQP1 increases the CO_2 permeability of *Xenopus* oocytes. The AQP1-dependent increase in CO_2 permeability is prevented by the mercurial agent pCMBS, suggesting that CO_2 and H_2O share a common pathway through the channel. In the present study we have tested the hypothesis that some MIP proteins are permeable to the gas NH_3. We have focused upon two channels, MIP-MOD (a protein associated with the self-incompatibility response of field mustard, *Brassica campestris*) and Nodulin 26 (a protein expressed at high levels in peribacterial membranes of legumes).

We used ion-sensitive pH microelectrodes to measure the changes in intracellular pH (pH_i) upon exposing the oocytes to NH_3/NH^+_4 containing solutions. In control oocytes, exposure to a Na^+-free/$NMDG^+$ solution at pH_o 8 containing 20mM NH_3/NH^+_4 (19.5mM NH^+_4 and 0.5 mM NH_3) causes a brief decrease in pH_i (consistent with NH^+_4 influx) followed by a slow increase (consistent with NH_3 influx). In oocytes expressing nodulin-26 the same maneuver caused a rapid and sustained acidification. These results suggest that nodulin-26 is permeable to NH^+_4. Because the large acidification could be masking any changes in pH_i associated with increased NH_3 influx, we raised the extracellular pH to 10, replaced Na^+ with K^+ rather than $NMDG^+$ and lowered NH_3/NH^+_4 to 2 mM (0.5 mM NH^+_4 and 1.5 mM NH_3). In H_2O-injected oocytes, adding NH_3/NH^+_4 under these conditions caused a sustained acidification. However, in nodulin-26 expressing oocytes, the initial pH_i decrease was followed by a large increase, indicating an increase in NH_3 permeability.

In oocytes expressing MIP-MOD, exposure to NH_3/NH^+_4 under the high-pH conditions yielded results similar to those observed in nodulin-26-expressing oocytes (i.e., a decrease in pH_i, followed by a large increase). Thus, MIP-MOD also increases oocyte NH_3 permeability.

Because mercurial agents reduce the permeability of many MIP proteins, we examined the effect of the pCMBS on the increased NH_3 permeability of oocytes expressing MIP-MOD. Oocytes were pre-incubated in 1 mM pCMBS for 15 minutes, and then exposed to NH_3/NH^+_4 solutions at high pH. Whereas pre-incubation in pCMBS had no effect upon the pH changes observed in H_2O-injected oocytes, in oocytes expressing MIP-MOD, pCMBS abolished the secondary increase in pH_i and the cells acidified to the same extent as the H_2O-injected oocytes. These results indicate that the MIP-MOD-dependent increase in membrane NH_3 permeability is due to permeation through a channel, and are consistent with our hypothesis that MIP-MOD and maybe also nodulin-26 are permeable to the gas NH_3.

P 37

Desiccation and osmotic stress trigger upregulation of tonoplast aquaporins

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Land plants cope with different environmental stresses that have in common an effect on plant water status. The vacuolar system of plant cells consists of a large central vacuole and associated vesicles that carry out diverse functions, many of which being involved in cellular homeostasis. Additionally, vacuoles act in combination with the cell wall to modulate turgor pressure and cell volume. The single limiting membrane of the vacuole (= tonoplast) contains Tonoplast Intrinsic Proteins (TIPs) which regulate the transmembrane movement of water. This membrane which has a 100-fold higher osmotic water permeability compared with that of the plasma membrane is able to buffer osmotic fluctuations in the cytosol [1].

A monospecific antiserum raised against a 26 kDa intrinsic protein from the tonoplast was used to screen a cauliflower (*Brassica oleracea* L. var. *botrytis*) meristematic cell cDNA library. Two distinct cDNAs have been isolated and characterised as ã-TIP isoforms [2]. The first cDNA (*BobTIP26-1*) is full length and encodes a protein that forms water channels (= aquaporins) in *Xenopus* oocytes. The second cDNA (*BobTIP26-2)* is partial and shares 90.5% sequence identity with the corresponding *BobTIP26-1* coding region. Both genes have been shown by Northern blot analysis to be up regulated under desiccation conditions [3].

In order to find out whether or not both *BobTIP26* genes are up regulated during desiccation and osmotic stress, *in situ* hybridisations were carried out. We took advantage of the divergence between the 3'-untranslated sequences of the two clones to design gene-specific digoxigenin labelled probes. Firstly, oligodesoxynucleotide probes (24-25 mers) were used. Secondly, in order to increase the labelling, *in vitro*-transcribed cRNA probes (100-150 nucleotides) were generated. *In situ* hybridisation was performed on shoot apical meristems. Under physiological conditions, transcripts were detected in meristematic cells when vacuoles are formed as well as in elongating cells but they were less abundant in more differentiated cells. The expression of both TIP genes was markedly up regulated upon desiccation and osmotic stress and *BobTIP26-2* transcripts were consistantly found more abundant than the *BobTIP26-1* transcripts. In order to know whether the abundance of the tonoplast aquaporin BobTIP 26-1 can modulate plant cell resistance to different osmotic stresses, *BobTIP26-1* has been expressed in a heterologous system.

We are discussing the *in situ* hybridisation results as well as the response of cells over-expressing the aquaporin under various osmotic stresses.

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Functional analysis of four MIP-like proteins from the developing pea seed coat

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In the seed coat of developing legume seeds, phloem-imported nutrients move symplasmically to seed coat parenchyma cells, from which they are released into the apoplasmic space facing the cotyledons of the embryo. This release implies transport across the plasma membrane.. The similarity of the permeability coefficients of tested organic cations and neutral solutes, their uptake kinetics and the inhibition of their influx by pCMBS suggest the presence of a non-selective, pore-forming transport protein (Van Dongen *et al.* in prep.). Possible candidates for these pores may be found in the family of the Major Intrinsic Proteins. Members of this family are particularly known for their ability to transport water and/or small solutes like urea and glycerol. But AQP9, from rat liver and testis, is a broad selectivity neutral solute channel facilitating the membrane transport of various neutral solutes (carbamides, polyols, purines and pyrimidines) with minimal osmotic perturbation [1].

We have cloned four MIP-like proteins from a cDNA bank of the pea seed coat. Apart from PsTRG31 (EMBL Z18288), a turgor-responsive gene cloned by Guerrero and Crossland [2], we found three novel MIP-like proteins, now registered as PsPIP2 (EMBL AJ243307), PsTIP (EMBL AJ243309) and PsNLM (EMBL AJ243308). Based on amino acid sequence homology, MIP-like proteins can be divided into four subgroups [3, 4]. At least one representative of each subgroup is expressed in the seed coat. PsTRG31 and psPIP2, putatively located in the plasmamembrane, belong to the subgroups PIP1 and PIP2, respectively. PsTIP shows high similarity with the subgroup of MIP-like proteins located in the vacuole membrane, and psNLM can be classified into the NOD26-like subgroup. NOD26, cloned from *Glycine max*, which, like pea, belongs to the Fabaceae, has been shown to be a glycerol facilitator [5].

Northern blotting revealed that PsNLM is merely expressed in the seed coat; the localisation of the others was not organ specific. The expression level of the four MIPs increased during development of the seed coat. Functional analysis by heterologous expression in *Xenopus* oocytes revealed increasing water permeability after injection of 2ng RNA of PsPIP2 and 25 ng RNA of PsTIP and PsNLM. No effect on the water permeability could be detected after injection of 25 ng RNA of PsTRG31.

Whether the expression of these MIPs will result in an increased permeability of the oocyt membrane for various solutes, is currently investigated. Additionally, antibodies are being raised against protein specific epitopes.

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P 39

Modulation of Aquaporins genes expression in *Arabidopsis* leads to altered membrane water permeability

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Aquaporins are water channel proteins found in plant vacuolar and plasma membranes. We presume that the permeability of the plant plasma membrane (PM) can be modulated by the abundance of aquaporins and by their activity but there are very few in planta studies confirming this idea. We expressed the entire coding region of the cDNA encoding the PM aquaporin RD28 in the sense or antisense orientation in *Arabidopsis thaliana.* Expression was driven by a double CaMV 35S promoter. Plants were selected on kanamycin and screened with an antiserum to RD28. After several generations of selfing, overexpressors (OE) and underexpressors (UE) in the T3 generation were obtained. The OEs have about four times more RD28 protein and the UEs have about 1/4 of the RD28 protein found in WT plants. Protoplasts were prepared from WT, OE and UE plants. The osmotic water permeability of protoplasts can be measured by changing the osmotic strength of the bathing medium and determining the rate of volume change. Protoplasts from WT plants had water permeabilities (Pos) ranging from 1 to 500 mm per sec. In the OE plants the distribution was similar with a higher proportion of protoplasts at the high end. In the UE plants most of the protoplasts were in the 1 to 10 mm per sec range and protoplasts with high permeabilities were absent. The plants did not have any obvious phenotypes when grown under normal laboratory conditions. These results demonstrate that changing the abundance of aquaporins changes the water permeability of plant cell membranes.

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Poster Presentations

Projection structure of the glycerol facilitator at 3.5 Å resolution

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The glycerol facilitator protein (GlpF) from E. coli is an archetypal member of the GLP subcluster of the aquaporin superfamily. Transmission electron microscopy of the solubilized negatively stained GlpF protein showed a tetrameric structure of approximately 80 Å sidelength. Mass measurements by scanning transmission microscopy yielded a mass of 194 kDa corroborating the tetrameric nature of GlpF. Two-dimensional crystallization in the presence of lipids produced highly ordered crystals, which diffracted electrons to 3.3 Å resolution. The 3.5 Å projection map revealed a unit cell comprised of two tetramers. In projection, GlpF is similar to AQP1, a member of the AQP subcluster of the aquaporin superfamily. However, the major density minimum central to each monomer and presumed to represent the pore is distinctly larger in GlpF than in AQP1.

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Isolation and characterization of two aquaporins from spinach plasma membranes

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Two major intrinsic proteins, PM28A [1] and a new isoform, were isolated from the spinach leaf plasma membrane. The membranes were solubilized with octyl-thioglucoside and the two isoforms were copurified by cation exchange chromatography. Their masses were determined by MALDI-TOF. To identify and partially sequence the new isoform, the mixture of both was digested with LysC. Fragments were separated by reverse-phase chromatography and analyzed by electrospray ionization mass spectrometry or N-terminal sequencing. Single particle analysis of solubilized mixed isoforms revealed two distinct sizes and shapes. The smaller was square-shaped while the larger exhibited a rather circular shape. The crystallization of the two isoforms into two dimensions yielded two different packing arrangements, reflecting the shape of the solubilized complexes.

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P 42

The sidedness of aquaporins determined by atomic force and transmission electron microscopy

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Aquaporins contain an internal sequence repeat forming two obversely symmetric hemichannels predicted to resemble an hourglass. This unique arrangement of two highly related protein domains oriented at 180E to each other poses a significant challenge to structural studies, especially the determination of sidedness. Aquaporin Z (AqpZ) from Escherichia Coli was reconstituted into highly ordered two dimensional crystals. They were freeze-dried and metal-shadowed to establish the relationship between surface structure and underlying protein density by electron microscopy. The shadowing of some surfaces was prevented by protruding aggregates. Thus, images collected from freeze-dried crystals that exhibited both metal-coated and uncoated regions allowed surface relief reconstructions and projection maps to be obtained from the same crystal. Cross correlation peak searches along lattices crossing metal-coated and uncoated regions allowed an unambiguous alignment of the surface reliefs to the underlying density maps. AqpZ topographs previously determined by AFM could then be aligned with projection maps of AqpZ, and finally with human erythrocyte aquaporin-1 (AQP1). Thereby features of the AqpZ topography could be interpreted by direct comparison to the 6Å three dimensional structure of AQP1. We conclude that the sidedness we originally proposed for aquaporin density maps was inverted [1].

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Probing AQP1 topology and structure by mutagenesis of loop B

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Purpose. AQP1 has two characteristic NPA (Asn-Pro-Ala) box repeats, and predictions place them on loops B and E on opposite sides of the membrane. However, the hourglass model predicts those two loops meeting inside the monomer to form the pore (2), and the latest structural findings at 4.5 Å resolution (5) are consistent with that view. To test such ideas, we have done cysteine-scanning mutagenesis on three residues (71-73) near the first NPA repeat (76-78), predicted to form part of intracellular loop B, to assess whether they are accessible or not to extracellular mercurial (pCMBS) as assayed by osmotic permeability of oocytes expressing AQP1. **Methods.** 1).Oligonucleotide-mediated mutagenesis: AQP1 site-directed mutants were constructed with the Altered Sites II in vitro Mutagenesis Systems. AQP1-pBluescript was digested with Kpn I and Bam H I. The resulting 2630-base pair full length clone was ligated into pAlter-1 cut with those same enzymes (AQP-pAlter). Mutation oligonucleotides were synthesized to replace or substitute individual residues by cysteine. 2) Expression and assay of mutant function: AQP mutants were excised from AQP-pAlter and ligated into AQP-pBluescript to assay mutant function by expression in Xenopus laevis oocytes. For in vitro mRNA transcription, AQP-pBluescript or AQP-pBluescript were linearized with Kpn 1. mRNA was synthesized using T3 RNA polymerase. Osmotic permeability (P_f) values were calculated (1) from osmotically-induced changes in the volume of oocytes injected with mRNA encoding AQP or AQPmut. **Results.** We examined the following gene products: AQP1 wild-type (C189); C189S; S71C/C189S; G72C/C189S; and A73C/C189S. We determined P_f (in units of (îm/sec) in groups of 5 oocytes per condition. The results are given in the adjoining Table.

As seen priorly (3), the mutant C189S exhibited wild-type P_f but was no longer sensitive to mercurials. All three C189S mutants had a P_f lower than that of wild-type AQP ($p < 0.04$ for res.72, and < 0.01 for res.73), and in all three cases the P_f expressed was inhibited by 1 mM $HgCl_2$ and 1 mM pCMBS. **Conclusions.** The results are in line with prior reports locating loop B at or near the water pore (2-5), as in the houglass model (2). The fact that all mutations decrease P_f strongly suggests that the segment 71-73 borders the pore; as C is bulkier that the residues replaced (S, G, A), the lower P_f of the mutants might be simply due to steric hindrance by C, or to more complex structural changes. The inhibitions by $HgCl_2$ and especially by the larger mercurial pCMBS acting on the C residue once more suggest as the simplest explanation that even though loop B is intracellular, its residues 71-73 are at the pore directly, since for pCMBS to be able to reach them across a pore of the dimensions of water, secondary structural modifications would have to occur.

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P 44

The fold of human Aquaporin 1

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The fold of human aquaporin 1 is determined from cryo-electron microscopic data at a resolution of 4.5 Å. The monomeric structure consists of two transmembrane triple helices arranged around a pseudo-twofold axis that are connected by a long flexible extracellular loop. Each triplet contains between its second and third helix a functional loop containing the highly conserved so-called fingerprint NPA motif. These functional loops are assumed to fold inwards between the two triplets, thereby forming the heart of the water channel. The helix topology was determined from the sidedness pattern of each of the six transmembrane helices with respect to the membrane, together with constraints defined by the sequence and atomic force microscopy data. The directionality of the helices was determined by collecting the best-fitting orientations resulting from a search through the three-dimensional experimental map for a large number of alpha helical fragments. Tests on cryo-electron crystallographic Bacteriorhodopsin data suggest that our method is generally applicable to determine the topology of helical proteins for which only medium-resolution electron microscopy data are available.

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Expression and purification of functional human AQP2

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Aquaporin-2 (AQP2) is the vasopressin-regulated water channel in the collecting duct cells of the kidney. Without stimulation, AQP2 is localized in subapical storage vesicles in these cells. Upon binding of vasopressin to its V2-receptor, vesicles containing the homotetrameric AQP2 fuse with the apical plasma membrane, thereby redistributing AQP2 to this membrane. Overexpression of AQP2 at the apical membrane in diseases such as congestive heart failure, pre-ecclampsia and liver cirrhosis has been shown to increase renal water reabsorption, resulting in life-threatening hyponatremia**.** Therefore, identification and development of AQP2 blockers is desirable, for which elucidation of the AQP2 structure is essential. For this, a large amount of pure and functional AQP2 is needed. To obtain this material, it was decided to express AQP2 as a recombinant protein using the baculovirus/insect cell expression system. The coding region of human AQP2 was cloned into the pFastBac-HT vector, in such a way that it was N-terminally flanked by a poly-histidine tag for easy purification using Ni-agarose beads, which can be removed by rTEV protease digestion. After digestion, full-length AQP2 would be obtained, just preceded by a glycine. After generation of the recombinant HT-AQP2 baculovirus DNA, insect cells were transfected to generate high amounts of virus. Immunoblot analysis revealed high expression levels of HT-AQP2. Confocal laser scanning microscopy and cell fractionation revealed that HT-AQP2 was localized at the plasma-membrane and to vesicles just below the plasma-membrane, which indicated that both fractions should be used for purification. To optimize HT-AQP2 purification, different detergents (Triton X-100, Dodecyl-Maltoside [DoM], CHAPS, Octyl-Glucoside [OG], Nonyl-Glucoside [NG], Decyl-Maltoside [DM]) were tested for effects on the solubilization of the protein and their effects on the binding of HT-AQP2 to Ni-beads. Of these, only DoM solubilized HT-AQP2 and did not interfere with its binding to Ni-beads. After urea/alkaline stripping, however, OG performed just as well. Since OG has a higher critical micel concentration value than DoM and sucrose gradient centrifugation of insect cells membranes solubilized in OG revealed that HT-AQP2 was expressed as a tetramer (which has also been shown for renal AQP2), OG was the detergent of choice. Using this detergent and isolation of HT-AQP2 with Ni-beads, Coomassie-stained gels together with immunoblotting revealed that 0.5 mg pure HT-AQP2 could be isolated from 1 liter of insect cell culture. Upon reconstitution into proteoliposomes at protein lipid ratios of 0.01, 0.02 or 0.1 followed by stopped-flow analysis, HT-AQP2 was shown to be a functional water channel. Preliminary results by Transmission Electron Microscopy and Atomic Force Microscopy revealed that using the isolated HT-AQP2, 2D crystals could be formed. In conclusion, the successful purification of high amounts of functionally active and structurally intact HT-AQP2 protein from insect cells now allows analysis of the three-dimensional structure of AQP2.

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Coupling between transports in the Na^+/glucose transporter (SGLT1) and the AQP1 coexpressed in *Xenopus* oocytes

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We have established that the human SGLT1 cotransports 210 water molecules per 2 Na^+ and 1 glucose molecule (Meinild *et al.*, 1998, J. Physiol. **508.1** pp.15-21), and that SGLT1 is a water channel (Loo *et al* 1999, J. Physiol. **518.1** pp.195-202). Since the cotransport is hypertonic this will in turn result in additional osmotic water flow into the cell. The goal of this study was to define the role of the cotransporter in generating water transport in two situations: when only SGLT1 was expressed in *Xenopus laevis* oocytes and when SGLT1 and AQP1 were coexpressed.

When glucose was added to oocytes expressing only SGLT1 under voltage clamped conditions, cotransport of water revealed itself as an initial rate of volume increase of about 20 pl sec^{-1}, for clamp currents of 1000 nA. After about 10 min the total water transport attained a steady state value of 2.2 " 0.2 times the initial rate (paired experiments in 6 oocytes). At this point the transport is isotonic to the intracellular solution which could be estimated to be about 7 mosm l^{-1} hyperosmolar relative to the external solution. Removal of glucose reduced the rate of swelling by 42 " 6%, the amount being identical to the rate of cotransport of water observed initially. Addition of of Phlorizin (100 ìM) reduced the rate of swelling by 75 " 5% and the passive water permeability by 39 " 3%. Phlorizin abolishes both the cotransport component of water transport and the water permeability of the SGLT1. We conclude that cotransport of water provided 42% of the total water flow, while osmotic flow was divided with about 23 % via the SGLT1 and 35 % via the membrane.

Coexpression of AQP1 with the SGLT1 increased the water permeability about 20-fold above that obtained with SGLT1 alone. Activation of the SGLT1 induced a rate of water flow similar to the one achieved after 10 min in the oocytes expressing only SGLT1. Given the rate of cotransport and the water permeability we conclude that about one third of the water transport is cotransported while two thirds are osmotic and mediated by a small osmotic gradient of about 0.5 mosm l^{-1} via the AQP1. This result was confirmed by experiments in which Na^+- currents were mediated via gramicidin channels inserted in oocytes co-expressing SGLT1 and AQP1 but with the cotransport modality of the SGLT1 inactivated by the absence of sugar.

Our data show that transport in SGLT1 can induce water transport in AQP1. The two situations investigated may serve as models for isotonic transport across apical (brush border) membranes of leaky epithelia. For membranes with relatively low passive water permeability (i.e. small intestine) it requires relatively large osmotic gradients to render the transported solution isotonic. For membranes with relative high water permeability (ie kidney proximal tubulus epithelium) it only requires small gradients. The model would predict the transportate across leaky epithelia of AQP1 -/- animals to be hypertonic relative to that found in AQP1 +/+ animals.

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Aquaporin-1 and Na^+,K^+-ATPase á-expression in human proximal tubule epithelial cells exposed to hyperosmolarity and cholera toxin

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Primary cells isolated from proximal tubule epithelium of human kidney (HRPTE cells) up-regulate aquaporin-1 (AQP-1) expression and localize in the membrane fraction of the cells in response to hyperosmolarity. NaCl and D(+)-raffinose increased (2-2.5 fold) AQP-1 protein expression when medium osmolarity was 400 and 500 mOsm/kg.H_2O. Urea did not have this effect. We demonstrated that vasopressin (AVP, 10^{-8} M) enhanced AQP-1 expression in mouse inner medullary collecting duct epithelial (mIMCD-3) cells exposed to hyperosmolar NaCl. However, both AVP and atrial natriuretic peptide á-ANP (1-28) showed no effect on the AQP-1 expression in HRPTE cells. Similarly, angiotensin II showed no effect on NaCl-induced AQP-1 up-regulation in HRPTE cells. Nevertheless, a 3 to 4- fold AQP-1 increase was observed when cells were exposed to contrast agents (CA) Reno-60 and Hypaque-76 at 400 and 500 mOsm/kg.H_2O. Isosmolar (290 mOsm/kg H_2O) Visipaque at 25% v/v also rendered cells a 3-fold increase in AQP-1 expression. This might have effect on CA-induced nephrotoxicity. In addition, D-glucose supplemented medium (500 mOsm/kg.H_2O) enhanced a 2-fold AQP-1 expression as compared to control (290 mOsm/kg.H_2O). Interestingly, cholera toxin (CT, 10^{-7} M) abolished at least 50% of AQP-1 expression in either isosmolar control cells or hyperosmolar NaCl, D-glucose, or CA treated cells. On the contrary, CT increased at least a 2-fold Na^+,K^+-ATPase á_expression in HRPTE cells without or with hyperosmolar (NaCl or D-glucose) exposure. However, this Na^+,K^+-ATPase á_augmentation by CT was not observed in cells exposed to contrast agents, although CA showed in the absence of CT, a 1.5 to 2-fold increase of Na^+,K^+-ATPase á_expression when compared to the control. Furthermore, at 500 mOsm/kg.H_2O medium osmolarity, D(+)-raffinose, NaCl, D-glucose, Visipaque (25% v/v), Reno-60 and Hypaque-76, but not urea, promoted cellular Na^+,K^+-ATPase á_expression. Our RT-PCR of HRPTE cells exposed to various hyperosmolar conditions demonstrated up-regulation of AQP-1 mRNA as well as protein levels. Our results also indicated that CT antagonized NaCl, D-glucose, or CA-induced AQP-1 up-regulation, but increased the Na^+,K^+-ATPase á_expression in HRPTE cells. However, CT has no effect on CA-induced increase of Na^+,K^+-ATPase á_expression in the cells. According to our data and others it is important to investigate hyperosmolarity- or stress-related signaling pathway that may involve protein kinase C and MAP kinases to regulate AQP-1 expression. In addition, the increase of Na^+,K^+-ATPase á_expression in osmo-stressed HRPTE cells and its further enhancement by CT may also merit a further study into cAMP, G protein, and G-protein coupled receptor.

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PKC and ERK dependent hypertonic induction of AQP1

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In response to hypertonic stress, a limited number of genes have been demonstrated to be upregulated. Much of this focus has been on compatible osmolyte transporters, but we have recently reported that AQP1 is also upregulated in a dose- and solute-dependent fashion when Balb/C fibroblasts are incubated in hypertonic medium. Furthermore, MAP kinases have been demonstrated to participate in regulation of the osmolyte transporters. In Balb/C fibroblasts, extracellular signal regulated kinase (ERK) was activated by osmotic stress. Blockade of ERK activation using the pharmacological inhibitors, PD098059 and U0126, blocked AQP1 induction in response to hypertonic stress. Transient transfection of dominant negative mutants of ERK1 and ERK2 into Balb/C fibroblasts also partially blocked hypertonic induction of AQP1. As protein kinase C (PKC) can lead to the activation of ERK, its potential involvement was also investigated. Downregulation of PKC by chronic exposure to low level TPA inhibited AQP1 induction in response to hypertonic stress, as did PKC inhibition with Ro-31-8425. Chronic, low level TPA exposure also negatively affected ERK activation by osmotic stress. Finally, AQP1 was upregulated in the lung of rats made hyperosmolar with hypertonic saline injection and dehydration. This study has demonstrated that AQP1 is dynamically regulated by osmotic stress both *in vitro* and *in vivo* and provides a basis for further exploration of mechanisms regulating AQP1 expression in the respiratory tract.

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Aquaporin-1 mediates fluid transport in human ciliary epithelial monolayer

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Water channel proteins (aquaporins) are important pathways for water movements across cell membranes, including those in the ciliary epithelium, which is the major site of aqueous humor secretion. Most of aqueous humor secretion is driven by the active transport of ions from plasma into the posterior chamber followed by water movement. Both the outer nonpigmented (NPE) and inner pigmented (PE) ciliary epithelial layers exhibit properties of transporting epithelia, however, the NPE layer is thought to provide the direct driving force for aqueous humor formation. Recently, we identified the functional expression of aquaporin-1 (AQP1) in the cultured human NPE cells [1]. The knockout mice studies with AQP1 indicate a role for AQP1 in kidney and lung fluid transport [2, 3]. We report here for the first time that cultured human NPE cell layers transport fluid. Cells were grown to confluence on permeable membrane inserts and fluid transport across cultured layers was determined by a volume clamp at 37 ^{0}C. Cultured NPE cell monolayers transported fluid from their apical to basal side and the rate of fluid transport was 3.6 ìl/h/cm (n = 4). Mercury chloride, a nonspecific but potent blocker of Hg-sensitive aquaporins, and AQP1 antisense oligonucleotides inhibited fluid transport across the NPE cell monolayers suggesting that water channels in the NPE cells may be central to fluid transport by these cells. These results demonstrate the physiological role of AQP1 in the fluid transport in ciliary epithelial cells, thus providing new insights into the mechanisms that regulate aqueous humor secretion in vivo.

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P 50

Development of an immunoisolation technique for preparing vesicles from rat cholangiocytes enriched in the water channel protein, Aquaporin-1

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We previously reported that cholangiocytes express aquaporin 1 (AQP1), a channel protein through which water moves selectively and passively in response to osmotic gradients (JBC 272:12984-12988, 1997). We recently proposed the HYPOTHESIS that key solute transporters/exchangers necessary to establish osmotic gradients and drive passive channel-mediated water transport may cluster in a subset of AQP1-containing vesicles. To begin to test this hypothesis, our AIM here was to establish a technique to isolate vesicles from cholangiocytes enriched in AQP1. METHODS: Highly purified rat cholangiocytes were isolated by enzymatic digestion and counterflow elutriation, sonicated, and a vesicle fraction prepared by isopycnic centrifugation on a linear sucrose gradient. The purified vesicle fraction was mixed with magnetic beads coated with an antibody to AQP1 linked to the beads by a secondary antibody. The vesicle fractions were characterized using specific organelle marker enzyme assays. Electron microscopy was performed to confirm the specific attachment of the vesicles to the beads. Immunoblotting was performed on a 10% SDS-polyacrylamide gel using a commercially available specific antibody to AQP1 on fractions of the microsome vesicles, the vesicles attached to the beads (bound) and those not bound to the beads (unbound). RESULTS: Organelle specific marker enzyme assays revealed an enrichment in microsomal esterase and glucose-6-phosphatase (microsome markers), of 3.5"0.63 and 4.06"0.26, respectively, in the vesicles bound to the beads versus the post nuclear supernate. Electron micrographs revealed numerous vesicles specifically attached to the magnetic beads in the presence of primary and secondary antibodies; no vesicles attached to magnetic beads in the absence of primary antibody. Immunoblotting showed the presence of a 28 kD band for AQP1 which was enriched approximately two-fold in the vesicle fraction bound to the beads compared to the microsome fraction and was absent in the vesicles not bound to the beads. CONCLUSIONS: We have developed and fully characterized a technique to immunoisolate AQP1-containing vesicles from rat cholangiocytes. This technique will allow future studies on passive channel-mediated water transport to test the concept of a membrane microdomain transporting complex.

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Colocalization of AQP1 and CFTR in cholangiocytes: evidence for a novel transporting organelle involved in ductal bile secretion

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We previously reported that ductal bile formation is regulated by secretin-responsive relocation of aquaporin 1 (AQP1), a water selective channel protein, from an intracellular vesicular compartment to the apical cholangiocyte plasma membrane. Because water moves passively through AQPs in response to osmotic gradients established by solutes and ions, we proposed the hypothesis that AQP1-containing vesicles also contain transporters for osmotically active ions (e.g., Cl^-) forming a membrane microdomain transporting complex. METHODS: AQP1- containing vesicles were isolated from highly purified rat cholangiocytes using a novel immunoisolation technique developed by us. Immunoblotting was performed on subcellular cholangiocyte fractions using specific antibodies against AQP1 and CFTR. Morphologic analysis of individual cholangiocytes in the presence and absence of dibutyryl cyclic AMP (d-cAMP), a secretin-responsive second messenger, was performed using confocal immunofluorescence microscopy and antibodies against AQP1 and CFTR with different fluorescent secondary antibodies. Cellular distribution of AQP1 and CFTR were determined using laser confocal microscopy. Immunoblotting of CFTR and AQP1 was performed using plasma membrane fractions isolated from bile duct ligated rats after IV infusion of the choleretic hormone, secretin (10^{-7}M), or vehicle. RESULTS: Immunoblotting of vesicles immunoisolated using a highly specific AQP1 antibody demonstrated both a 28 kD band for AQP1 and 170 kD band for CFTR that were enriched approximately 2-fold relative to microsomes. Immunoisolation using an antibody to CFTR also yielded a 2-fold enrichment of both proteins in the vesicle fraction. Immunofluorescence of cholangiocytes in the absence of d-cAMP displayed considerable colocalization of AQP1 and CFTR in a punctate, vesicular distribution. Exposure of cholangiocytes to d-cAMP (100ìM) resulted in co-redistribution of both AQP1 and CFTR from within the cell to the cholangiocyte plasma membrane. Immunoblots of organelle fractions of cholangiocytes isolated from rats before and after IV secretin demonstrated a secretin-induced relocation of AQP1 and CFTR to the plasma membrane, principally the apical domain. CONCLUSIONS: Our results using biochemical and morphologic techniques demonstrate that: (i) in the basal state, AQP1 and CFTR colocalize to an intracellular vesicular compartment in cholangiocytes; (ii) after exposure of cholangiocytes to d-cAMP in vitro or after secretin infusion *in vivo*, AQP1 and CFTR redistribute together to the cholangiocyte plasma membrane. These data support the hypothesis that a novel transporting organelle containing channel proteins for both water and chloride exists in cholangiocytes. We propose that this novel transporting organelle is involved in hormone induced ductal bile secretion.

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Ion and water transport in a new rat cortical collecting duct cell line ($RCCD_1$): regulation by AVP

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The purpose of this work was to characterize water and ion coupling and its regulation by arginine vasopressin (AVP) in a high transepithelial resistance cell line established from the rat cortical collecting duct ($RCCD_1$) [1]. Transepithelial net water fluxes (J_w) and short-circuit current (I_{sc}) were minute by minute recorded in $RCCD_1$ monolayers cultivated on permeable supports. A net water secretion was observed in the absence of any osmotic or chemical gradient and even against a hydrostatic pressure difference, which is characteristic of energy-requiring mechanisms for the transport of fluid. Moreover, when transport stopped in response to temperature or inhibitors, the fluid movement reversed its direction. Since it is well established that different types of aquaporins are present in the kidney [2], the first candidates as water pathways in $RCCD_1$ were water channels. However, RT-PCR and immunoblotting experiments demonstrated that the until present cloned renal aquaporins were not expressed in $RCCD_1$ cells. The inhibitory effects of chloride and potassium transporter blockers on the I_{sc} and J_w observed, is consistent with specific coupling of KCl and water in the here reported secretion. It was previously reported that AVP stimulates cAMP production and sodium reabsorption in $RCCD_1$ cells [3]. We have now observed that the amiloride-sensitive AVP-induced increase in I_{sc} was paralleled by a simultaneous modification of the observed J_w. Both responses had similar time courses and half-times (about 4 min). On the other hand, AVP did not modified the osmotically driven J_w induced by serosal hypertonicity. These results led us to conclude that the natriferic response to AVP, preserved in $RCCD_1$ cells, was associated to a "net water absorptive component" observed even in the absence of AQP2, AQP3 or AQP4 but the hydrosmotic response to AVP was completely lost.

As $RCCD_1$ cells also conserve feature of intercalated cells (secretoty cells), responsible of acid-base transport in the native tubule, we have studied intracellular pH regulation in these cells. Proton transport was determined using fluorescent pH-sensitive BCECF following an acid load. Our results show that pH_i recovery kinetics is quite different if acidification is undertake from serosal or mucosal bath. AVP induce an immediate alkalization sensitive to 10^{-3} M apical amiloride. However, pH_i recovery kinetics after serosal acidification is sensitive to basolateral, but not apical, amiloride. Taking in account these results, we propose the existence of different isoforms of the Na^+-H^+ transporter in the basolateral membrane (confirmed by RT-PCR and immunolocalization studies) which may be involved in cell volume regulation and in consequence in fluid secretion.

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P 53

Biosynthesis and sorting of aquaporin-2 in Madin Darby canine kidney cells

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Aquaporin-2 (AQP2) is an integral membrane protein expressed in renal epithelial cells of the collecting duct. AQP2 is localized in a subapical storage compartment. Upon stimulation with vasopressin, eighter the storage compartment itself or vesicles originating from this compartment fuse with the plasma membrane, thereby expressing the tetrameric AQP2 water pore at the cell surface. This reversible translocationg mechanism depends on an increase in cAMP levels and phosphorylation of AQP2 by PKA.

Although a number of mutations in AQP2 have been identified and shown to cause Nephrogenic Diabetes Insipidus, surprisingly little is known about folding, synthesis and intracellular transport of wild type and mutant AQP2. Questions that remain to be solved include the decoding of the signal(s) in AQP2 that regulate(s) the transport of the newly synthesized protein to the apical plasma membrane, the signals in AQP2 that are required for its reversible translocation, the relationship of the AQP2 storage compartment to other organelles of the central vacuolar compartment and the proteins involved in docking and fusion of these storage vesicles with the plasma membrane.

Glycosylation has been shown to play a critical role in apical targeting of proteins in polarized cells. Since AQP2 contains a putative glycosylation site, we first investigated the biosynthesis and targeting of AQP2 to the apical plasma membrane. MDCK cells expressing AQP2 were pulse-labeled for 20 min with ^{35}S-Trans label and chased for different periods of time. Cells were lysed and AQP2 was immunoprecipitated from detergent lysates. At early chase times, a nonglycosylated precursor and an Endoglycosidase H sensitive form were easily detected. The high mannose form disappeared within 1 hour chase, at which time AQP2 reached the Golgi complex as evidenced by the appearance of a complex type glycosylated form that is sensitive to Peptide-N-glycosidase F. Interestingly, a limited amount (< 20 %) of AQP2 is glycosylated. Consistent with this, cell surface biotinylation showed that the nonglycosylated AQP2 precursor is the major molecular species on the plasma membrane. The halflife of nonglycosylated AQP2 was 10 hr. In contrast for the E258K and S256D AQP2 mutants, this was decreased to 2.5 hr. Immunogold electron microscopy of ultrathin cryosections showed a shifted distribution of the mutant proteins to multivesicular bodies, providing a possible explanation for the reduced half life of the mutants as compared to wild type AQP2.

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Bidirectional regulation of AQP2 phosphorylation and subcellular distribution

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Arginine vasopressin (AVP) increases kidney collecting duct water permeability by phosphorylation and redistribution of aquaporin-2 (AQP2) from intracellular vesicles (V) to plasma membrane (M). *In vivo*, certain hormones counteract AVP effects on water permeability, enabling precise regulation of water reabsorption according to the particular needs of the organism. Prostaglandin E_2 (PGE_2) is one of the factors counteracting AVP effects on the level of collecting duct. We have recently shown [1] that PGE_2 does not prevent AVP-stimulated phosphorylation and translocation of AQP2 in rat renal inner medulla (IM). However, PGE_2 is able to reverse AVP-stimulated redistribution of AQP2, when added after AVP. The retrieval of AQP2 from M to V was not coupled to AQP2 dephosphorylation.

Dopamine is also known to have a diuretic effect, and has been shown to decrease AVP-stimulated water permeability in collecting ducts [2, 3]. The aim of the present study was to determine whether dopamine regulates AQP2 phosphorylation state and subcellular distribution. Experiments were performed on rat IM tissue incubated *in vitro*. The experimental animals were pretreated with indomethacin to avoid endogenous prostaglandin production. IM fragments from the same animal were incubated with (*a*) vehicle for 30 min, (*b*) AVP 10^{-8} M for 30 min, (*c*) AVP 10^{-8} M for 30 min and dopamine 10^{-5} M that was added after 15 min of incubation, (*d*) vehicle for 15 min and dopamine 10^{-5} M for 15 min. After the incubation the level of AQP2 phosphorylation was assessed using Ser^{256} phosphorylation state-specific antibodies [4], and AQP2 distribution was studied by comparison of AQP2 abundance in M- and V-enriched fractions of the tissue. We have found that dopamine significantly decreased both basal and AVP-stimulated level of AQP2 phosphorylation. It also significantly decreased M/V ratio of AQP2 abundance increased by AVP.

In conclusion, we have shown that AQP2 is a target for bidirectional regulation by AVP and endogenous diuretic factors, such as dopamine and PGE_2. The results are of potential clinical importance. Excessive water retention is a common problem in acutely ill patients, and there is a great need for new therapeutic strategies to overcome the water retention effects in syndrome of inappropriate excretion of antidiuretic hormone.

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P 55

Mutated AQP2 (E258K), lacking the concensus site of the Golgi casein kinase, is retained in the Golgi apparatus and does not redistribute after forskolin stimulation

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The water channel Aquaporin 2 (AQP2) possesses at ser-256, a consensus sequence for cAMP-dependent protein kinase A (PKA) which is involved in the vasopressin-induced exocytosis of AQP2 to the apical membrane in collecting duct principal cells. A casein kinase resident in the Golgi apparatus, termed Golgi casein kinase (G-CK) phosphorylates serine residues followed by an acidic residue at position +2, making AQP2 a potential substrate for either G-CK and PKA. In this study we tested the hypothesis that AQP2 might be phosphorylated within the Golgi network by the G-CK. *In vitro* phosphorylation studies of AQP2 immunoprecipitated from a Golgi-enriched fraction obtained from AQP2-transfected CD8 cells, demonstrated that in the presence of Mn^{2+}, a G-CK activator, caused an strong increase in AQP2 phosphorylation even in the presence of 30 FM H89, a PKA inhibitor, thus excluding any contribution of PKA in the phosphorylation state of AQP2. Treatment of intact CD8 cells with 30FM H-89, a specific PKA inhibitor resulted in a dense accumulation of the AQP2 protein in the Golgi region indicating a requirement of PKA activity for the budding of AQP2-containing vesicles from the Golgi network. In this experimental condition, immunofluorescence studies using an anti-AQP2 antibody which specifically recognizes the ser256-phosphorylated AQP2 (p-AQP2), revealed that AQP2 is blocked in the Golgi in a phosphorylated state. Similar results were obtained when AQP2 blocking was induced by temperature shift from 37EC to 20EC. Interestingly, an autosomal dominant form of inheritance NDI caused by a change of glu-258 into lys in AQP2, affects an amino acid that is irrelevant to PKA targeting, while it is essential to make ser-256 a good substrate for G-CK. Expression studies in oocytes have shown that AQP2-E258K is retained in the Golgi complex. This might suggest that the absence of residue glu-258 in this AQP2 mutant impairs proper phosphorylation by G-CK, causing retention of AQP2-E258K in the Golgi complex. To test this hypothesis under physiological conditions, wild type CD8 cells were transfected with the E258K-AQP2 mutant. Western blot analysis revealed the expression of the 29kDa band as well as the complex-glycosylated form of the AQP2-E258K. Immunofluorescence studies showed that mutated AQP2 was concentrated in a perinuclear region, probably corresponding to the Golgi apparatus as assessed by co-localization with the Golgi marker â-COP. Stimulation with forskolin did not cause any redistribution of the E258K-AQP2, indicating that mutated AQP2 did not enter the regulated compartment.

We conclude that phosphorylation of AQP2 in the Golgi, likely by G-CK, might be essential for normal budding of AQP2-containing vesicles from the TGN to the vasopressin-regulated compartment.

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Expression of SNAREs proteins in AQP2-transfected collecting duct renal cells

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SNAREs proteins are part of the membrane fusion machinery thought to act as membrane receptors during the process of vesicle docking and fusion. Closely related SNAREs homologues of the synaptobrevin-2, syntaxin-4 and SNAP-23 have been identified in rat kidney. To evaluate the possible role of SNAREs in regulating vasopressin-induced AQP2-containing vesicles exocytosis, we tested AQP2-transfected renal CD8 cells for the endogenous expression of SNAREs proteins. Degenerate RT-PCR with total RNA extracted from CD8 cells showed the presence of a synaptobrevin-2-like transcript whereas no apparent signals related to synaptobrevin-1 and 3 were detected. Western blotting analysis using specific antibodies against human synaptobrevin-2 confirmed the expression of a protein of about 18 kDa in AQP2-purified vesicles from CD8 cells.

To test whether tetanus neurotoxin (TeNT) was efficient in cleaving the synaptobrevin-like protein expressed in CD8 cells, AQP2-containing vesicles were incubated with the TeNT (500nM) for 1h. After TeNT treatment, the staining for the 18 kDa band was abolished suggesting that the synaptobrevin-like protein expressed in CD8 cells is a synaptobrevin-2-like protein. Immunfluorescence studies in CD8 cells showed the expression of synaptobrevin 2 in a vesicular compartment. Synaptobrevins are proposed to bind to cognate vesicles-targeting receptors in target membranes, which include several members of the syntaxin family. By degenerate RT-PCR, we detected the presence of syntaxin-1, -3 and -4 whereas no apparent syntaxin-2 bands were observed. The identity of the detected syntaxins was confirmed by sequencing. Western blotting of a membrane fraction enriched in plasma membrane from CD8 cells, confirmed the presence of a 36 kDa protein stained by specific antibody recognizing the syntaxin 1A and B. Moreover, treatment of CD8 plasma membrane with botulinum neurotoxin C (BoNT/C, 100nM, 3h) which inactivates syntaxin 1 and not syntaxin-4, caused the cleavage of syntaxin 1 expressed in CD8 cells, removing a fragment of 4 kDa from the C-terminus. Among the known syntaxin isoforms, only syntaxin-1 and syntaxin-4 are known to bind synaptobrevin 2 with high affinity. To define the functional role of SNAREs proteins in CD8 cells, we applied a fluorescence dequencing assay for membrane fusion. Purified AQP2-containing vesicles were labeled with the lipid-soluble fluorescence probe octadecylrodamine R18. When labeled vesicles were incubated with unlabeled target plasma membranes, fusion between the two membranes resulted in a dilution-dependent dequencing of the fluorescence probe R18. Virtually no dequencing signal was seen when Ca was buffered with 1mM EGTA. In contrast, in the presence of Ca (1mM) a clear time-dependent dequencing signal was observed. This indicates that AQP2-containing vesicles undergo Ca-dependent fusion *in vitro* with plasma membrane. When unlabeled target plasma membranes were incubated with labeled vesicles pretreated with TeNT, the fusion between the two membranes was inhibited by about 50%. In contrast, pretreatment of either plasma membrane or AQP2-containing vesicles with BoNT/C did not affect the fusion between the two interacting membranes suggesting that syintaxin-1 is not involved in this process. Altoghether these results suggest that synaptobrevin 2, probably through the interaction with the counterpart t-SNARE protein syntaxin-4, mediates the fusion between AQP2-containing vesicles and apical plasma membrane in CD8 cells.

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Vasopressin V_2-receptor dependent regulation of collecting duct AQP2 expression in Brattleboro rats

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The purpose of this study was to evaluate the role of AVP-V_2-receptor dependent regulation of AQP2 expression in vasopressin-deficient Brattleboro (BB) rats. AQP2 levels were high in BB rats corresponding to 52 " 8% of levels in normal Wistar rats. Treatment of BB rats with an AVP-V_2-receptor antagonist (SR 121463A, 0.8 mg/day) for 48 hours resulted in a significant increase in urine output to 170 " 9% of control levels. Moreover the treatment was associated with a marked reduction in total kidney AQP2 protein levels (42 " 10% vs 100 " 8%), inner medullary AQP2 protein levels (53 " 8% vs 100 " 20%) and AQP2 mRNA levels (36 " 7% vs 100 " 21%). In addition the levels of AQP2 phosphorylated in the PKA consensus site (Ser-256 of AQP2) was dramatically reduced to 3 " 1% of control levels (100 " 17%). Lithium-treatment for 1 month, known to reduce adenylyl cyclase activity, also resulted in a very extensive downregulation of total AQP2 protein levels in BB rats to 15 " 6% of levels in control BB rats (100 " 10%) and was associated with an increase in urine output to 220% of control levels. The marked downregulation of AQP2 levels in response to AVP-V_2-recepor antagonist treatment or lithium treatment strongly indicates that the high AQP2 expression in BB rats depends in part on activation of AVP-V_2-receptors and that the signaling cascade(s) involve adenylyl cyclase and hence cAMP. BB rats subjected to complete water restriction produced only a small but significant increase in AQP2 mRNA levels (235 " 33%) and total AQP2 protein levels (156 " 22%). Semiquantitative immunoelectron microscopy confirmed the increase in AQP2 abundance but revealed no significant change in the AQP2-labeling density of the apical plasma membrane of collecting duct principal cells in untreated and thirsted BB rats. Thus despite significant AVP-V_2-receptor dependent regulation of AQP2 expression in BB rats there was no AVP-V_2-receptor dependent induction of trafficking to the apical plasma membrane. In conclusion, the expression and phosphorylation of AQP2 in BB rats is in part dependent on AVP-V_2-receptor signaling, and the AVP-V_2-mediated regulation of AQP2 trafficking and AQP2 expression is effectively decoupled in BB rats indicating that there are differences in the AVP-V_2-receptor mediated regulation of AQP2 trafficking and expression.

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NHE proteins involved in the long-term regulation of aquaporin2 in primary cultured IMCD cells

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Our recently established primary cultured IMCD cell model [1] maintains long term expression of the endogenous water channel protein aquaporin2 (AQP2) without transfection of the cells with a plasmid encoding this protein. Instead, AQP2 expression in IMCD cells is stimulated by activation of protein kinase A (PKA). IMCD cells thus constitute an appropriate *in vitro* model to study the long-term regulation of AQP2. The aims of this study were to investigate culture conditions that modulate the expression of the water channel protein AQP2, and, to identify possible targets which might suggest pharmacological approaches aimed at the regulation of AQP2 expression. Two modifications of standard cell culture conditions were used for the cultivation of IMCD cells, leading to increased or decreased AQP2 expression levels respectively. We observed an upregulation of AQP2 expression under extracellular acidification (cell culture media pH 6.4). In contrast, cells cultured in media containing elevated Ca^{2+} concentrations (5 mM) had significantly reduced AQP2 expression. These findings were investigated at the protein level by Western-blot analysis and immunofluorescence microscopy, as well as at the RNA level by northern-blot analysis.

The increased AQP2 protein expression under acidic conditions was reversed back to the control levels by the inhibition of sodium proton exchangers (NHE´s) with the non-subtype-selective inhibitor ethylisopropylamiloride (EIPA). This indicates the involvement of NHE´s in the long-term regulation of AQP2 under acidic extracellular conditions. The subtypes NHE1, 2 and 4 were detected in IMCD cells by RT-PCR analysis as could be expected [2], and a subtype specific classification of the NHE´s involved in this process will follow. A decrease of AQP2 expression caused by elevated Ca^{2+}-concentrations, observed in rats after the application of dihydrotachsterole [3], was confirmed *in vitro* in IMCD cells by elevation of the extracellular Ca^{2+}-concentration from 1.8 mM to 5 mM. The signalling cascade whereby extracellular Ca^{2+} leads to decreased AQP2 expression remains elusive, but probably does not involve the inhibition of adenylyl cyclase by the G-protein $G\acute{a}_i$ *via* the Ca^{2+} sensing receptor. Interestingly, AQP2 expression was normalized to control levels when IMCD cells were challenged with 5 mM extracellular Ca^{2+} under acidic conditions, indicating the involvement of NHE proteins under such pathological conditions. The influence of lowered extracellular pH as well as of elevated Ca^{2+} concentration on the trafficking of AQP2 (short term regulation) were also investigated, but in contrast to the long-term regulation of AQP2 experiments, no effect of the above mentioned conditions on the trafficking was detectable.

Taken together these findings may establish a basis for the treatment of edematic states due to pathologically increased AQP2 expression by the pharmacological inhibition of NHE´s.

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The study of the AQP3 basolateral targeting by the two hydrid system

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The targeting of the AQP3, as well as the AQP4, in principal cells of the kidney collecting duct is constitutive and polarised to the basolateral membrane. Conversely, the AQP2 apical sorting is well known to be regulated by vasopresine [1, 2]. This different targeting pattern suggests that unknown and distinct mechanisms could be involved in the trafficking of each aquaporin to the plasma membrane.

By the two hybrid system, several reports have recently shown that the routing of different membrane proteins is accomplished by motor proteins of the cell [3,4, 5].

In this study we have envisaged the two-hybrid system in yeast in order to search for protein-protein interactions involved in AQP3 sorting. Previous results from our laboratory on chimeric constructs AQP2/3, suggested that both the amino- and carboxyl-termini might carry potential basolateral signals. Both two AQP3 cytoplasmatic termini were employed as baits in a two-hybrid system.

Firstly, a kidney cDNA library screening was conducted with a bait expressing the Cterminus, and potential interactions were found with a dynein light-intermediate chain 2 (LIC2), a synaptobrevin like protein and a kinase-anchoring like protein. The *in vivo* study of those potential AQP3-interacting proteins is now being undertaken.

On the other hand, as the N-terminus contains a potential tyrosine-based motif (19YRLL22), we study the possible protein-protein interactions with the medium subunits of the adaptor proteins AP1, AP2 and AP3, using a simple two hybrid assay. With a bait expressing the AQP3 N-terminus (1ML27) we found specific interactions with the subunits ì1 and_ì2, but not with ì3A nor ì3B.

Point directed mutagenesis have been performed in the AQP3 Nt bait (Tyr19Ala Leu22Ala), in order to know what are the essential aa for those interactions. As expected, in a two hybrid assay, the Y19A mutation abolished any interaction with both subunits, ì1 and_ì2. Interestingly, L22A substitution also resulted in a complete loss of interaction with ì2 subunit, and slightly with the ì1 one. The expression of the AQP3 Y19A single mutation does not alter significantly the basolateral targeting of the protein, but interestingly the L22A substitution directs AQP3 to the apical membrane of MDCK stably-transfected cells as well. The expression of the double mutant AQP3 Y19A/L22A is now under study, as well as pull-down assays, to demonstrate the role of the Nt, in AQP3 sorting and the *in vitro* interactions with ì1 and_ì2,_respectively.

As far as the function of these assembly proteins in the intracellular trafficking is concerned, our results suggests a crucial role for that tyrosine motif, and consequently the AQP3 tail, in the basolateral trafficking of the AQP3, as a whole or by a single di-leucine sequence (*YRLL*).

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P 60

Functional expression of aqp3 in human epidermis and keratinocyte cell cultures

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The purpose of this study was to examine the presence of aquaporin water channels in human skin and to assess their functional role. On Western blots of human epidermis obtained from plastic surgery, a strong signal was obtained with polyclonal anti-AQP3 anti-peptide antibodies, similar to the staining of human red blood cells, where AQP3 is also expressed. In indirect immunofluorescence, on 5µm cryo-sections, the anti-AQP3 antibodies strongly stained keratinocyte plasma membranes in the epidermis, whereas no staining was observed in the dermis or the stratum corneum,or when anti-AQP3 antibodies were pre-absorbed with the peptide used for immunization. Similarly, a strong signal with anti-AQP3 antibodies was observed on keratinocytes grown in culture at the air-liquid interface for up to 3 weeks. Transepidermal water permeability was measured on intact human skin, on stripped human skin and on keratinocytes in culture. Whereas intact skin was highly impermeable to water, stripping increased its water permeability. The water permeability of keratinocytes grown at the air-liquid interface decreased sharply from 0 to 21 days. Water transport across both stripped human skin and 3 week keratinocyte cultures was comparable and inhibited by ~50% in the presence of 1mM $HgCl_2$; an inhibitor of AQP3 and other aquaporins. By stopped-flow light scattering, human keratinocyte plasma membrane vesicles had a high water permeability, consistent with the presence of functional water channels. These results suggest that although human skin is highly impermeable to water, water transport in human epidermis is facilitated by the presence of AQP3 in keratinocyte plasma membranes, which may contribute to the hydration of skin.

P 61

Composition and subcellular localization of aquaporin-4 water channels

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Aquaporin water channel proteins are tetrameric assemblies of individually active ~30 kDa subunits. In mammalian brain, the predominant water channel protein is Aquaporin-4. *AQP4* encodes two distinct mRNAs with different translation initiating methionines, M1 or M23, but the expression and distribution of different AQP4 polypeptides within the brain are unknown. We identified four AQP4 polypeptides with the following levels of expression: 32 kDa > 34 kDa > 36 kDa > 38 kDa. Based on immunoreactivity, the 34 and 38 kDa polypeptides represent the predicted M1 isoform, and 32 kDa and 36 kDa correspond to the shorter M23 isoform. Immunogold electron microscopy of rat cerebellum cryosections demonstrated that both polypeptides are expressed in perivascular astrocytes in membrane regions apposed to blood vessels. Velocity sedimentation and immunoprecipitation analyses revealed that the 32 kDa and 34 kDa polypeptides reside within heterotetramers and that heterotetramer formation reflects the relative expression levels of the two polypeptides. Colocalization of AQP4 polypeptides with the PDZ domain-containing, dystrophin-associated protein complex and recognition of a potential C-terminal PDZ-binding motif in AQP4 supported the idea that dystrophin-associated proteins may constitute important determinants of AQP4 subcellular localization. Direct evidence of physical interaction between AQP4 and dystrophin-associated proteins, including the PDZ protein syntrophin, was provided by chemical crosslinking and coimmunoprecipitation. Analyses of AQP4 in transfected cells are being used to resolve the question of whether AQP4 binds directly to syntrophin through a C-terminal SSV/PDZ interaction. These studies demonstrate that AQP4 in brain is composed of heterotetramers formed from two overlapping polypeptides that differ at their amino-termini. Anchorage of AQP4 tetramers to the extracellular matrix through the dystrophin-associated protein complex may explain their perivascular organization in astrocytes and constitute a key factor in regulating the vectorial movement of water at the blood-brain interface.

P 62

Identification of a new form of AQP4 mRNA that is developmentally expressed in brain

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The water channel aquaporin 4 (AQP4) is abundantly expressed in the brain, and also in lung and kidney. Previous studies have suggested that there are at least two AQP4 mRNA. The two mRNA encode for two AQP4 proteins that differ with regard to the length of the N-terminal, AQP4 M1 and M23. Here we report, by use of RT PCR and comparison of genomic and cDNA structures, the presence of a third form of mouse AQP4 mRNA. The upstream sequence of this form of mRNA originates from an additional exon, interspaced between exon 0 and exon 1, and an alternatively spliced form of exon 1. Analysis of nucleotide sequence suggests that this new form of AQP4 mRNA also encodes for the AQP4 M23 protein. The two forms of AQP4 mRNA, that both encode for M23, have a tissue- and age-specific expression. The new AQP4 mRNA was predominately expressed in brain. The expression was approximately two-fold higher in the adult brain than in the infant brain. In contrast, the expression levels of the new mRNA were low in both infant and adult lung and kidney. The previously described mRNA encoding for AQP4 M23 was predominantly expressed in lung and kidney. In lung the expression of this form was higher in infancy than in adulthood. In conclusion, we have identified a new form of AQP4 mRNA that is predominantly expressed in the brain and that is developmentally regulated.

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Changes in AQP4 expression in rat skeletal muscle during development and after hindlimb suspension

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As we previously reported AQP4 is expressed in tipe II fibres of mammalian skeletal muscle [1]. In order to understand the physiological role of AQP4 in mammalian skeletal muscle and to determine neuronal influences in its expression pattern we investigated AQP4 expression during the first few weeks of postnatal rat muscle development and in adult rats. Semiquantitative RT-PCR and immunofluorescence experiments were used to analyze AQP4 expression of fast-twitch extensor digitorum longus (EDL) and slow twitch Soleus at days 3,6,12,18, 24, 35 and 49 after birth. Results showed that AQP4 expression in EDL increased systematically during maturation until about day 24 when approximate adult values are attained. Contrary analysis of data collected from developing Soleus revealed fluctuations in AQP4 expression. In fact, AQP4 mRNA and protein rapidly increased from day 3 to day 24 when AQP4 expression is in excess in respect of adult values. From day 24 to day 49 AQP4 expression decreased to reach adult value. During development, EDL is innervated almost entirely by fast motor units. In contrast, the Soleus is innervated by a more heterogeneous population of motor units. Thus, we suggest that AQP4 expression is different in the EDL and Soleus as a result of different types of innervating motor units in the two muscle. This conclusion is further confirmed by using the hindlimb rat suspension model in which after few weeks of tail suspension the Soleus manifests a slow to fast-twitch fiber conversion [2]. By immunofluorescence experiments we found that fast type IIA positive fibers increases dramatically in Soleus (35.3 +/- 1.6 %, n = 3, Vs 15 +/- 1 %, n = 3) after 1 week of hindlimb suspension. Conversely, the number of type I fibers decreases after suspension. IF, western blot and RT-PCR experiments showed an increases of the AQP4 expression in Soleus after hindlimb suspension parallel with the increase of fast-twitch fibers. Our results indicate that AQP4 expression in skeletal muscle can be regulated by neurotrophic elements by functional innervation.

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P 64

Two distinct signals determine the basolateral targeting of AQP4 in the renal epithelial cell line MDCK

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To fulfill their physiological role in water handling by the kidney, aquaporins are specifically expressed in either the apical and/or the basolateral membranes of kidney epithelial cells. However, the cellular mechanisms involved in the polarized membrane distribution of aquaporins in these cells remain largely unknown. To address this question we have studied the targeting of the basolateral aquaporin AQP4 in MDCK cells.

The rat AQP4 cDNA was cloned in the pcB6 expression vector and stably transfected in MDCK cells. Confocal microscopy analysis showed that AQP4 was specifically localized in the basolateral membranes, indicating that MDCK constitute an appropriate cellular model system to analyze AQP4 targeting.

Previous studies identified targeting signals in the cytoplasmic carboxy-terminal amino acid (a.a) sequences of basolateraly targeted membrane proteins. We thus focused our attention on AQP4 C-terminus. In a first step, we introduced a VSV tag on the extreme amino-terminus of the protein to enable the analysis of deletions in its C-terminal part that constitutes the epitope of AQP4 directed antibody. Confocal analysis showed that the VSV tag did not modify the basolateral targeting of AQP4. Then, several deletion mutants were established and analyzed by confocal microscopy using anti-VSV antibody. The results are summarized in the following table:

250				323	AQP4	Basolateral
250				321	AQP4-321 Stop	Baso + Intracellular
250			303		AQP4-303 Stop	Baso + Intracellular
250		282			AQP4-282 Stop	Baso + Intracellular
250	272				AQP4-272 Stop	Intracellular
250	272		302	323	AQP4-Ä272-302	Apical
250		282	302	323	AQP4-Ä282-302	Basolateral
250	272	286		323	AQP4-Ä272-286	Basolateral

The deletion of the last three a.a (321 stop) led to an increased labeling of intracellular compartments without modifying AQP4 basolateral expression. Additional deletions did not significantly modify AQP4 expression pattern until the last 52 a.a (272 stop) were deleted. In fact, AQP4-272 stop was retained in an intracellular compartment presumably the Golgi apparatus. On the other hand, the intra-molecular deletion of 31 a.a (from Q272 to I302) resulted in the complete re-routing of AQP4 to the apical membranes of the cells, whereas the deletion of either 15 or 21 a.a (AQP4 Ä272-286 and Ä282-302, respectively) did not modify the basolateral targeting of AQP4.

We conclude from these experiments: 1) the last three a.a (SSV) of AQP4, that constitute a putative PDZ interaction motif, may participate in the basolateral stabilization of AQP4 presumably through interactions with basolateral scaffolding proteins. 2) the a.a stretch between Q272 and I302 contains two distinct basolateral targeting signals. The role of potential tyrosine and/or leu-iso motifs present in these regions is now investigated.

P 65

Water permeability of aquaporin 4 expressed in renal epithelial cells is regulated by protein kinase C

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Aquaporin 4 (AQP4) is expressed in collecting ducts, in brain and in lung epithelial cells. Two isoforms of AQP4 have been identified, M1 and M23 [1,2]. We have shown [3] that AQP4 M23 is encoded by two mRNAs, one of which is mainly expressed in kidney and lung. AQP4 can be phosphorylated by protein kinase C (PKC) [4]. Here we have examined whether the water permeability (P_f) of AQP4, expressed in a kidney epithelial cell line, LLC-PK1, can be regulated by PKC. The cells were transiently transfected with AQP4 tagged with GFP at N- or C-terminus using the cDNA that encodes for kidney AQP4 M23. Confocal microscopy showed that N-tagged AQP4-GFP was almost exclusively located in the basolateral plasma membrane (BLPM). C-tagged AQP4-GFP was mainly in BLPM, but there was also a signal from the cytoplasm. Activation of PKC with the phorbol ester, PDBu (10^{-6} M), did not change the distribution of neither N- nor C- tagged AQP4. We have developed a method by which P_f can be measured in individual cells within a monolayer [5]. An image showing GFP signal distribution was recorded to identify cells that expressed AQP4. The cells were then loaded with calcein and exposed to a hypotonic shock. Dilution of calcein in the cytoplasm during cell swelling resulted in a decrease in fluorescence that was measured at 2 s intervals. The first 10 s were used for P_f calculation. P_f was compared between cells that did and that did not express AQP4. AQP4 expressing cells had significantly higher (2-fold) P_f than untransfected cells. PDBu significantly decreased P_f of AQP4 expressing cells, but had no effect on untransfected cells. These effects on cell permeability were similar for N- and C-tagged AQP4. Conclusion: Water permeability of AQP4 M23, expressed in renal epithelial cells, is decreased by PKC activation. The effect is likely mediated by gating of the channel via phosphorylation reactions.

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P 66

Co-expression studies in oocytes suggest that the basolateral localization of Aquaporin-2 in renal collecting ducts is not mediated by hetero-tetramerization with Aquaporin-3 or Aquaporin-4

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Aquaporin-2 (AQP2) is the vasopressin-regulated water channel in the collecting duct cells of the kidney. Upon binding of vasopressin to its V2 receptor, adenylate cyclase is activated, cAMP is formed, resulting in an activation of protein kinase A. Consequently, vesicles containing AQP2 fuse with the apical membrane, rendering these cells water permeable. Driven by an osmotic gradient, water that apically enters the cell via AQP2, exits the cell via AQP3 and AQP4, which are located in the basolateral membrane. Recent immuno-histochemical data revealed that sometimes AQP2 is also found in the basolateral membrane, which might indicate that an alternative targeting mechanism is employed. Of potassium channels it well established that they are expressed as homotetramers or heterotetramers. In the latter case, members of different subfamilies of potassium channels might be involved. Of AQP2, and AQP4 it is known and for AQP3 it is likely that they form homotetramers.

Therefore, basolateral routing of AQP2 could be caused by hetero-tetramerization with AQP3 and AQP4. To test this hypothesis, oocytes were first injected with cRNAs coding for AQP2, AQP3 or AQP4. Functional analysis showed that all aquaporins conferred water permeability to the oocytes. Sucrose gradient centrifugation of total membranes of these oocytes solubilized in 4% desoxycholate and subsequent immunoblotting revealed that each AQP sedimented as a homotetramer. This indicated that in 4% desoxycholate AQP2, AQP3 and AQP4 are present as tetramers. Subsequently, oocytes were injected with cRNAs encoding AQP2, AQP3 and AQP4, total membranes were solubilized in desoxycholate and subjected to immunoprecipitation with antibodies specifically recognizing AQP2, AQP3 or AQP4. Immunoblotting of these immuno-precipitates with antibodies recognizing AQP2, AQP3 or AQP4 revealed that neither AQP co-precipitated with an AQP from another subfamily. These results suggest that heterotetramerization of AQP2 with AQP3 or AQP4 is not the mechanism for basolateral location of AQP2.

P 67

Persistent increase in the amount of AQP5 in the apical plasma membrane of rat parotid acinar cells induced by the muscarinic receptor agonist SNI-2011

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We demonstrated that acetylcholine (ACh) and epinephrine (Epi), acting at M_3 muscarinic and á$_1$-adrenergic receptors, respectively, induced the increases in the amount of AQP5 in the apical plasma membrane (APM) [1, 2]. The intravenous administration of SNI-2011, (")-cis-2-methylspilo(1,3-oxathiolane-5,3')quinuclidine, was reported to induce long-lasting salivation in rats and dogs [3], but the mechanisms have not been clarified. We have now investigated the effect of SNI-2011 on the amount of AQP5 in the APM in rat parotid cells and compared it with that of ACh or pilocarpine [4]. The treatment of rat parotid tissues with SNI-2011 or pilocarpine increases in the amount of AQP5 in the APM in a dose-dependent manner. SNI-2011- or pilocarpine-induced increase in the amount of AQP5 was maximal at 10 min or 3 min, respectively, and persisted for longer than 30 min. This effect was inhibited by *p*-F-HHSiD, showing that these drugs act at M_3 muscarinic receptors. SNI-2011-induced the increase in the amount of AQP5 in the APM was also inhibited by U73122, dantrolene, or TMB-8, but not GF109203X or H-7, suggesting that activation of phospholipase C but not protein kinase C lead to this induction via the elevation of $[Ca^{2+}]i$. This finding was supported by the result that the treatment of the tissues with phorbol 12-myristate 13-acetate did not induce it. Cytochalasin D and tubulozole-C also inhibited SNI-2011- and pilocarpine-induced translocation of AQP5 to APM. In the presence of extracellular $CaCl_2$, SNI-2011 induced marked and persistent oscillations in $[Ca^{2+}]i$ in isolated parotid acinar cells.

These results suggest that SNI-2011 induces a persistent increase in the amount of AQP5 in the APM of rat parotid cells by acting at M_3 muscarinic receptors to activate the IP_3 and ryanodine receptors and results in the long-lasting elevation of $[Ca^{2+}]i$. In addition, cytoskeleton elements involved in this induction, but protein kinase C did not have a regulatory role on AQP5 trafficking in rat parotid cells.

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P 68

Coupling of aquaporin-5 and lipocalin controls tear secretion

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Tear production is drastically increased by emotional excitation or reflex response to nociceptive stimulation in the eye. The most abundant secretory "product" in the tear is water. This rapid and abundant transcellular water transport is thought to be mediated by AQP5, which is a functional water-selective molecular channel in the apical membrane of lacrimal cells. However, its gating mechanism is not known. Here we show that odorant-binding protein-1 (OBP-1), a lipocalin-like protein, controls AQP5-mediated water transport in mouse lacrimal glands. OBP-1 and its associated protein binds reversibly to the intracellular C-terminal domain of AQP5, and causes closure of the channels. Cyclic AMP stimulation, which causes rapid tear secretion, induces phosphorylation of the AQP5 followed by its dissociation. Evidence for this gating function is that reconstitution of the complex is blocked by AQP5-mimetic peptides and (-)-carvone, mint-like odorant, which produces lacrimation in mice. Thus, gating of AQP5 water channel is a consequence of changes in response to PKA-mediated phosphorylation, and is regulated by the functional inhibitory subunits. These results provide a new model to the gating mechanism of the water channels for fluid secretion.

P 69

Aquaporin 5 is phosphorylated in response to interferon-á treatment of human parotid gland cells *in vitro*

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Aquaporins are a large and exceptionally conserved family of homologous membrane proteins that function as highly selective water channels. Aquaporin-5 (AQP5) is unique in that it is the only aquaporin present in lacrimal and salivary glands, where it accounts for normal tear and saliva production. Our studies suggest that oral interferon-á (IFN-á) may benefit patients with xerostomia by upregulating the transcription and translation of AQP5 in epithelial cells of parotid gland. We have shown previously that IFN-á consistently augmented AQP5 transcription and translation in a concentration dependent manner in cells obtained from parotid glands of three different patients [1]. Using cells from human parotid glands (three different patients), we now show that in response to 2 hr incubations in1,000 IU/ml of human lymphoblastoid IFN-á, AQP5 is phosphorylated. Phosphorylation was studied via 2-dimensional polyacrylamide gel electrophoresis followed by fluorography as well as by Western Blot.

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P 70

The kidney in the inner ear: aspects of the endolymph regulation

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The tight regulation of the endolymph fluid of the inner ear is of utmost importance for sensing sound as well as gravity and acceleration. The endolymph is an extracellular fluid with high potassium (150mM) and low sodium (5mM) content which is trapped in a closed compartment of about 40ìl in human (2ìl in rat). Disturbances in the production or resorption of the endolymph resulting in an overpressure (endolymphatic hydrops) can lead to typical symptoms of Ménière's disease, such as tinnitus, hearing loss and vertigo attacks.

Using RT-PCR we quantitatively determined the expression pattern of aquaporin water channels (AQP) in functionally distinct tissues of the inner ear [1]. AQP1 was ubiquitous. AQP2, AQP3, and AQP4 were present in the endolymphatic sac. AQP5 appeared in the organ of Corti and Reissner's membrane. The regulation of AQP2 by vasopressin and the resulting vesicle transport are well understood in the kidney [2]. By RT-PCR, we were able to identify the vasopressin V_2-receptor as well as the vesicle transport components VAMP-2, syntaxin 4 and rab3a in the endolymphatic sac [1,3]. In physiological studies using primary tissue cultures of the endolymphatic sac vasopressin was found to have an opposite effect on the membrane turnover compared to the kidney collecting duct [3]. This was shown by a reduced uptake of FITC-dextran from the culture media after addition of 1nM vasopressin or 50ìM forskolin. Finally, guinea pigs treated systemically with vasopressin developed a massive endolymphatic hydrops and suffered from vertigo.

In order to study the AQP regulation of the endolymphatic sac in greater detail, an immortalized cell line (mES12) from the transgenic Immorto mouse was established. The characterization of the cell line is in progress.

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P 71

Expression and localization of aquaporins in rat exocrine pancreas

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The exocrine pancreas secretes a bicarbonate-rich fluid containing digestive enzymes. Given the fluid secreting capabilities of the pancreas, the expression of one or more aquaporins within this tissue would be expected. As the exocrine pancreas is composed of acinar and ductal cells that contain both apical (luminal) and basolateral membranes, expression of aquaporins on these membranes could contribute to an transepithelial route of water transport. While Koyama *et al*, (1997) reported the cloning of AQP8 from the rat pancreas, its exact localization within this tissue has not been determined.

The aim of this study was to use molecular, immunohistochemical and immunoelectron microscopy techniques to investigate the expression and localization of aquaporins in rat pancreas. RT-PCR analysis resulted in the amplification of four aquaporins, AQP1, AQP4, AQP5 and AQP8 from rat pancreas cDNA. High stringency Northern analysis was carried out using ^{32}P labeled probes generated from tissues know to express specific aquaporins. Transcripts of the expected size were detected for AQP8 (~1.45 kb) and AQP1 (~2.6 kb) but not AQP4 and AQP5. Immunohistochemistry of the rat pancreas demonstrated the discrete localization of AQP1 to microvasculature between pancreatic acini whereas AQP8 localized to the apical regions of pancreatic acinar cells. Further experiments carried out using immunoelectron microscopy defined AQP8 labeling at the apical plasma membrane of the acinar cells, with considerable labeling within intracellular vesicles in the subapical part of the acinar cell. The localization of AQP8 within intracellular vesicles suggests a possibility for recycling of AQP8 from apical plasma membranes following exocytosis of pancreatic secretions.

Koyama, Y., Yamamoto, T., Kondo, D., Funaki, H., Yaoita, E., Kawasaki, K., Sato, N., Hatakeyama, K. and Kihars, I. (1997) *J. Biol. Chem.*, 272 (8), 30329-30333.

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P 72

Complementary expression of AQP8 and AQP7 in rat testis

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The process of spermatogenesis by which spermatogonia differentiate to mature spermatozoa is characterized by a striking reduction in germ cells volume. This major morphological event has been explained as mostly due to a conspicuous efflux of water out of the cells. A strong support to this assumption was provided by the recent identification of the AQP8 aquaporin water channel and the AQP7 and rAQP9L aquaglyceroporins in rat testis. Aquaporin-7 has been suggested to contribute to the volume reduction of round spermatids during rat spermiogenesis, however, the role of a major water channel such as AQP8 in testis physiology is still unknown. Hence, we evaluated the possible functional involvement of AQP8 in spermatogenesis by studying the specific expression of the *AQP8* mRNA and protein in rat seminiferous tubule and epididymis. Moreover, the expression of *AQP8* was compared to that of *AQP7*. Immunoblotting of rat seminiferous tubule membranes tested with a polyclonal antibody specific for the C-terminal of the rat AQP8 revealed a band of about 25 kDa and a 32-38 kDa component likely corresponding to the expected glycosylated form of AQP8. A similar pattern was also obtained by immunoblotting of mouse seminiferous tubule membranes. Immunofluorescence using affinity purified anti-AQP8 antibodies showed intracellular immunoreactivity in all stages of spermatogenesis although a stronger staining was observed at the basal portion of all tubules where spermatogonia and primary spermatocytes are located. This result is in agreement with previous *in situ* hybridization data by Ishibashi *et al.* (1997). Faint AQP8-immunoreaction was associated to mature spermatozoa in epididymis. No apparent staining was observed in control experiments using preimmune serum instead of primary antibody. Interestingly, a nearly complementary pattern of immunostaining was observed with affinity purified anti-AQP7 antibodies since an intense plasma membrane labeling was seen in the most advanced spermatids and a faint immunoreaction at the early stages of spermatogenic cycle. In epididymis, the anti-AQP7 antibodies stained intensely the plasma membrane enveloping the head, middle piece, and principal piece of mature spermatozoa. These immunocytochemical data were fully consistent with immunoblotting analyses by which the AQP8 detection was strong in seminiferous tubules but very weak in epididymis whereas AQP7 bands of comparable intensity were detected in both locations. RT-PCR and RNase protection studies showed that the distribution of the *AQP8* and *AQP7* mRNAs in rat testis reflects that of the related proteins. Although the role of aquaporins in spermatogenesis is not yet elucidated, definition of the expression and distribution of aquaporins in testis represents a fundamental step for future functional studies. Useful information on the physiological significance of aquaporins in male reproductive tract may also derive from the development of targeted gene disruption in mice.

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P 73

Localization of aquaporin-8 (AQP8) mRNA and protein in rat kidney and other organs as determined by RT-PCR, immunoblotting and immunocytochemistry

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Discovery of aquaporin water channel proteins has provided insight into the molecular mechanism of membrane water permeability. The aim of this study was to determine the cellular and subcellular localization of aquaporin 8 in rat kidney and other organs by RT-PCR, immunoblotting and immunohistochemistry. To analyze this we developed peptide derived rabbit antibodies to rat AQP8 (amino acids 249 - 263). Affinity purified antibodies to AQP8 revealed a predominant band of 27-28 kDa in membranes of total rat kidney. Immunoblotting using membrane fractions revealed modest AQP8 labeling in cortex,outer stripe and inner stripe of outer medulla and inner medulla.

Membrane fractionation revealed predominant AQP8 labeling of membrane fractions enriched with intracellular vesicles. In contrast AQP1 was mainly present in membrane fractions enriched with plasma membrane. Peptide-absorption of the antibody eliminated the labeling. Immunoblotting also revealed labeling of AQP8 in liver, testes, epididymis, small intestine and colon.

RT-PCR data showed AQP8 to be localized in cortex, outer stripe and inner stripe of outer medulla and inner medulla.

Immunohistochemistry revealed modest labeling of proximal tubules in cortex and medulla of rat kidney. The labeling was confined to cytoplasmic areas of the proximal tubule cells with no labeling of the brush border. Antibody preabsorped with the immunizing peptide revealed no labeling. Further more immunohistochemistry revealed AQP8 to be localized in the hepatocytes, the surface epithelial cells in the colon and small intestine, the spematogenic cells in testes and in the duct cells of epididymis.

In conclusion , AQP8 proteins appears to be expressed in rat kidney, liver, small intestine, colon, testes and epididymis.

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Localization of aquaporin-9 (AQP9) protein in rat liver, testes, epididymis and spleen as determined by immunoblotting and immunuhistochemistry

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P 75

Localization of AQP7 in rat and mouse kidney using RT-PCR, immunocytochemistry and immunoblotting

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P 76

Water permeability in rat oocytes at different maturity stages: Aquaporin-9 expression

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Important functional and structural modifications occur in oocytes during their arrival to maturity. In this process, oocytes switch from a high activity level, implying an important metabolic rate and a coordinated movement of water and solutes, to a lower functional state. We have previously demonstrated that a water pathway is present in *Bufo arenarum* ovarian oocytes [1]. This water pathway is regulated *in vitro* by progesterone at the transcription level [2]. The aim of our present work is to study the mechanisms involved in water movements during mammalian oocyte arrival to maturity. For that purpose volume changes, induced by an osmotic gradient, were followed by video microscopy in rat oocytes. The water osmotic permeability (P_{osm}) of immature oocytes (proestrus) was sensitive to $HgCl_2$ and phloretin. In contrast, mature oocytes (estrus) had a reduced P_{osm} that was not sensitive to these compounds. When proestrus oocytes were incubated *in vitro* at 37ºC they spontaneously arrived to maturity and its P_{osm} decreased between four and six hours of incubation. RT-PCR experiments were performed using specific primers for all rat aquaporins at present cloned. We found that aquaporin-9 transcript (AQP9) is present in proestrus oocytes but not in estrus ones. As AQP9 has been recently described as a "broad selective channel" responsible for solute and water transfers in highly active cells, we had also studied the mannitol permeability in the two stages of maturation and we found difference between proestus and estrus oocytes. Immature oocytes appear to be more permeable than matured ones. The disappearance of AQP9 mRNA at later developmental stages was also observed by Tsukaguchi [3] in spermatocytes, indicating a parallelism in AQP9 mRNA expression between female and male germinal cells. We can speculate that AQP9 expression in immature stages would be necessary during oocyte growth. At the moment of ovulation the lost of AQP9 may serve as defense mechanisms to protect the delicate oocyte from the acute changes that occur during follicular wall disruption.

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P 77

The identification of new superfamilies related to MIP proteins has expanded the mammalian aquaporins to AQP12

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MIP proteins typically have two relatively hydrophobic domains named NPA boxes (NPA-NPA). The amino acid sequences around NPA are well conserved, which are the signature sequences for MIP proteins. Here we identified new proteins with six transmembranes related to MIP proteins. However, they have poorly conserved NPA boxes. They include AQP10, AQP12 from mammals, Ce-1, Ce-2 from C. elegans, and Dros2 from Drosophila (arbitrarily named). Their corresponding NPA boxes are NPC-NPA, NPT-NPA, SPL-DPL, NCA-NPI, and CPY-NPV, respectively. None the less, they should be grouped as new branches of MIP family because the transmembrane domains (especially TM3 and TM6) are homologous to MIP proteins. Moreover, they share some functional similarities with MIP proteins. AQP10 functions as a water-selective aquaporin when expressed in Xenopus oocytes (JASN 10: 16A, 1999). Ce-2 functions as an acid-sensitive anion channel when expressed in CHO cells similar to AQP6. As the homology between these new proteins are less than 30 %, each may represent a new superfamily. These new proteins will expand the frame of references for MIP proteins to discard NPA boxes as the signature sequences. The more general signature sequences will be TM3 and TM6. Interestingly, no homologs of these superfamilies are found in bacteria and yeasts, suggesting the relatively recent deviation from MIP family, possibly first in multicellular organisms. The list of new members are now increasing with on-going genome projects. Further screening and characterization of these superfamilies will lead to deeper understandings of water and solutes transports. For example, AQP12 is selectively expressed in pancreas and will be a bona fide aquaporin for pancreatic secretion regulated by GI hormones. These superfamilies will herald the diversity of MIP family as is the case with potassium channels, and shed light on the search for functional domains and on the evolution of MIP proteins.

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A mercury-sensitive water channel in the spinal cord revealed by optical imaging

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Several different water channels are known to exist in the central nervous system, but their physiological roles are not clear [1, 2]. We applied an optical imaging technique to a slice preparation to measure intrinsic optical signals (IOSs), which reflected cell swelling, and investigated the localization and the function of water channels in rat spinal cords. Brief perfusion with hypotonic solutions (omission of mannitol) induced IOSs that were restricted in the superficial layers of the dorsal horn. This observation showed that these areas of the spinal cord were very sensitive to osmotic changes, and suggested the presence of a water channel. Incubation of slices with the water channel blocker Hg^{2+} or *p*-chloromercuribenzenesulfonate inhibited the hypotonic-induced IOSs. The inhibition of IOSs by Hg^{2+} was reversed by the addition of mercaptoethanol. Repetitive stimulation (20 Hz for 1 s) of primary-afferent fibers in the dorsal root elicited IOSs in the superficial layers of the dorsal horn. When examined on the same slices, both stimulation- and hypotonic-induced IOSs were inhibited by Hg^{2+} in a mercaptoethanol-sensitive manner. These results suggest that a mercury-sensitive water channel exists in the spinal dorsal horn and that high afferent input triggers water influx through the channel.

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P 79

Epithelial layers could transport fluid via cyclic volume regulatory changes

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How epithelia couple solute and water transports is a key question remaining in epithelial physiology. In classical models, steady state solute transport results in local osmotic gradients. However, this fails to account for transport of isotonic fluid. Ussing and Eskesen (APS, 1989;136:443-54) stated that "isotonic transport via a cell is impossible". Alternative proposals involve electroosmosis and molecular osmotic engines [1]; however, some results appear inconsistent with electroosmosis, and there is still no sufficient experimental evidence to assess molecular engines. In spite of recent contributions [4], uncertainties persist.

We are proposing a novel model [2, 6] in which fluid transport may result from oscillatory cell volume regulatory changes under isotonicity. In our view, plasma membrane transporters and channels involved in volume regulation of epithelial cells are polarized so that, as a rule, in secretory epithelia, activated basolateral transporters would elicit RVI and apical ones RVD (and conversely for absorptive epithelia), adding up to vectorial transfer of fluid and electrolytes. The presence of RVI and RVD processes implies that the cell transport and water permeability parameters depend on the cell volume and exhibit bistability and hysteresis. This is sufficient to give rise to a cyclic mechanism. Epithelial cells with volume-sensitive solute and water channels (displaying bistability and hysteresis) can behave as a relaxation oscillator resulting in transepithelial pulsatile transport of fluid with a wide osmolarity range. We have solved numerically and analytically equations for time-dependence cell volume and concentration. Oscillations or relaxations (i.e. simple volume regulation) develop when activation reaches a critical value (periodic attractor). We analyze the quantitative dependence of oscillation period, fluid transport and solute fluxes on the activation parameters. A representative rate of fluid transport is 3.5 ìL/(hr Hcm^2), which is consistent with experimental measurements in epithelia.

Consistent with these ideas, induced osmotic swelling led to cyclic vectorial flow across a tandem of coupled pigmented-non pigmented cells from ciliary body [5]. There are also many reports of cellular oscillatory phenomena in fluid transporting layers, such as oscillations for $[Ca^{2+}]_i$ and its mobilizing agents, K^+ currents, resting cell membrane potential oscillates in response to cell shrinkage, protein phosphorylation (in conjunction with cell volume regulatory). Plus following anisosmotic challenge, small amplitude volume oscillations are visible in recordings from our laboratory and another one. Similarly, activation (~1.34X) of osmotic permeability of oocytes expressing AQP1 have been described by another laboratory and ourselves [3].

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Molecular characterization of a new UTA urea transporter isoform (UTA-5) in testis

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Urea movement across plasma membranes is modulated by specialised transporter proteins that play key roles in fluid homeostasis. In mammals they are derived from two genes, UTA (*Slc14a1)* and UTB (*Slc14a2)*. These genes give rise to proteins which share a high degree of homology and are functionally similar. Recent studies in our group have identified a unique mRNA species homologous to UTA in mouse testis. The protein this transcript encodes may play a role in urea movement across the blood-testis barrier and in so doing influence fluid movement into the seminiferous tubules (SMT). Our aim was to determine the molecular structure of the transcript, the cells in which it is located, and to see whether it is implicated in the intratubular accumulation of urea in the SMT.

High-stringency screening of a mouse testes cDNA library with a full-length mouse UTA-1 cDNA probe yielded a 1,446-bp cDNA. Analysis of the nucleotide sequence revealed a putative open reading frame (ORF) that predicts a 323-residue protein which we named UTA-5. The ORF is flanked by a 237bp 5N UTR with no homology to the known UTA transcripts and a 235bp 3N UTR which is identical to the 3' UTR of mouse UTA-3 (Howorth, unpublished data).

Expression of cRNA encoding UTA-5 in *Xenopus* oocytes increased urea uptake by ~3.25 fold above water injected levels. Characteristically, phloretin reduced urea uptake in oocytes expressing rat UTA-2 or UTA-5. Expression of rat UTA-2 or UTA-5 had no effect on oocyte osmotic water permeability. These properties are consistent with those of previously characterised mammalian, phloretin-sensitive, facilitative urea transporters.

High stringency northern analysis of mouse poly A+ RNA using the unique 5' UTR of UTA-5 as a probe gave a strong signal at 1.4kb in testes. This transcript was not detected in any other tissue analysed, including epididymis. *In situ* hybridisation with a UTA-5 antisense probe revealed strong signals in SMTs. Closer examination showed the signal resided in the peritubular cells that form the outermost layer of the tubules. All tubules were heavily stained, indicating that expression of UTA-5 mRNA, unlike UTB expression in the Sertoli cells, is not associated with the stage of spermatogenesis of the SMT.

Global representative PCR amplification of poly A+ RNA from testis of mice from 0 to 35 days post partum showed that UTA-5 mRNA appeared between 15 and 20 days post partum and was fully expressed at 30 days. This pattern of expression correlates with formation of the blood-testis barrier and initiation of fluid movement into the SMTs.

In summary, we have isolated and characterized a novel isoform (UTA-5) of the UTA family of urea transporters. UTA-5 expression is restricted to the testes, specifically the peritubular cells of the SMTs. Appearance of UTA-5 mRNA in the testis correlates with the initiation of fluid movement into the SMTs, implicating UTA-5 in this process.

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Expression and regulation of aquaporin-1 and endothelial nitric oxide synthase in relationship with water permeability across the peritoneum

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In uremic patients treated by peritoneal dialysis (PD), net ultrafiltration (UF) is the difference between transcapillary UF (TCUF) and reabsorption of water through lymphatics. According to the three-pore model [1], TCUF is mainly due to a water transfer across transcellular, ultrasmall pores, driven by the osmotic agent present in the dialysate (e.g. glucose). The ultrasmall pores, permeable only to water, are now widely accepted to be aquaporin-1 (AQP1). In situations such as acute peritonitis or long-term PD, a decrease in TCUF is observed. That UF failure might be due either to a decreased transcellular water permeability or, alternatively, to an increase in glucose permeability secondary to augmented endothelial surface area. To substantiate the molecular mechanisms involved in normal and altered UF across the peritoneum, we investigated the expression of AQP1 and endothelial nitric oxide synthase (eNOS) in human and rat peritoneum, in correlation with peritoneal permeability parameters and NOS activities.

Immunoblot analyses identified both AQP1 (28 and 35-50 kDa) and eNOS (140 kDa) in normal human and rat peritoneum extracts. Immunostaining showed that AQP1 was specifically located in endothelia lining peritoneal capillaries and venules but not in small arteries. In contrast, eNOS was located in all types of endothelia. No specific staining for AQP2, AQP3 and AQP4 was documented in peritoneal endothelia. The staining for AQP1 and its expression pattern on immunoblot were remarkably stable in a large series of peritoneal samples obtained from controls, uremic patients treated or not by PD, and patients with ascites and/or peritonitis. That stability contrasted with eNOS, which was upregulated in case of peritoneal inflammation [2]. The differential regulation of AQP1 and eNOS was further illustrated in a long-term PD patient, in whom TCUF failure (documented by a loss of sodium sieving) was associated with an apparently normal expression of AQP1 together with eNOS upregulation [3]. We next used a rat model to show that the UF failure induced by acute peritonitis did not correlate with changes in AQP1 expression but rather with a major increase in peritoneal NOS activity, the latter being mediated by both inducible and endothelial NOS [4]. Increased NOS activity was reflected by a significant staining for nitrotyrosine along the endothelium [4]. By inference, it was postulated that increased NOS might induce nitrosylation of critical residues within AQP1. Preliminary experiments have shown indeed a substantial decrease of water permeability following exposure of AQP1-expressing *Xenopus* oocytes to NO donors.

These data (i) support AQP1 as the molecular counterpart of the ultrasmall pore in normal and diseased human peritoneum; (ii) show that AQP1 and eNOS are differentially regulated in clinical conditions associated with a loss of of water permeability across the peritoneum; (iii) suggest that changes in NO-mediated vascular tone and permeability or, alternatively, in AQP1 structure might be involved in the loss of UF.

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P 82

Expression of aquaporin and urea transporter proteins in renal medulla during cisplatin-induced polyuria in rats

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Cisplatin (*cis*-dichlorodiammine platinum II; CP) is a potent antineoplastic agent, whose clinical use is limited by its renal toxicity, leading to acute renal failure. Patho-logicallly, CP toxicity in both humans and laboratory animals is characterized by damage to the S3 segment of the proximal tubule. Functionally, CP nephrotoxicity manifests as a reduction in glomerular filtration rate (GFR), paradoxically, associated with a persistent polyuria and marked defect in urine concentrating ability. CP-induced polyuria is attributed to an end organ resis-tance to vasopressin (reduced cAMP generation) as well as to a reduction in medullary hyper-tonicity. The maintenance of medullary hypertonicity requires efficient recycling of urea and the generation of urea concentration gradient. However, the most prominent abnormality of solute transport during CP-induced polyuria is the lack of addition of urea to fluid in the loop of Henle. Instead, urea is reabsorbed from the loop fluid, resulting in a reversal of urea concentration gradient in the medulla. So, in order to understand the molecular basis of CP-induced end organ resistance to vasopressin, and the reversal of urea concentration gradient, we determined the protein abundance of AQP1, AQP2 and AQP3 water channels, and urea transporters, UT-A (expressed in the loop of Henle and collecting duct), and UT-B (expressed in the descending vasa recta) in the renal medulla of rats during CP-induced polyruria. Groups of male Sprague-Dawley rats (250-310 g bw) received either a single injection of CP (5 mg/kg; n = 4 or 6) or saline (n = 4 or 6) IP, and were sacrificed on day 5. CP-treated rats showed a 4.5-fold increase in urine volume associated with a 4-fold decrease in urine osmolality as compared to the controls. Blood urea nitrogen showed a 4-fold increase in CP-treated rats. Immnoblots using peptide-derived polyclonal antibodies showed that AQP2 and AQP3 protein abundance was significantly reduced to 33% ($P < 0.001$) and 69% ($P < 0.05$) respectively in the inner medulla of CP-treated rats as compared to controls. AQP1 protein abundance was modestly (67%; $P = 0.057$) and significantly (53%; $P < 0.007$) decreased in outer and inner medullae respectively of CP-treated rats. Immunoblots revealed no significant alterations in the abundance of UT-A1, UT-A2 and UT-A4 proteins in the outer and inner medullae. However, the band pattern of UT-A2 or UT-A4 protein in the outer medulla of CP-treated group markedly differed from that of controls, suggesting a qualitative change or modification of these proteins. UT-B protein abundance in the outer medulla was increased by 31% in CP-treated rats as compared to the controls ($P = 0.053$). These data indicate that (i) CP-induced polyuria in rats is associated with a significant decrease in the abundance of collecting duct (AQP2 and AQP3) and proximal nephron and microvascular (AQP1) water channels in the inner medulla, and (ii) the reversal of urea concentration gradient seen during CP-induced polyuria is not associated with significant quantitative alterations in the expression of UT proteins, but suggest that it may be related to qualitative changes in UT-A2 and/or UT-A4 proteins.

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Downregulation of AQP1, AQP2, and AQP3 in rats with bilateral ureteral obstruction (BUO) is associated with polyuria

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Previously we have shown that BUO and release of BUO (BUO-R) is associated with persistant downregulation of AQP2 and postobstructive polyuria [1]. To further examine the role of renal aquaporins in postobstructive diureses the effects of a 24 h bilateral ureteral obstruction (BUO), and (BUO-R) for 5 and 48 h on renal aquaporins (AQPs) and urine output (UO) were examined in a rat model. Membranes from kidney inner medulla and whole kidney were prepared and AQP2, AQP3 and AQP1 were determined by immunoblotting. Twenty-four hours of BUO resulted in a marked downregulation of AQP2 (13"4% of sham levels, $p < 0.05$), AQP3 (19"4% of sham levels, $p < 0.05$) and AQP1 (48"7% of sham levels, $p < 0.05$). Consistent with previous observations release of BUO was associated with a dramatic polyuria (125"17 vs. 25"4 Fl/min/kg, $p < 0.05$) and reduced urine osmolality (559"38 vs. 2262"232 mOsmol/kgH_2O in controls, $p < 0.05$). Immunoblotting demonstrated a significant downregulation in protein expression of all 3 AQP´s 5 hours after release of BUO: AQP2 (32"11% vs. 100"4%), AQP3 (14"5% vs.100"14%), and AQP1 (7"2% vs.100"22%). Forty-eight hours after release all 3 AQPs remained significantly downregulated: AQP2 (13"5% vs. 100"1%), AQP3 (10"5% vs.100"1%), and AQP1 (24"5% vs.100"10%) concurrent with a persistant polyuria (104"10 vs. 30"3 Fl/min/kg, $p < 0.05$), and a marked reduction in free water clearance (-64"11 vs. -181"8 Fl/min/kg, $p < 0.05$) suggesting a defect in the water reabsorptive capacity at the collecting duct level. In conclusion, BUO and BUO-R is associated with a marked dysregulation of AQP1, AQP2 and AQP3, coincident with the development of postobstructive polyuria suggesting that aquaporins located both to the collecting duct and the proximal tubule and descending thin limb of Henle all are involved in the development of postobstructive polyuria.

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Dysregulation of renal aquaporins and sodium transporters in Brattleboro rats with bilateral ureteral obstruction

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We have previously shown that bilateral ureteral obstruction (BUO) is associated with downregulation of AQP1, AQP2 and AQP3 which persists after release of obstruction concommittant with a dramatic polyuria. In order to further examine the mechanisms involved in regulation of renal aquaporin expression the effect of 24 hours BUO was studied in Brattleboro rats which manifest diabetes insipidus due to a complete lack of vasopressin secretion. Membranes from kidney inner medulla and whole kidney were prepared and expression of AQP1, AQP2 and AQP3 was determined by immunoblotting. The expression of AQP2 and AQP3 in inner medulla was markedly decreased (AQP2: 33"9% vs. 100"9%, $p < 0.05$; AQP3:19"7% vs. 100"6%, $p < 0.05$) and the expression of AQP1 in whole kidney was also significantly reduced (32"4% vs. 100"8%, $p < 0.05$). Furthermore, Na-K-ATPase, BSC1 and Tamm Horsfall Protein (THP) was examined in membranes from whole kidney. Na-K-ATPase expression in whole kidney decreased (61"4% vs. 100"7%, $p < 0.05$), and BSC-1 expression in inner stripe of outer medulla was dramatically reduced (7"2% vs. 100"5%, $p < 0.05$). In contrast, the expression of THP was significantly inceased compared with sham control rats (124"6% vs. 100"4%, $p < 0.05$). BUO was associated with increased plasma osmolality (362"6.5 mOsm/kgH2O vs. 311"1.6 mOsm/kgH2O), and plasma creatinine increased dramatically (360"2.2 vs. 39"1.5 Fmol/l, $p < 0.05$). Plasma sodium decreased and plasma potassium increased markedly. In conclusion, renal aquaporins (AQPs) and solute transporters in BB rats with BUO are downregulated similar to levels observed in Wistar rats with BUO suggesting that 1) both AQPs and solute transporters are downregulated in response to BUO and 2) downregulation of AQP1 and BSC1 is independent of vasopressin levels.

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Altered expression of renal aquaporins and Na^+ transporters in rats with lithium-induced nephrogenic diabetes insipidus

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Lithium (Li) treatment is often associated with nephrogenic diabetes insipidus (NDI). Previously we demonstrated that Li-induced NDI in rats is associated with severe downregulation of inner medullary AQP2 (Marples et al, *J.Clin.Invest*., 1995). However it is unknown whether other renal aquaporins or Na^+ transporters may also play important roles in Li-induced NDI. We examined the changes in whole kidney expression of AQP1, -2, -3, and -4 as well as Na,K-ATPase, NHE-3, NaPi-2, BSC-1, and TSC in rats treated with Li orally for 4 weeks in two protocols: *Protocol 1)* high doses of lithium (access to solid NaCl). *Protocol 2*) low doses of lithium (Li-treated rats received same amount of food resulted in equal sodium intake compared to controls). Both protocols resulted in a severe polyuria. Consistent with this, urine osmolality, urine-to-plasma osmolality ratio, and solute-free water reabsorption were markedly reduced. Semiquantitative immunoblotting revealed that whole kidney abundance of AQP2 was dramatically reduced in both protocols to 6 " 1% (protocol 1, n = 14) and 27 " 5% (protocol 2, n = 6) of control levels(protocol 1, n = 13 and protocol 2, n = 6), respectively, $P < 0.05$. In addition, whole kidney AQP3 abundance was also dramatically reduced in Li-treated rats to 6 " 2% (protocol 1) and 14 " 4% (protocol 2) of control levels, respectively, $P < 0.05$. In contrast, the abundance of the proximal nephron water channel AQP1 was not decreased in either protocols. Immunoelectron microscopy confirmed the dramatic downregulation of AQP2 and AQP3 whereas AQP4 labeling was not reduced in rats with Li-induced NDI. Li-treated rats had a marked increase in urinary sodium excretion in both protocols, even when sodium intake was matched. However, the expression of several major Na^+ transporters in the proximal tubule, loop of Henle, and distal convoluted tubule was unchanged in protocol 2, whereas in protocol 1, significantly increased NHE-3 and BSC-1 or reduced NaPi-2 expression was associated with chronic Li therapy. In conclusion, severe downregulation of AQP2 as well as AQP3 appears to play crucial roles for the development of Li-induced NDI. Reduced NaPi-2 expression may also contribute to the development of polyuria. In contrast, the increased or unchanged expression of NHE-3, BSC-1, Na,K-ATPase and TSC indicates that these Na^+ transporters do not participate in the development of Li-induced polyuria, but rather, the increased expressions may reflect secondary compensatory mechanisms in the setting of marked polyuria and natriuresis induced by chronic Li treatment.

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Altered expression of renal AQP2 and Na,K-ATPase mRNA in rats with lithium-induced nephrogenic diabetes insipidus

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Lithium treatment is commonly used against bipolar affective disorders. A side effect is the development of nephrogen diabetes insipidus (NDI) in up to 50% of the patients. In a rat model, lithium treatment is associated with a severe downregulation of Aquaporin-2 (AQP2) protein (Marples et al., J. Clin Invest., 1995). However, it is not known to what extent this downregulation occurs at the mRNA level and if the main sodium transporter of the nephron, Na,K-ATPase, is regulated simultaneously. Moreover, it is unclear whether lithium treatment induces zonal or segmental differences in AQP2 and Na,K-ATPase mRNA levels. We examined the change in mRNA for AQP2 and Na,K-ATPase in rats treated with lithium for 10 and 28 days orally using standard protocols (free access to food, water and solid NaCl). This resulted in severe polyuria after 10 days: 57"10 ml/24hr/100g in lithium treated rats ($n = 8$) vs. 6"1 ml/24hr/100g in control rats ($n = 8$, $p < 0.05$). AQP2 and Na,K-ATPase mRNA levels in kidney cortex (C), inner stripe of outer medulla (ISOM), and inner medulla (IM) were determined by semiquantitative Northern blot analysis. AQP2 mRNA decreased significantly ($p < 0.001$) in lithium treated rats to 33"9% in C, 17"4 in ISOM, and 23"5 in IM while Na,K-ATPase mRNA levels were not altered in C and ISOM, but significantly ($p < 0.003$) reduced to 63"8% in IM. The localization of mRNA for the two proteins were determined by *in-situ* hybridization using radioactive or digoxigenin labeled RNA probes at the light and electron microscope levels. In rats lithium-treated for 10 days the AQP2 mRNA level was dramatically reduced in all zones of the kidney. The Na,K-ATPase mRNA level was found slightly decreased only in IM tubules. The results demonstrate substantial downregulation of AQP2 at the mRNA level throughout the nephron in experimental NDI, while Na,K-ATPase mRNA is moderately decreased only in the inner medulla suggesting that the sodium-potassium pump has a minor role in the development of lithium-induced NDI.

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Altered expression of renal aquaporins and Na-transporters in rats treated with the calcium blocker nifedipine

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Nifedipine, a calcium antagonists, has diuretic and natriuretic properties. However the molecular mechanisms by which these renal effects are produced are poorly understood. We examined the abundance of renal aquaporins (AQP1, AQP2 and AQP3) and major renal sodium transporters (NHE-3, NaPi-II, Na-K-ATPase, BSC-1, and TSC), moreover, levels AQP2 that is phosphorylated in the PKA phosphorylation consensus site (serine 256) and calcium-sensing receptor were evaluated. Rats were treated with nifedipine orally (700mg/kg) for 19 days. After 19 day of treatment, urine output was significantly increased, wheras urine osmolality and solute-free water reabsorption were significantly decreased. Consistent with this, semiquantitative immunoblotting of total kidney membrane fractions revealed a significant decrease in AQP2 abundance in nifedipine-treated rats to 47 " 7% of control rats (100 " 16% $p<0.05$). In contrast, AQP3 abundance was significantly increased (344 " 13% of control levels) and AQP1 abundance was not altered. In inner medulla, there was a significant decrease in AQP2 expression in nifedipine treated rats to 60 " 7% of control levels (100 " 16%). Moreover, p-AQP2 expression was dramatically decreased in nifedipine treated rats (17 " 6%) compared with control (100 " 23%). Immunoblots also showed calcium-sensing receptor expression was significantly increased in inner medulla of nifedipine treated rats to 295 " 25% of control rats (100 " 25%). The fractional excretion of sodium was significantly increased in nifedipine treated rats to 1.5 " 0.2% of control rats (0.4 " 0.1%), semiquantitative immunoblotting revealed significant reductions in proximal tubule Na transporter abundance compared to control group: NHE-3 (3"1%), NaPi-II (53"12%), and Na-K-ATPase (74"5%). In contrast, there was a significant increase in the whole kidney levels of the distal tubule NaCl transporter TSC (240 " 29%), and maintained expression in BSC-1 expression levels in thick ascending limb. In conclusion: 1) The increased urine output and decreased solute-free water reabsorption in nifedipine treated-rats may be in part be due to downregulation of AQP2 expression; 2) The increased expression of calcium-sensing receptor suggested that it may be involved in water metabolism in IMCD; 3) The reduced expression of proximal tubule Na transporter (NHE-3, NaPi-II, and Na-K-ATPase) may be involved in the increased fractional urinary sodium excretion; 4) The increase in TSC expression in distal tubules is like to represent a compensry phenomenon. Thus, the diuretic and natriuretic effects of nifedipine treatment may be produced due to alterations in the expression of several aquaporins and sodium transporters in renal tubules.

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Altered expression of renal aquaporins and Na-transporters in the rats with vitamin D-induced chronic hypercalcemia

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Chronic hypercalcemia (HC) induced by dihydrotachysterol (DHT) is associated with a nephrogenic diabetes insipidus. Previous studies have demonstrated a reduction in kidney AQP2 expression in rats with DHT induced NDI [1,2]. The purpose of the present study is to assess the effects of DHT treatment on the expression of renal aquaporins and several major renal sodium transporters in different parts of the kidney. Oral administration of DHT (8.5mg/kg diet) to rats was associated with an increase in urine production from 44 " 4 to 93 " 8 ul/min/kg, whereas urine osmolality decreased from 1617 " 117 to 888 " 59 mosmol/kgH_2O. Immunoblotting and densitometry of membrane fractions revealed a significant decrease in AQP2 abundance in inner medulla of HC rats to 52 " 6% (n = 14) of control levels (n = 12), consistence with previous studies [1,2], more over, there was also a marked decrease of AQP2 abundance in hypercalcemic rats to 57 " 11% of control rats (100 " 13%). Next the levels of AQP2 that is phosphorylated in the PKA phosphorylation consensus site (serine 256) of AQP2 was evaluated. The results demonstrated that the abundance of phosphorylated AQP2 was significantly decrease to 36 " 8% of control levels (100 " 8%). AQP3 expression was decreased in inner medulla to 45 " 7% of control levels (100 " 8%), in contrast, AQP1 abundance was not altered. The urinary sodium excretion rate increased significantly in rats treated with DHT (1.2 " 0.03Fmol/min/100g) compared with control rats (0.4 " 0.09). Semiquantitative immunoblotting revealed reduced abundance of two sodium transporters abundance: NaPi-II in proximal tubules (25 " 4%) and BSC-1 in thick ascending limbs (36 " 5%) in response to DHT treatment. In addition, the abundance of NaPi-II and BSC-1 were also markedly reduced in whole kidney of hypercalcemic rats corresponding to 22 " 5% and 50 " 11%, respectively. In contrast, there was a significant increase in the expression of NaCl transporter TSC (272 " 22%) in the distal convoluted tubule and 182% increase in whole kidney. In summary: 1) The downregulation of AQP2, AQP3 and p-AQP2 expressions in inner medulla play important roles in the development of polyuria in association with hypercalcemia. 2) Hypercalcemia was associated with downregulation of NaPi-II and BSC-1, which may participate in the reduction in proximal sodium reabsorption. In contrast, TSC expression was increased indicate a compensary mechanism.

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Aquaporin-2 (AQP2) expression and vasopressin-mediated renal water reabsorption in rats with liver cirrhosis

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Initial studies in rats with liver cirrhosis induced by chronic CCl_4 administration showed an increased expression of AQP2 (Hepatology 21:169-73, 1995; Am J Physiol 269:F926-31, 1996). However, studies from our laboratories in rats with liver cirrhosis induced by common bile duct ligation (CBL) showed a significant downregulation of AQP2 (Am J Physiol 275:F216-25, 1998; Am J Physiol 278:F246-56, 2000). The rats had sodium retention but no sign of ascites and plasma levels of vasopressin were slightly increased. Renal function studies in conscious, chronically instrumented rats showed that the decreased AQP2 expression was associated with a significantly decreased aquaretic response to selective V_2-receptor blockade. Together these results indicate that the vasopressin-mediated renal water reabsorption is decreased in rats with liver cirrhosis induced by CBL. Accordingly, Fernandez-Llama and coworkers (J Am Soc Nephrol 10:1950-7, 1999) recently reported a significant downregulation of AQP2 in rats with liver cirrhosis induced by CBL where decompensation was induced by giving the rats free access to sweetened water.

One question to be answered is whether the reported differences in AQP2 expression in experimental cirrhosis is due to the model used, or due to the fact that the rats were investigated at different stages of the disease, i.e that AQP2 expression may be downregulated in the preascitic state and during early decompensation, and then increased in terminal conditions with severe decompensation.

We have therefore made a number of experiment in rats with CCl_4 induced liver cirrhosis. The rats showed marked alterations in water homeostasis: Urine production was decreased, urine osmolality increased, and the rats had hyponatremia and ascites. Plasma vasopressin concentrations were increased and the rats showed an impaired ability to excrete an i.v. water load. Renal function studies in conscious, chronically instrumented rats showed that renal plasma flow, GFR and lithium clearance were significantly decreased. The aquaretic response to selective V_2-receptor blockade was unchanged compared to control rats. However, when expressed as a fraction of GFR or distal delivery the aquaretic effect of OPC-31260 was significantly enhanced in the cirrhotic rats: ÄV/GFR: +24% (7.99 " 0.56 *vs.* 6.46 " 0.64 %; $p < 0.05$), $ÄV/C_{Li}$: +41% (35.37 " 2.66 *vs.* 25.04 " 2.97 %; $p < 0.01$).

In parallel series of animals, semiquantitative immunoblotting revealed that the expression of AQP2 was unchanged in membrane fractions of both whole kidney and inner medulla from cirrhotic rats.

In summary, the present study demonstrates that rats with CCl_4 induced liver cirrhosis develop hyponatremia and ascites in the absence of changes in the renal expression of AQP2. Functional *in vivo* studies indicate that the vasopressin-mediated distal water reabsorption is increased despite an unchanged expression of AQP2. Together these results suggests a Arelative escape@ from vasopressin as a compensatory mechanism aimed to limit excessive collecting duct water reabsorption and thereby preventing avid water intoxication. The mechanism behind such a compensatory Aescape@ from vasopressin stimulation in the collecting ducts is however unknown.

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Aquaporin-2 (AQP-2) expression is increased in rat kidneys after ovariectomy (OVX)

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Recent data suggest that female sex hormones are directly involved in renal water handling and solute transport in rats [1,2]. The collecting duct water channel aquaporin-2 (AQP2), is known to play a key role in the regulation of renal water handling. Ovariectomy (OVX) is known to be associated with weight gain, which may in part be a result of water retention and we therefore hypothesized that OVX may be associated with dysregulation of AQP2 expression and changes in renal water handling. Female Wistar rats were monitored for 7, 14 and 21 days after OVX or sham operation and expression of AQP2 in inner medulla (IM) and whole kidney samples were examined. Immunoblotting revealed a persistant and significant increase in AQP2 expression levels in the kidneys of OVX rats to 332"10% (n=6) at day 7, 353"37% (n=13) at day 14 (n=13) and 165"22% (n=13) at day 21 as compared to the time matched sham operated controls, $P<0.05$. Morover, the abundance of AQP2 that is phosphorylated in the PKA phosphorylation consensus site (serine 256 of AQP2) was evaluated. In kidney IM the abundance of phosphorylated AQP2 levels were significantly higher 14 days after OVX (265"41% (n=8) of sham levels), demonstrating an increase in the levels of PKA-induced phophorylated AQP2. In parallel OVX was associated with a significant increase in body weight (OVX: 240.7"1.7 g (n=12) vs Sham:230.1"2.2 g (n=12) at day 7, OVX:254.9"1.6 g (n=13) vs Sham:228.5"2.2 g (n=14) at day 14 and OVX:267.8"6.6 g (n=13) vs Sham:239.9"4.8 g (n=13) at day 21, $P<0.05$). Water intake and urine output were slightly reduced in all groups and urine output was significantly reduced 14 days after OVX (OVX:43"4 $Fl \cdot min^{-1} \cdot kg^{-1}$ (n=13) vs Sham: 66"7 $Fl \cdot min^{-1} \cdot kg^{-1}$ (n=14), $P<0.05$). Consistent with this, urine osmolality values were slightly increased in OVX rats and solute-free water reabsorption was significantly decreased at day 14 (OVX:187.2"12.8 $Fl \cdot min^{-1} \cdot kg^{-1}$ vs Sham:226.9"13 $Fl \cdot min^{-1} \cdot kg^{-1}$, $P<0.05$). In conclusion, OVX is associated with a significant increase in AQP2 expression levels, increased abundance of phosphorylated AQP2, unchanged or marginal reductions in urine output and a significant increase in body weight, suggesting that AQP2 may play an important role for the known postmenopausal changes in renal water and sodium metabolism.

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P 91

Misrouting to the basolateral membrane of an AQP2 mutant with a C-terminal frame-shift explains dominant Nephrogenic Diabetes Insipidus

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The homotetrameric aquaporin-2 (AQP2) water channel is localized to the principal cells of the renal collecting duct. Upon binding of arginine vasopressin (AVP) to its V2-receptor in these cells, an intracellular signaling cascade is initiated, resulting in redistribution of AQP2 from intracellular vesicles to the apical plasma membrane, the rate-limiting barrier for water transport. Consequently urine is concentration. After removal of AVP, AQP2 is re-distributed again to intracellular vesicles, rendering the cells water impermeable. Nephrogenic diabetes insipidus (NDI) is a disease characterized by the inability of the kidney to concentrate urine upon stimulation with AVP. Several mutations in the AQP2 gene have been reported to cause autosomal recessive NDI, whereas one AQP2 mutation (AQP2-E258K) has been shown to be involved in autosomal dominant NDI. Here, we report another case of dominant inheritance of NDI, caused by an insertion of an adenosine at position 872 in the AQP2-gene (AQP2-872insA). This insertion causes a C-terminal frame-shift, which, compared to wild-type (wt) AQP2, changes the last 12 amino acids and extends the protein by 7 amino acids before a stop codon is encountered. Expressed at low levels in *Xenopus* oocytes, this mutant was indistinguishable from wt-AQP2 in that it conferred a similar water permeability (Pf), and was only expressed at the plasma membrane. In contrast to oocytes, Madin Darby Canine Kidney (MDCK) cells are polarized and, when stably-expressing heterologous AQP2 (wt10 cells), reveal a vasopressin-regulated AQP2 shuttling to the apical membrane as found in renal principal cells[1]. Since a side-specific routing of AQP2-872insA would not become apparent in oocytes, MDCK cells were stably-transfected with an AQP2-872insA expression construct. As found for AQP-872insA expressed in oocytes, immunoblotting of lysates of selected clones revealed a representation in nonglycosylated/glycosylated AQP2 bands as found for wt-AQP2. Confocal laser scanning microscopy, however, revealed that AQP2-872insA was routed to the basolateral plasma membrane. Solubilization of total membranes if MDCK cells stably-transfected with AQP2-872insA and wt-AQP2 followed by specific immunoprecipitation for AQP2-872insA and immunoblotting for wt-AQP2 revealed that wt-AQP2 co-precipitated with AQP2-872insA, indicating that wt-AQP2 and AQP2-872insA formed heterotetramers.

In conclusion, heterotetramerization of wt-AQP2 with AQP2-872insA and the subsequent routing of these complexes to the basolateral membrane of principal cells might preclude routing of sufficient wt-AQP2 to the apical membrane, thereby providing an explanation for dominant NDI in this particular family. In addition, the basolateral routing of AQP2-872insA also indicates that important information for routing to the apical membrane is contained in the C-terminal end of wt-AQP2, which will be further investigated.

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A new AQP2 mutant in dominant Nephrogenic Diabetes Insipidus, which encodes a different C-tail, confers a dominant phenotype upon expression in oocytes

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Nephrogenic Diabetes Insipidus (NDI), a disease in which the kidney is unable to concentrate urine in response to vasopressin. Recently a family was identified in which NDI was presented as autosomal dominant. To define the molecular cause of NDI in this family, genomic DNA was subjected to sequence analysis. In the proband and affected father, a guanosine was missing at position 820 (AQP2-820delG), which is located in the C-terminus and leads to a frame shift. To determine the full coding sequence of this mutant AQP2, the sequence of the 3'-untranslated region (3'-UTR) of the proband was determined, which appeared to encode the same amino acids (aa) as that found in healthy persons, but was different from the reported 3'-UTR of the AQP2 gene[1]. In the patient, the mutant AQP2 gene encodes a 333 aa AQP2 protein (35 kDa), which exchanges 29 aa and is extended for 62 aa compared to wild-type (wt) AQP2. In oocytes, AQP2-820delG did not confer a water permeability (Pf) at low expression levels, while at high expression levels some Pf was obtained. Immunoblotting of total and plasma membranes revealed that AQP2-820delG migrated as a protein of about 35 kDa and that it was absent (low injection) or reducedly (high injection) expressed at the plasma membrane. Immunocytochemistry revealed that AQP2-820delG was dispersed thoughout the cell. To test whether AQP2-820delG can confer a dominant negative phenotype on wt-AQP2, oocytes were injected with wt-AQP2 cRNA alone or together with cRNA coding for AQP2-820delG or AQP2-R187C, a mutant in recessive NDI. Analysis revealed that the Pf (mean " SEM; in Fm/s) of wt-AQP2 (116 " 29) was reduced upon co-expression with AQP2-820delG (56 " 18), whereas no reduction was obtained upon co-expression with AQP2R187C (111 " 23). Immunoblotting revealed that the expresion levels of wt-AQP2 and the AQP2 mutant were similar and that the co-injection with AQP2-820delG, but not AQP2-R187C, reduced the amount of wt-AQP2 at the plasma membrane.

In conclusion, the oocyte expression studies reveal that AQP2-820delG is impaired in its routing to the plasma membrane, but the immunocytochemistry indicates that this mutant is retained in another organelle than AQP2-E258K (another mutant in dominant NDI; Golgi complex) or mutants in recessive NDI (Endoplasmic reticulum). In addition, AQP2-820delG confers a dominant-negative effect on wt-AQP2, which presumably occurs by heterotetramerization with wt-AQP2. On basis of these results, it can be concluded that AQP2-820delG causes dominant NDI in this particular NDI family.

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P 93

Urinary excretion of Aquaporin-2 (UAQP-2) water channel is dominant in pathological state of Arginine vasopressin (AVP)-induced impaired water excretion

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The present study was undertaken to determine whether hydroosmotic action of AVP is exaggerated in pathological state of impaired water excretion. Fourteen hyponatremic patients with impaired water excretion and 7 control subjects were studied an acute water load. Serum Na levels were 128.6?3.4 and 142.2?0.8 mEq/l in the patient group and the control subjects, respectively ($p < 0.01$). Plasma AVP levels of 1.3?0.4 pg/ml were not sufficiently suppressed despite hypoosmolality in the hyponatremic patients. Urinary excretion of AQP-2 (UAQP-2) under ad libitum water drinking was 429.3?24.3 fmol/mg creatinine in the patient group, a value greater than that of 153.3?28.1 fmol/mg creatinine in the control subjects ($p < 0.05$). Water (20ml/kg BW) was ingested for 30min, and then urine and blood collections were made at 30 and 60 min intervals for 4h, respectively. The acute water load verified the impairment in water excretion in the patient group, as the percent excretion of the water load was only 29.9?4.6 % (control, 78.6?5.1 %, $P < 0.05$) and minimal urinary osmolality was as high as 387.8?61.1 mOsm/kgH_2O (control, 94.4?9.8, $p < 0.05$). The minimal levels of plasma AVP and UAQP-2 were 1.0?0.1 pg/ml and 87.6?10.4 fmol/mg creatinine in the control subjects, respectively, when Posm was decreased to 272.9?3.7 mOsm/kg H_2O. In the patient group the minimal plasma AVP levels were 1.1?0.1 pg/ml with hypoosmolality of 268.0?3.6 mOsm/kgH_2O, a value comparable to that in the control. In contrast, the minimal UAQP-2 was as high as 232.5?43.2 fmol/mg creatinine, which was significantly greater than the control($P < 0.05$). There was a positive correlation between plasma AVP levels and UAQP-2 in the control subjects ($r = 0.64$, $n = 52$, $P < 0.01$). However, UAQP-2 was relatively high as compared to an increase in plasma AVP in the patient group, and thus the positive correlation disappeared. These results indicate that UAQP-2 accounts for the hydroosmotic action of AVP in renal collecting ducts cells, and that hydroosmotic action of AVP is exaggerated in the pathological state of impaired water excretion dependent on nonsuppressible release of AVP despite hypoosmolality.

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Role of aquaporin 2 in the osmoregulatory function of the kidney in enuretic children

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In mammals, vasopressin induces urine concentration through Aquaporin 2 (AQP2), the vasopressin-dependent water channel. In mammalian kidney and urine, two forms of AQP2 are detected: a nonglycosylated form of 29kDa and a glycosylated form of 40-45kDa. In this study we examined the hypothesis that primary nocturnal enuresis, a pathological state more frequent in 5 to 10 years old children, might be paralleled by AQP2 urinary excretion. 80 children suffering from nocturnal enuresis were studied and compared with 9 healthy children. The 24 h urine samples were divided into two portions: collected during the night and during the day. Creatinine equivalents of urine samples from each patient were analyzed by Western blotting. AQP2 levels were semiquantified by densitometric scanning and reported as a ratio between the intensity of the signal in the day urine sample *vs* the night urine sample (D/N AQP2 ratio). The D/N AQP2 ratio was 0.59" 0.11 (n=9) in healthy children and increased to 1.27 " 0.24 (n=10) in a subpopulation of enuretic children having low nocturnal vasopressin levels. In enuretic children displaying hypercalciuria and having normal vasopressin levels, the D/N AQP2 ratio was 1.05 " 0.27 (n=8). These data indicate that reduced secretion of vasopressin and absorptive hypercalciuria are independently associated with an approximately twofold increase in the urinary D/N AQP2 ratio. When low nocturnal vasopressin levels were associated with hypercalciuria a nearly threefold increase in the D/N AQP2 ratio was observed (1.67"0.41, n=11). In addition, in all enuretic patients tested, the urinary D/N AQP2 ratio correlates perfectly with the severity of the disorder (nocturnal polyuria). The finding reported in this study indicate that AQP2 plays a crucial role in nocturnal enuresis.

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Aquaporin-4 deficiency in the neuromuscular system of dystrophic MDX-mice

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In this study we investigated the expression of AQP4 in the neuromuscular system of mdx mice, used as experimental model of Duchenne Muscular Dystrophy. Immunocytochemical analysis performed by double immunostaining, revealed that mdx mice manifested a progressive reduction in AQP4 at the sarcolemmal level of the fast skeletal muscle fibers and that type IIB fibers were the first to manifest the reduction of AQP4 expression. By Western blot and RT-PCR analysis we found that, while the total content of AQP4 protein decreased (by 80% in adult mdx mice), mRNA levels for AQP4 remained unchanged. Interestingly, a similar age-related reduction in AQP4 expression has been found in brain astrocytic end-feet surrounding capillaries. Morphometric analysis performed after immunogold electron microscopy indicated a reduction of ~90% in gold particles. Western blot experiments revealed a strong reduction (of 70 %) in AQP4 protein in adult animals and RT-PCR experiments demonstrated that the reduction was not at transcription level. More interesting was the finding that AQP4 reduction occurs in parallel with the presence of swollen perivascular astrocytic endfeet. The involvement of the endothelial component in the blood-brain barrier (BBB) alterations was also investigated. In mdx mice, the endothelial tight junctions appeared modified by microdetachements of the external membrane leaflets. Moreover, ZO-1 protein, located in the endothelial tight junction in the control brain, was found diffusely distributed in the cytoplasmic endothelium indicating that BBB structural modifications at vascular and perivascular level occur in mdx mice.

Our data provide evidence that dystrophin deficiency in mdx mice leads to disturbances in AQP4 assembly in the plasma membrane of fast skeletal muscle fibers and brain astrocytic end-feet, suggesting that changes in the osmotic equilibrium of the neuromuscular apparatus may be involved in the pathogenesis of muscular dystrophy.

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P 96

Assessment of aquaporin-4 in rat brain in response to systemic hyponatremia

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Aquaporin-4 (AQP4) is abundant in astroglial cells and has been suggested to be involved in the regulation of brain water homeostasis. This study was undertaken to assess whether AQP4 protein and AQP4 mRNA expression levels undergo downregulation and whether there is a subcellular redistribution of AQP4 protein in rat brain in response to systemic hyponatremia and brain edema. Systemic hyponatremia was induced by combined administration of hypotonic dextrose i.p. and 8-deamino-arginine vasopressin (dDAVP) s.c. and rats were followed for 4 or 48 hours. Semiquantitative immunoblotting of membrane enriched fractions, using an antibody against the C-terminal of the protein showed significantly increased immunoreactivity to 164 " 12% ($n=6$) and 153 " 12% ($n=6$) of control levels in brain after 4 or 48 hours of systemic hyponatremia, respectively. Similarly, immunoblots of cerebellar samples revealed an increase in AQP4 immunoreactivity to 136 " 6% ($n=6$) and 218 " 44% ($n=6$) of control levels, after 4 or 48 hours of systemic hyponatremia, respectively. Blots of samples from rats treated with s.c. dDAVP alone (i.e. without hypotonic dextrose) did not produce any increase in AQP4 immunoreactivity, indicating that the increased AQP4 immunolabeling is associated with severe systemic hypoosmolarity. In contrast, AQP4 mRNA levels were unchanged in response to 4 hours of severe hyponatremia (104"14% vs. 100"8% in sham controls; $n=17$), indicating that there are no changes in AQP4 expression level in response to systemic hypoosmolality. Immunocytochemistry and high resolution immunogold electron microscopy revealed highly polarized labeling of AQP4 to astrocyte end-feet surrounding capillaries and forming the glia limitans. There was no change in the subcellular distribution of AQP4 protein in response to hyponatremia.

We conclude, that there was i) no downregulation of AQP4 expression and ii) no subcellular redistribution of the protein in response to hyponatremia. iii) there was a pronounced and rapid increase in AQP4 immunoreactivity, which may reflect secondary conformational modifications of AQP4 protein leading to increased immunoreactivity. This post-translational modification of AQP4 may participate in the adaptation of the cerebral tissue to systemic hyponatremia.

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P 97

Tumor necrosis factor alpha decreases aquaporin 5 expression in murine lung epithelial cells

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Inflammation is often associated with aberrant fluid handling in the lung and other organs resulting in tissue edema. In the distal lung, water movement through the airspace-capillary barrier is thought to be facilitated by two aquaporins: AQP1 which is expressed in the capillary endothelium, and AQP5, which is expressed in alveolar epithelium. Previously, we investigated whether distal lung aquaporins undergo altered regulation *in vivo* under conditions of aberrant fluid handling in the lung namely intratracheal infection of mice with adenovirus which is associated with subsequent pulmonary inflammation and edema [1]. We showed that mRNA and protein expression for AQP1 and AQP5 were decreased in the lungs of mice 7 and 14 d following adenoviral infection. Immunohistochemical analyses of lung sections from adenovirus infected and control mice showed that the decreased expression of AQP1 and AQP5 were not localized to regions of overt inflammation but were found throughout the lung. This observation suggested the possibility that the decreased AQP expression could be mediated by systemic factors released during the inflammatory response. As the cytokines are likely candidates for such factors, we used RT-PCR to estimate the levels of expression of tumor necrosis factor á (TNFá) and interferon ã (INFã) in total RNA from infected and control lungs, and demonstrated that the expression of both TNFá and INFã was increased in the infected lungs. Murine lung epithelial (MLE12) cells, which express AQP5 at high levels, were utilized in *in vitro* analyses to determine whether treatment of cells with TNFá results in decreased expression of AQP5. MLE12 cells were treated with various concentrations of murine TNFá and mRNA and protein were analyzed at a range of time points after the addition of TNFá. Dose response studies determined that 100 U/mL of TNFá was optimal for reducing AQP5 mRNA and protein. AQP5 mRNA expression was analyzed after 1, 2, 4, 8, 16, 24, and 48 hours of TNFá treatment. AQP5 mRNA expression was significantly decreased after 4 hours of TNFá treatment, maximally decreased 2-3-fold ($p < 0.002$, $n = 6$) after 8 hours of treatment and returned to baseline levels within 48 hours of TNFá treatment. AQP5 protein expression was maximally decreased 10-fold ($p < 0.0003$, $n = 6$) 24 hours after treatment and remained significantly decreased after 48 hours of treatment. Decreased AQP1 and AQP5 levels during adenoviral infection suggest a role for AQP1 and AQP5 in the abnormal fluid fluxes detected during pulmonary inflammation, and decreased expression of AQP5 in MLE12 cells after treatment with TNFá suggests a role for TNFá in mediating this response.

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P 98

Expression levels of aquaporin-5 in the lacrimal glands of dacryoadenitis model mice

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Purpose. It is reported that some kind of dacryoadenitis model mouse shows a decrease in tear volume and gradual disappearance of localized aquaporin-5 from lacrimal cell membranes. To investigate whether such defective tear secretion is caused by a decrease in the water-channel proteins in lacrimal glands, we measured the amount of aquaporin-5 in the lacrimal glands of dacryoadenitis model mice.

Methods. Female MRL/lpr, MRL/gld, and male NOD mice were used as dacryoadenitis model mice. These mice are known to suffer gradual damage to the lacrimal glands with age. Control mice were female BALB/c mice. Lacrimal glands were removed from 5, 10, and 15-week-old mice and homogenized in buffer solution. After centrifugation, the supernatants were collected and the amount of aquaporin-5 was determined by enzyme linked immunosorbent assay (ELISA).

Results. The expression levels of aquaporin-5 in the lacrimal glands varied according to the strain of mice (BALB/c: 450.0-508.4, MRL/lpr: 404.4-470.9, MRL/gld: 583.1-613.4 and NOD: 201.2-257.3 ng/mg of proteins). However, in all strains, there was no significant difference in the expression levels of aquaporin-5 according to the age of mice.

Conclusions. To quantify the amount of aquaporin-5, we developed an ELISA method and established the expression levels of aquaporin-5 in the lacrimal glands of dacryoadenitis model mice. Our results indicate that the expression level of aquaporin-5 is not changed even when the lacrimal gland is damaged. In this study, it was demonstrated that in some kind of lacrimal gland disorder, sorting of aquaporin-5 into lacrimal cell membranes is impaired and it leads to defective tear secretion.

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Yeast aquaporins: expression, localization, function

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The analysis of the genome of *Saccharomyces cerevisiae* indicates the presence of four ORFs encoding MIP proteins, two of which having a high similarity to aquaporins from plants, mammals and *E. coli*. Among *S. cerevisiae* strains, a genetic heterogeneity was observed for *AQY*1 (YPR192W) and *AQY*2 (YLL052c-053c) genes when DNA sequences from different strains were compared ([1,2]; http://genome-www.stanford.edu/Saccharomyces). Strain Ó1278b may possess two aquaporins, Aqy1-1p and Aqy2-1p, whereas most of the laboratory strains, such as W303-1A, may possess only one aquaporin, Aqy1-2p. In W303-1A, *AQY*2-2 has an internal deletion, producing 2 ORFs that may not code for a functional protein.

Specific antibodies were used to follow the expression of the two aquaporins in the Ó1278b, and the W303-1A strains. Aqy1p was never detected in either Ó1278b and W303-1A strains whatever the growth phase and culture conditions tested (exponential and stationary growth phase, on rich or minimal medium, or after hyper- or hypo-osmotic shock). In contrast, in Ó1278b, Aqy2-1p was detected only during the exponential growth phase on rich medium containing glucose as carbon source (YPD). It was not produced on rich medium containing galactose or ethanol as carbon source, nor on minimal medium, nor after growth in hyper-osmotic conditions. In fact, as demonstrated by the disappearance of Aqy2-1p after addition of 0.5 M KCl or 1 M sorbitol to YPD grown cells, hyper osmotic conditions repressed *AQY*2-1 expression. This effect is reversible as shown by the reappearance of Aqy2-1p when cells grown on YPD + KCl were placed in fresh YPD. Thus, the expression of *AQY*2-1 is not constitutive but is tightly regulated by growth conditions.

We isolated the different membrane compartments from Ó1278b grown on rich medium and tested them to localize and investigate the function of Aqy2-1p. The Aqy2-1p is abundant on microsomal vesicles, which are being used to test the water channel activity of this protein using stopped-flow spectrophotometry. The selectivity of the channel against various solutes is also being investigated.

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P 100

Differential behaviour of aquaporins and GlpF in N-lauroyl sarcosine detergent

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Purification of MIP proteins is of fundamental interest for further reconstitution in artificial lipid bilayers and for structural analysis by electron microscopy of 2D-crystals. It has been shown that native or recombinant AQP1 can be purified by a few steps procedure using N-lauroyl sarcosine (NLS) as detergent. A first extraction with NLS solubilizes almost all the membranous proteins, except AQP1. The aquaporin can then be obtained at good degree of purity by resuspending the NLS insoluble extract by n-octyl glucoside (OG). We have successfully used this procedure to purify AQPcic expressed in yeast[1]. This protocol allows the aquaporins to remain in their homotetrameric native state.

In the present study, we have firstly attempted to purify the glycerol facilitator GlpF. By treating cell membranes by NLS, GlpF was always found in the NLS soluble fraction with other membrane proteins. We then observed that AQPcic mutants in which amino acids were replaced by corresponding amino acids of GlpF behave like the glycerol facilitator toward NLS extraction. Furthermore, all these mutations abolish the water channel function of the protein and affect the oligomerization state of the protein, except for AQP-A209K or AQP-W223L which remain homotetrameric.

Moreover, we show that i) the cytoplasmic C-terminal part of AQPcic is not essential for this particular behavior. ii) the 6th transmembrane domain of AQPcic is necessary for the NLS resistance but can be partially replaced by the GlpF one.

This different comportment of an aquaporin and a glycerol facilitator suggests a distinct structural organization of the two kinds of channels proteins in the lipid bilayer. This study shows that despite their high degree of homology, MIP proteins adopt different topology in the cell membranes.

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Overexpression and purification of the glycerol transport facilitators, Fps1p and GlpF, in *S. cerevisiae* and *E.coli*, respectively

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Fps1p is an osmoregulated glycerol transport facilitator, located in the *Saccharomyces cerevisiae* plasma membrane, where it is mainly used for efflux of glycerol in the adaptation to lower external osmolarity. Fps1p is an unusual member of the MIP(Major Intrinsic Protein)-family. The protein is much larger than most of the other proteins in the family, due to long hydrophilic extensions at both termini. The N-terminus (~250 aa) is involved in the regulation of the channel, but the function of the C-terminus (~150 aa) is not yet known. Fps1p is also one of the few members that differs in the well-conserved NPA-motifs in the two putative channel forming loops B and E; serine instead of alanine in Loop B and leucine instead of proline in Loop E.

Because of the special features of Fps1p and the fact that the channel is regulated, we are interested in studying the structure-function relationship of this MIP-protein in more detail. Our goal is to obtain pure Fps1p for two purposes; to obtain 2D crystals for structural studies and to reconstitute protein into liposomes and measure function with stopped-flow techniques. We are also interested in exploring the structure of the hydrophilic N-terminus by NMR.

Fps1p has been overexpressed in *S.cerevisiae*, *E.coli* (lac-promoter) and *Pichia pastoris*. Fps1p is expressed and localised to the membrane when expressed in all three systems. Although the expression level is rather low, a band corresponding to Fps1p has been detected on a Western Blot. *Pichia pastoris* has been the most successful system so far. Fps1p is expressed from the strong AOX1 promoter and the protein is localised to the plasma membrane. Fps1p is tagged with 6xHis residues at the C-terminus and the protein can be purified on a Ni-column after solubilisation in 1% Triton X-100.

We are also interested in comparing Fps1p with its closest homologue, GlpF, the glycerol transport facilitator from *E.coli*. GlpF can replace the transport function of Fps1p in yeast, but the transport is not regulated. GlpF is expressed in *E.coli* from the T7 promoter and the protein is tagged with a 6xHis residue at the N-terminus. The protein is solubilised in 1 % Triton X-100 and purified on a Ni-column. The estimated yield is about 1 mg/L culture. The Fps1p N-terminus is expressed in the same system and the yield of this soluble peptide is about 4-5 mg/L after the Ni-column.

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Regulated trafficking of the Pho84 phosphate transport protein

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Uptake of phosphate in the yeast *Saccharomyces cerevisiae* has been shown to be mediated by two different systems (reviewed in [1]). The high-affinity uptake of phosphate is mediated via the Pho84p which catalyzes a phosphate uptake in symport with H^+ at acidic conditions or by Pho89p which is Na^+-coupled and active at more alkaline conditions. These proteins are synthesized and active when the cell meets limitations in available external phosphate. Besides this derepressible system, the cells maintain a low-affinity transport system believed to be constitutively expressed.

In a previous study we have visualized *in vivo* expression and trafficking of the Pho84p fused with the green fluorescent protein (GFP) [2]. At derepressing conditions the Pho84-GFP fusion protein is expressed and localizes to the plasma membrane where it catalyzes active phosphate uptake. Once the external phosphate is fully depleted the Pho84-GFP chimera is removed from the plasma membrane and re-localized to the vacuole for degradation, possibly preventing efflux of internal phosphate. The underlying mechanism for the removal of the Pho84p from the plasma membrane is presently not known.

The á-factor pheromone receptor, Ste2p, is rapidly internalized upon ligand binding in a ubiquitin-dependent manner and transported to the vacuole for degradation. As a first step in this internalization and degradation process, a 9 amino acid sequence, SINNDAKSS, in the C-terminal tail of the Ste2p becomes phosphorylated at its serines and subsequently ubiquitinated at the lysine [reviewed in 3].

The Pho84p contains a partly conserved SINNDAKSS sequence, nkNNDieSS, at its C-terminus. We are analyzing the roles of the serines and lysines within and in close vicinity to this sequence in the deactivation process of the Pho84 phosphate transporting protein. Deletion and site-directed mutagenesis of the residues have been carried out, and the consequences of the mutations on protein localization under defined growth conditions will be presented.

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P 103

A test system to identify solutes transported by MIP channels

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Under hypertonic conditions *Saccharomyces cerevisiae* adapts to the stress by accumulating glycerol. When the cells experience a drop in osmolarity accumulated glycerol is quickly exported from the cells. This glycerol release is mediated by a member of the MIP family, Fps1p. Fps1p is known to be a regulated channel, which means that it is closed under hyperosmotic conditions when glycerol needs to be accumulated, and it opens when the cell experiences a drop in osmolarity and glycerol has to be released. A domain in front of the first TMD involved in this regulation has previously been identified. If it is deleted Fps1p becomes a constitutively open channel.

A *gpd1 gpd2* yeast mutant is unable to produce glycerol and is therefore very sensitive to high osmolarity. In a growth assay on plates containing 3 M glycerol, the *gpd1 gpd2* double mutant experiences hyperosmotic stress and has severe growth problems. However if the same cells express the yeast glycerol channel Fps1p lacking its N-terminus (i.e. the channel is constitutively open), the cells grow almost as well as wild type. This is because the glycerol equilibrates and hence the concentration gradient collapses.

Since other polyols can also act as compatible solutes, the system can be used to characterise the polyol substrate spectrum of glycerol facilitators from other organisms expressed in the yeast *gpd1 gpd2* mutant. We demonstrate this with Fps1p as an example, which apparently transports polyols up to a C5 carbon backbone. The system will even allow selection for mutants of facilitators with an altered substrate spectrum and hence the system will be a useful tool to study substrate specificity of MIP channels. In addition, the system allows cloning of glycerol transporting MIP channels from cDNA libraries and hence is a useful tool to identify such proteins from different organisms.

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MIP channel proteins Fps1p and yfl054p influence the accumulation of compatible solutes in recombinant strains of Saccharomyces cerevisiae

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The Fps1p membrane channel protein is a regulated glycerol export protein involved in yeast osmoregulation [1]. Besides glycerol other metabolites can also procure stress tolerance in *Saccharomyces cerevisiae*. Trehalose is an effective protection agent against thermal shock and dehydration in yeasts [2], and xylitol was shown to protect xylose utilising yeasts during dehydration and rehydration [3]. These metabolites accumulate under the stress conditions.

To establish weather Fps1p and yfl054p , a distant yeast homoloque of Fps1p, also transport trehalose and xylitol and whether one compatible solute can substitute for another compatible solute under stress conditions recombinant strains of *S. cerevisiae* were constructed. In these strains synthesis of glycerol, trehalose and xylitol was deleted individually and in pairs. In addition, Fps1p and yfl054p were constitutively open [4] and closed as well as regulated. During aerobic fermentation substrate consumption, product formation, cell growth and intracellular content of compatible solutes were determined. The intracellular content of glycerol, trehalose and xylitol was also determined under hyperosmotic conditions.

Fps1p regulated the transport and intracellular accumulation for glycerol and xylitol. When Fps1p lacks its N-terminus the transport channel is constitutively open [4] and glycerol and xylitol release became deregulated. Under hyperosmotic conditions Fps1p does not regulate the xylitol accumulation. yfl054p does not influence the xylitol transport during fermentation and hyperosmotic conditions. The trehalose accumulation was under no conditions influenced by the function of either Fps1p or yfl054p.

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Phylogenetic relationships of microbial MIP channels

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The ability to regulate water and solute flux across cell membranes is an intrinsic feature of all living organisms which ensures a constant turgor pressure as well as the proper functioning of biochemical processes. This process appears to be mediated by a ubiquitous family of transmembrane water channels and glycerol facilitators. A significant number of these MIP channels have been characterised in higher organisms and their roles in mediating water and solute flux have been established. Recently, and mainly by systematic genome sequencing, about 100 MIP channels have also been identified in unicellular organisms where they appear also to fulfil roles in osmoregulation as well as in nutrition. However, there is still very little information about microbial MIP channels with regard to their physiological roles. Phylogenetic analysis reveals two major groups, the glycerol facilitators and the aquaporins, but further divides the glycerol facilitator into two subfamilies. Water channels have been identified in, archaea, yeasts as well as in Gram-negative bacteria and seem to be important for growth after drastic changes in medium osmolarity, especially to lower osmolarity. With some exceptions, water channels (aquaporins) have not been observed in many Gram-positive bacteria whose genome has been fully sequenced. The reason for such a restricted distribution of aquaporins in bacteria is not yet clear. However, glycerol facilitators appear to exist in all bacterial groups, where they may function in the uptake of glycerol and related compounds for their catabolism. Apparently, there are some microorganisms which lack MIP channels altogether. In this paper, we discuss the distribution and phylogenitic relationships of microbial MIP channels in detail. It is anticipated that phylogenetic analysis of MIP channels may provide insight into their physiological roles.

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Role of the MIP channel (Fps1p) of *Saccharomyces cerevisiae* on membrane composition and glycerol release during hypoosmotic stress

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Saccharomyces cerevisiae accumulates the compatible solute glycerol intracellularly in response to hyperosmotic stress, which is then released upon hypoosmotic stress. Coupled with these responses, rapid out- and inflow of water occurs with cell shrinkage and swelling. These observations indicate that the regulation of solute flux across the cell membrane plays a fundamental role in osmoadaption. *S. cerevisiae* controls the glycerol flux across the plasma membrane via the serpentine protein Fps1p, and via passive diffusion [1]. The Fps1 protein is closed during hyperosmotic stress, thereby conserving glycerol, and opens upon hypoosmotic stress, releasing glycerol. Deletion of this protein results in mutants sensitive to hypoosmotic stress [2]. The gene encoding this protein, *FPS1*, is a member of a large family of membrane proteins known as major intrinsic proteins (MIPs) that occur in plants, animals, bacteria and *S. cerevisiae*; little is known about its occurrence in other yeasts. Most MIPs appear to control water flux but others are involved in the movement of glycerol and other solutes. Fps1p may also be involved in regulating passive diffusion as glycerol diffusion is lower in an *fps1* deletion strain than the wild-type strain [3]. We have found that the lipid composition of the plasma membrane differs between the wild-type and *fps1* deletion strain of *S. cerevisiae*. Specifically, the ergosterol level of the deletion strain is approximately half that of the wild-type strain when cultivated in a rich medium. The difference is less marked in strains cultivated in a defined medium. The levels of intra- and extracellular glycerol apparently influence plasma membrane ergosterol levels as shown by the analysis of various strains with deletions in their ability to synthesise or accumulate glycerol. This observation implies that *S. cerevisiae* may alter ergosterol levels in order to control passive diffusion of glycerol. Furthermore, strains with lower ergosterol concentrations were found to be more sensitive to hypoosmotic shock than the isogenic wild-type strain. And this defect can be partially restored by supplementation with ergosterol.

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Genetic analysis of plasma membrane aquaporin function in *Arabidopsis*

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In plants, MIP homologues define a large gene family with up to 27 members in the model species *Arabidopsis thaliana*. The Plasma membrane Intrinsic Protein (PIP) subfamily comprises 13 members which correspond to putative plasma membrane aquaporins. A large body of molecular data has been described on plant aquaporins but informations about their function at the whole plant level are still scarce. This question was addressed by a reverse genetic approach. Our objective is the isolation and characterisation of *pip* knock-out mutants in *Arabidopsis*. We searched for aquaporin insertion mutants in a library of more than 20,000 *Arabidopsis* lines independently transformed by the *Agrobacterium tumefaciens* transferred DNA (T-DNA). This library was screened by PCR using a combination of primers specific for *pip* genes and T-DNA border sequences, respectively. Using this strategy, six T-DNA insertion mutants were identified; the targeted genes belong to the *pip1* subfamily (2 mutants) and to the *pip2* subfamily (4 mutants). Whereas some of these mutants still need to be genetically characterised, plant lines homozygous for a single T-DNA insertion within a *pip* gene could be obtained and their phenotypic characterisation is in progress. In soil, *in vitro* and hydroponic cultures were used to screen a broad range of culture conditions and environmental stresses. The parallel study of *pip* gene expression will help in revealing specific functions for these genes. A *pip* mutant showed a normal growth and development when grown in soil in standard conditions. However, this mutant showed an altered root growth, as compared to the wild type, when grown *in vitro* in the presence of NaCl. These preliminary observations need to be confirmed in a second, independently isolated mutant carrying a T-DNA insertion within the same *pip* gene. They suggest a role for aquaporins in the response of plants to salinity.

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Distribution and regulation of plant aquaporins

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There are at least 30 MIPs in Arabidopsis and most functionally tested genes have been shown to be aquaporins. Based on sequence similarities, they are divided into three subgroups; PIPs for plasma membrane intrinsic proteins, TIPs for tonoplastic intrinsic proteins and NLMs for NOD26-like-MIPs. Plant aquaporins are regulated at the transcription level, by hormones and by drought stress, and at the post-translational level, by phosphorylation. Knowledge concerning expression pattern and water transport activity regulation for different aquaporins is important for understanding the physiological role of aquaporins at the whole plant level.

We have cloned and characterised three spinach aquaporins. The plasma membrane aquaporins PM28A and PM28B are expressed in leaf, stem, and root as analysed by western blots and nordern hybridisation. The cell-type specific expression of the vacuolar aquaporin So-äTIP has been determined by immunogold labelling, and the protein is abundant in most vacuolated cells in all vegetative organs, but excluded from the leaf epidermis as well as from the root phloem parenchyma and meristem. No alteration in protein or RNA levels could be seen for these proteins under drought stress conditions.

The activity of PM28A has been shown to be regulated by phosphorylation *in vivo* and in an oocyte-swelling assay. Ser-274 in the C-terminal end of PM28A was phosphorylated *in vivo* in a turgor dependent manner. The water channel activity of PM28A when expressed in oocytes increased when Ser-274 and an additional site in the first cytoplasmic loop, Ser-115, were phosphorylated. In order to characterise these phosphorylation events, the phosphorylation sites around Ser-274 and around Ser-115 were expressed in *E. coli* as fusion-proteins with a maltose binding protein. The kinase phosphorylating Ser-274 is calcium dependent and membrane associated. The C-terminal phosphorylation site is conserved in the PIP2 subgroup of aquaporins and the cytoplasmic loop phosphorylation site is conserved in all PIPs and in most TIPs.

Recently, a third plasma membrane aquaporin named PM28C has been purified and cloned.

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Characterization of plasma membrane MIP proteins from maize

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A great number of MIP homologs have been identified in plant species. They are classified in two main groups according to their sequence identity with MIPs localized in either the plasma membrane (PIP, plasma membrane intrinsic protein) or the vacuolar membrane (TIP, tonoplast intrinsic protein). Sequence analysis show that PIPs cluster in two sequence subgroups. We are currently characterizing three maize PIPs, belonging to the PIP1 and PIP2 subfamilies (ZmPIP1a, ZmPIP1b and ZmPIP2a). Xenopus oocytes injected with *ZmPIP2a* cRNAs exhibit high osmotic water permeability coefficients (P_f), whereas those injected with *ZmPIP1a* and *ZmPIP1b* cRNAs exhibit low P_f values. A review of the literature shows that most PIP1 proteins identified in other plants have no- or a very low aquaporin activity in oocytes. Arabidopsis PIP1 proteins are the only exception. Control experiments show that this lack of activity of maize PIP1 proteins is not caused by their failure to reach the plasma membrane of the oocytes. ZmPIP1b also does not appear to facilitate the transport of any of the small solutes tested (glycerol, choline, ethanol, urea, amino acids). Experiments in which ZmPIP1b and ZmPIP2a proteins are co-expressed in oocytes show cooperativity between them: P_f of oocytes co-expressing ZmPIP1b and ZmPIP2a increases significantly in comparison with P_f of ZmPIP2a injected oocytes and this increase is proportional to the amount of injected ZmPIP1a cRNA. Altogether, these data indicate that when ZmPIP1a and ZmPIP1b are expressed in Xenopus oocytes, they reach the plasma membrane but do not function as water channels on their own, suggesting that either they are transporters for solutes not identified yet, or they need to be regulated by components or active aquaporins not present in Xenopus oocytes.

The plasma membrane localization of ZmPIPs relies entirely on amino acid sequence comparisons with proteins previously localized in the plasma membrane and needs to be demonstrated. We fused *ZmPIP1b* and *ZmPIP2a* cDNAs to the reporter gene coding for the Green Fluorescence Protein (GFP) and introduced these constructs into tobacco. Strong green fluorescent signals were observed in the plasma membrane surrounding the cell and in the perinuclear region. Filaments extending from the nucleus to the plasma membrane were also labeled and these could be ZmPIP-GFP proteins travelling through the secretory pathway.

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Functional characterisation of *Arabidopsis thaliana* aquaglyceroporins

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The functional identification of a subset of the major intrinsic proteins (MIPs) as molecular water channels in cell membranes has opened new access to investigate water relation in more detail. These proteins are encoded in the large MIP gene family and present in most organisms. Although the majority of these transport proteins tested so far are relatively specific for water, some very long-known members of the MIP gene family (e.g. GlpF and FPS1) are glycerol permeases.

In plants, known MIP gene products fall in at least three major subgroups; the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs) and the NOD26-like-MIPs (NLMs) named after the first MIP, NOD26, identified from plants [1]. While for most PIPs and TIPs, water channel activity seems to be the only (physiologically relevant) transport activity (with only few exceptions), functional analysis of members of the NLM group clearly show water and glycerol (and perhaps ion) transport activities, too. Interestingly, plant NLMs have amino acid signatures which can be found both in "true" aquaporins *and* in "true" glycerol permeases [2]. These proteins are now called "aquaglyceroporins".

We have investigated the NLM gene family in *Arabidopsis* in more detail because a mixed transport activity for water and glycerol together with e specific expression profile of the genes tend us to speculate that these proteins might be involved in specific stress responses of plant roots.

From own results and from data from the *Arabidopsis* genome project we could identify at least eight genes (*AtNLM1* to *AtNLM8*) which fall into the NLM group of MIP genes. Although not all of the genes were investigated in detail to date, four of the genes are expressed in *Arabidopsis* since we could clone the corresponding intron-free cDNA. One of them, AtNLM1, has been shown to be a functional aquaporin [1] when expressed in *Xenopus* oocytes.

We then used *AtNLM1* and *AtNLM2* cDNA to test for the glycerol permease activity by heterologous expression in *Saccharomyces cerevisiae*. Yeast mutants without a functional FPS1 glycerol permease show a growth retardation under anoxia and hyposmotic stress [3]. AtNLM1 and AtNLM2 proteins present in the plasma membrane of yeast cells were (to a certain extent) able to revert the *fps1* growth phenotype. Direct transport studies with radiolabelled glycerol into *AtNLM1* and *AtNLM2* transformed yeast cells clearly showed the glycerol transport activity of the *Arabidopsis* proteins.

The *AtNLM* genes seem to be either exclusively (*AtNLM1* and *AtNLM5*) or predominately (*AtNLM2* and *AtNLM4*) expressed in roots. When thinking about the physiological role of glycerol in cells, some authors discuss this solute in conjunction with hypoxia and anoxia which occurs frequently in plant roots in wet soil or after flooding. Glycerol is thought to function as an alternative electron acceptor when oxygen is not available Our first results indeed indicate that *AtNLM1* gene expression is affected by hypoxia stress and therefore might play a role under hypoxia/anoxia.

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Progress in the structure of a plant vacuole membrane aquaporin

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The plant aquaporin alpha-TIP is a member of the MIP-family and is found in vacuole membranes of seed storage organs. This aquaporin is expressed during embryo maturation and germination and may play roles in seed dessication, cytoplasmic osmoregulation, and/or seed rehydration. alpha-TIP is phosphorylated after germination *in vivo* and its water channel activity can be regulated by phosphorylation *in vitro* [1]. Structures for the mammalian MIP-family proteins AQP1 (CHIP28) and AQP0 (MIP26), as well as the *E.coli* MIP-family protein AQPZ have recently been determined by electron microscopy and image analysis of 2D crystals. We have purified alpha-TIP from the common bean *Phaseus vulgaris*, grown tubular crystals and have used electron crystallography to explore the molecular architecture of this aquaporin.

Vacuole membranes were isolated from ungerminated seeds and solubilized in diheptanoylphosphatidylcholine. One step purification and detergent exchange into decanoylsucrose were accomplished by anion exchange chromatography. *In vitro* reconstitution of the protein into soybean polar lipid bilayers resulted in the formation of tubular crystals. Diffraction patterns of these crystals exhibited discrete layer lines, demonstrating that they are helical. Images of frozen-hydrated flattened tubes were recorded by electron cryomicroscopy [2]. The flattened tubes behave like 2-D crystals, and a projection density map at a resolution of 7.7 . revealed that alpha-TIP assembles as a 60 x 60 . square tetramer [3]. Each subunit is formed by a heart-shaped ring comprised of density peaks, which we interpret as alpha-helices. The similarity of this structure to mammalian and bacterial plasma membrane MIP-family proteins suggests that the molecular design of functionally analogous and genetically homologous aquaporins is maintained between the plant, animal, and bacterial kingdoms. To understand the unique gating properties of alpha-TIP, 3-D maps of the phosphorylated (open channel) and unphosphorylated (closed channel) states are being determined using helical image processing.

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Calcium-dependent regulation of water channels in the *Arabidopsis* plasma membrane

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The plasma membrane of plant cells exhibits a large range of water permeability values depending on either the cell type or the technique used for the measurements. For instance, plasma membrane vesicles purified from tobacco suspension cells exhibited a reduced water permeability together with a lack of active water channels (Maurel *et al.*, 1997, Proc Natl Acad Sci USA, 94: 7103) whereas evidence for active water channels in *Arabidopsis* leaf protoplasts was provided using plants expressing a *pip1b* antisense transgene (Kaldenhoff *et al.*, 1998, Plant J., 14: 121). In the present work, we used *Arabidopsis* suspension cells to investigate the mechanisms which may allow the regulation of plant plasma membrane permeability to water. This question was first addressed at the cell level by means of pressure probe measurements. Using this technique, we found that the cell hydraulic conductivity (L_p) of *Arabidopsis* suspension cells can vary up to 8-fold depending on the measuring conditions. When cells were impaled in the presence of 20 mM KCl, 3 mM $CaCl_2$, 20 mM Hepes-Tris, pH 7.5, in the pipette and in bathing solution, cell L_p was reduced 4-fold as compared to control values obtained in the same medium but in the absence of calcium. In contrast, a nearly 2-fold increase in L_p was observed when chloride ions were replaced by fluoride ions, the latter presumably acting as a non specific protein phosphatase inhibitor.

To investigate these changes in permeability at the membrane level, plasma membrane vesicles were purified by aqueous two phase partitioning either in standard conditions or in the presence of NaF and chelators of divalent cations such as EDTA. The latter conditions are referred to as protected conditions. The water permeability of purified vesicles was measured by stopped-flow spectrophotometry and the presence of active water channels was deduced from a low activation energy. Vesicles purified in standard conditions exhibited a reduced water permeability and no water channel activity. A 5-fold higher water permeability associated to the presence of active water channels was observed in plasma membrane vesicles purified in protected conditions. Furthermore, addition of calcium to these purified membrane vesicles reduced their water permeability down to the basal level of membranes isolated in standard conditions. A half reduction in membrane water permeability was observed for a free calcium concentration in the the range of 20 nM. The cellular and molecular mechanisms of this plasma membrane-delimited regulation of *Arabidopsis* water channels by calcium are under investigation.

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Functional complementation of the yeast *Saccharomyces cerevisiae* mutant SP1-Ä*fps1* by the plant aquaporin BobTIP26 gene

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In contrast to most animals, higher plants are nonmotile organisms. As a consequence, when plants encounter stress, physiological responses are the main forms of defense. At the cellular scale, plant vacuoles appear to house adaptative mechanisms in order to cope with various environmental stresses. They act as reservoirs for water, ions and metabolites, and contribute to cytoplasmic homeostasis. The passage of water and/or small uncharged solutes through the vacuole membrane (tonoplast) is facilitated by specific channels which belong to the major intrinsic protein (MIP) family. Analyses of sequences and transport selectivities have shown the genetic and functional groupings into aquaporins, selective for water and aquaglyceroporins, permeated by water and other small molecules [1].

In plant cells, proteins from the MIP family have been localized in the plasma membrane and in the tonoplast (and refered to as PIPs and TIPs accordingly) [2]. More recently, it was shown that plant aquaporins may have a dual function in water and solute transport [3].

We have previously cloned a full length cDNA from *Brassica oleracea* L. var. *botrytis* encoding a membrane protein which has a high sequence homology and shares functional similarities with aquaporins from bacteria, animals and other plants [4]. The gene product is localized in the tonoplast and has been called BobTIP26. Like other plant aquaporins, BobTIP26 does not contain the hallmark residues found in aquaglyceroporins from animals.

In order to know whether BobTIP26 has a dual function in water and solute transport, we have tentatively used the cloned cDNA to functionally complement the yeast mutant SP1- Ä*fps1* [5] which is osmosensitive and unable to transport glycerol. Our preliminary results show that transformants rescue the mutant phenotype and are no longer sensitive to hypoosmotic shocks.

Experiments are in progress in order to understand the transport mechanisms responsible for the recovered phenotype.

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P 114

Regulation of two plasmodesmata transport channels in plants. The role of actin-myosin sphincter

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The existence of two transport channels in plasmodesmata has been determined by confocal microscopy in the last few years. The first one is a free diffusion contact of cytoplasmic compartments of cells through outside ring section of plasmodesmata, and another one is through internal (desmotubula) – vacuolar ones. Thus, we can state the existence not only the cytoplasmic, but also vacuolar symplast in plant cells. The model with actin-myosin sphincter within a cytoplasmic ring has to be considering in this case. The participation of such sphincter in simultaneous regulation of two transport channels has not been investigated. In this work we studded the self-diffusion of water molecules along radius of wheat seedling's root (Triticum aestivum L.) by pulsed NMR-method. The diffusion attenuation of spin echo signal with complicate non-exponential shape was obtained. The successive computer decoding of the signal results into three exponents components, each with it's own coefficients of water self-diffusion (CSD). It is determined that two out of three CSD characterized the self-diffusion of water molecules within the cytoplasmic and vacuolar symplast. They depend on water-permeability of transport channels in plasmodesmata at the diffusion time greater then 100 ms. In order to prove the participation of ATP-dependent actin-myosin proteins in regulation of water-permeability of the transport channels of plasmodesmata we studied the influence of inhibitor of actin-myosin mobility of muscle cells – 2,3-butadion monoxime (10^{-2}M, 30 min) and under the action of anaerobic stress on the water CSD within cytoplasmic and vacuolar symplasts. Anaerobiosis was simulated by the presence of sodium azide (10^{-2}M, 30 min) which reduces the ATP level in the tissue by over 80%. Both treatments lead to the decrease of water-permeability of both channels in plasmodesmata (about 20% on value of corresponding CSD of water molecules). Such response of plasmodesmata on decrease of ATP level and direct inhibition of actin-myosin mobility witness that both transport channels in plasmodesmata are opened either by ATP or by actin-myosin-dependent processes.

P 115

The effect of abscisic acid (ABA) on water transport through maize roots

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Abscisic acid (ABA) promotes the hydraulic conductivity of young maize roots both at the tissue (root Lp_r) and cellular level (cell Lp). Using the cell pressure probe, it has been shown that ABA transiently increases the water permeability of root cortical plasma membranes. Increases in root Lp_r, however, were stationary within 70 to 150 min. Effects of ABA on root hydraulics were highly specific for the undissociated (+)-cis-trans ABA. Other phytohormones tested proved to be ineffective. Kinetin and indole-3-acetic acid even decreased Lp. A possible role of aquaporins is discussed. Water channels have been located in the cortex of maize roots. A survey of a possible ABA effect on aquaporin mRNA has been done.

P 116

An almost-complete guide to the MIPs of Arabidopsis

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By the end of this year we will have access to the complete genomic sequence of Arabidopsis. This will for the very first time allow identification of all the MIP encoding genes in a plant. So far over 30 MIP genes have been found in Arabidopsis. These genes will be presented and compared to each other. Evolutionary as well as functional implications will be discussed.

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The *Arabidopsis thaliana* aquaporin AtPIP2a is activated in syncytial root cells induced by the plant parasitic nematode *Heterodera schachtii*

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Cyst nematodes are obligate sedentary endoparasites. Second-stage juveniles penetrate the root and induce a specific syncytial nurse cell system within the vascular cylinder. Syncytia are formed by partial digestion of cell walls and cell hypertrophy [1]. They are metabolically highly active cell complexes and act as strong sinks within the root. Apart from assimilates, the nematodes need large amounts of water as a solvent of nutrients. The daily nutrient uptake of an adult female was calculated to resemble four times the syncytial volume [2]. Former investigations of water relation parameters showed that the turgor pressure and the absolute value of the osmotic potential within syncytia was much higher than in uninfected root cells [3]. Due to the specific water balance syncytia seem to be optimally supplied with water under almost any condition (e.g. during water-stress).

Specific water channels should be necessary to meet the high demands in water supply. In order to identify such channel proteins, transgenic *Arabidopsis thaliana* plants with promoters of several aquaporins fused to the reporter gene â-glucuronidase (GUS) were infected with the beet cyst nematode *Heterodera schachtii* [4]. It could be shown that the promotor of the aquaporin AtPIP2a is activated in syncytia. As feeding cell development is a dynamic process GUS expression was determined at different time points. The maximum GUS level was found two days after nematode infection with 91 % GUS-positive syncytia. Later, GUS-stained syncytia were found at much lower frequency. The specific expression of the gene product PIP2a was confirmed by RT-PCR. We suggest the aquaporin AtPIP2a to be an important link in the water balance during the syncytium differentiation and nematode development.

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P 118

Aquaporin regulation under salt and osmotic stress in the halophyte *Mesembryanthemum crystallinum*

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It is evident that plant aquaporins should play a dynamic role in maintaining cellular water homeostasis under conditions that necessitate modifications in water flux, including drought- and salt-stress. Changes in water uptake and allocation would be required to balance alterations in cellular osmotic potential and therefore, aquaporin activity and/or expression must be tightly regulated. Using the halophyte *Mesembryanthemum crystallinum* as a model plant system (1) we have initiated studies towards understanding the role played by plant vacuolar (tonoplast) and plasma membrane aquaporins during the adaptation of this plant to salt- and drought-stress. To date, nine MIP (major intrinsic protein/aquaporin) -like genes have been identified and characterized in *M. crystallinum*, and from these we have chosen four and generated peptide-specific antibodies (2). Based on phylogenetic classification MIP-A, -B and -C belong to the PIP (plasma membrane intrinsic protein) plant subfamily of MIP?s, while MIP-F represents the TIP (tonoplast intrinsic protein) subfamily (3). Membrane protein analysis confirmed MIP-F as tonoplast-located. Interestingly, MIP-A and MIP-B were found in tonoplast fractions and also in membrane fractions distinct from either the tonoplast or plasma membrane, while MIP-C was most abundant but not exclusive to the plasma membrane fraction (2).

MIP regulation by drought and/or salt-stress was complex. Concomitant with changes in MIP abundance and differential regulation by salt- and drought-stress, MIP membrane localization on sucrose density gradients changed with the stress conditions. We suggest that within the cell, stress-induced alterations in MIP trafficking is occuring, in analogy to aquaporin cycling through animal endosomes (4). MIP cycling, and changes in MIP abundance may be fundamental for the control of cellular/tissue water flux.

In order to corroborate salt-induced changes in MIP expression at the protein level with changes in membrane water permeability, we have measured changes in vesicle light-scattering employing a Stopped-flow spectrophotometer. These experiments have demonstrated that *M. crystallinum* tonoplast water permeability is higher than that reported for other plant species, but similarly inhibited by $HgCl_2$. Preliminary results indicate that salt-stress caused a 20% increase in tonoplast water permeability following a 24 h treatment period.

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P 119

Investigations of water transport in genotypes of winter wheat by the method of NMR: effects of cold acclimation, abscisic acid and oryzalin

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The water status of cells is of great importance in plant responses on different factors of surroundings. However, there is a little information about the change of water transport in plant tissues during the increase (cold acclimation, abscisic acid - ABA) or the decrease (oryzalin) of plant frost resistance. The aim of work was to obtain the information about water membrane transport on the base of the relaxation and the diffusion parameter registration in plant tissues treated with cold, ABA and oryzalin. For the experiments the seedling leaves and roots of three winter wheat (*Triticum aestivum L.*) cultivars differed in frost resistance were used: Bezostaya 1 - low frost-resistant, Mironovskaya 808 - frost-resistant and Albidum 114 - very frost-resistant. Non-hardened (23^0C) and cold-hardened (3^0C, 3 and 7 days) seedlings were grown in water culture as with ABA (30 mkM) as without ABA. The modification of the cytoskeleton in vivo was carried out with the help of the plant microtubule (MT) polymerization specific inhibitor - oryzalin (the leaves - 15 mkM, the roots - 10 mkM, exposure - 3 h). By the method of NMR with the impulse gradient of magnetic field the times of spin-spin relaxation (T_2) and effective coefficient of water selfdiffusion (D_{eff}) were measured. T_2 characterizes the rate of transmembrane change between intro- and extracellular water which depends on the permeability of plasma membrane [L.V. Gusta et al., Plant Physiol. 63, 627-634, 1979]. D_{eff} was used for the quantitative appraisal of water diffusion transport in tissues [A.V. Anisimov, Studia Biophysica 91, 1-8, 1982]. The frost resistance of leaves and roots was tested by LT_{50} which was determined by electrolyte leakage from tissues.

It was shown that the roots had more high values of T_2 (120-130 ms) than the leaves (70-85 ms). It testifies about the intensive water transport of the roots. The treatment of non-hardened plants with exogenous ABA as cold hardening (3^0C, 3 and 7 days) has increased T_2 and has decreased D_{eff} in leaves, it means that it has slowed down the water transport through the plasma membrane. Possibly, this result is the consequence of cold- and ABA-induced intensification of membrane "lipidization" due to the acclimation of phospholipids in them. Oryzalin decreased T_2 and increased D_{eff} in the leaves and the roots and least of all in very frost-resistant cultivar. Taking into consideration the physic contacts of transmembrane proteins with MTs [C.W. Lloyd et al., In: Interactions between the plasma membrane and the cytoskeleton in plants Jn: Membranes: specialized functions in plants (Smallwood M., Knox Y.P., Bowles D.Y. eds.), Oxford: Bios. Scientific, pp.1-20, 1996] one may suppose that oryzalin disturbs the interactions between water channels and cortical MTs and that's why the water permeability of plasma membrane increases. Separately ABA and cold decreased the sensitivity of T_2 and D_{eff} to oryzalin in the leaves and most of all in frost-resistant cultivars. This effect may be the result of stabilizing influenced these two factors on the plasma membrane water permeability due to the intensification of its interactions with the tubulin cytoskeleton. At the joint treatment of plants with ABA and low temperatures (3^0C, 3 days) the additivity of their actions on oryzalin-induced disturbance of water membrane transport in the leaves was discovered. However at the long cold hardening (3^0C, 7 days) this additivity was not revealed. In the roots the responses of T_2 and D_{eff} to oryzalin after the treatment of seedlings with ABA and cold were smaller. This result may serve as the evidence of more weak root ability to the adaptation with the involvement of "cytoskeleton - water transport" system. It is concluded that the responses of water membrane transport to the cold acclimation, treatment with exogenous ABA and the desorganization of tubulin cytoskeleton are organ-specific and genotypically determined and correlate with not the same ability of different organs and cultivars to the adaptation and the extent of their frost resistance.

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A nuclear magnetic relaxation study of water uptake in Barley kernels

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Water uptake by barley kernels as a function of time was followed by ^{1}H Nuclear Magnetic Resonance (NMR) Spectroscopy. While increasing during the first seven hours of imbibition, the water spin-lattice relaxation rate R_1 slowly, but continuously, decreased afterwards. The observed changes in R_1 with imbibition time coincide with the typical two-phase behaviour generally observed in germinating seeds[1]. NMR can thus be used to non-invasively distinguish these two phases during imbibition or general germination. The second phase was characterized by a slow but continuous decrease in R_1, indicating a re-distribution of water within the kernel. Magnetic Resonance Imaging experiments showed that during the first seven hours of imbibition, water uptake in the kernel was restricted to the embryo region.

The^{1}H NMR spectrum of imbibing barley kernels is dominated by the water resonance. ^{1}H Resonances from the starch reserves are significantly broadened in the solid state to become undetectable. Resonances from lipids in oil bodies can be observed, however, upon imbibition these signals quickly lose signal intensity compared to the ^1H_2O resonance amplitude. A simple model was used to interpret the observed relaxation rate R_1 of water in the kernels during the first 7 hours of imbibition. Measurement of the ^{1}H spin-lattice relaxation rate R_1 showed that during this period, water uptake is dominated by a purely diffusive process with a †(Dt) dependence. Observed spin-lattice relaxation rates were treated as a superposition of relaxation rates from water molecules in two different physical phases: a mobile and a less mobile phase. Each phase is characterized by a specific ^1H_2O spin-lattice relaxation rate. Within the framework of a two-phase model, predictions of collegative properties such as water viscosity in the kernel can be calculated. Using the Stokes-Einstein-Debye theory for viscosity, the overall water viscosity within the kernel was determined using the two-phase model to describe water mobility.

The observed continuous decrease in spin-lattice relaxation rate after 7 hours of imbibition coincides with the onset of major metabolic events in preparation for radicle emergence. Measurement of ^1H_2O spin-lattice relaxation rates can thus be used to study seed viability.

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