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Importance of the mercury-sensitive cysteine on function and routing of AQP1 and AQP2 in oocytes

SABINE M. MULDERS, JOHAN P. L. RIJSS, ANITA HARTOG, RENE J. M. BINDELS, CAREL H. VAN OS, AND PETER M. T. DEEN
Department of Cell Physiology, University of Nijmegen, 6500 HB Nijmegen, The Netherlands

Mulders, Sabine M., Johan P. L. Rijss, Anita Hartog, Rene J. M. Bindels, Carel H. van Os, and Peter M. T. Deen. Importance of the mercury-sensitive cysteine on function and routing of AQP1 and AQP2 in oocytes. Am. J. Physiol. 273 (Renal Physiol. 42): F451–F456, 1997.—To discriminate between water transport of aquaporin-2 (AQP2) mutants in nephrogenic diabetes insipidus and that of an AQP2 molecule used to drag them to the oolemma, we investigated the mercury sensitivity of wild-type and AQP2 C181S proteins in oocytes. Incubation with HgCl₂ inhibited the osmotic water permeability (Pf) of human (h) AQP2 by 40%, whereas inhibition of hAQP1 was 75%. Oocytes expressing hAQP1 C189S revealed a Pf comparable to wild-type hAQP1, but mercury sensitivity was lost. In contrast, no increase in Pf was observed when hAQP2 C181S was expressed. Also, expression of rat AQP2 C181A and C181S mutants did not increase the Pf, which contrasts with published observations. Immunocytochemistry and immunoblotting revealed that only AQP1, AQP1 C189S, and AQP2 were targeted to the plasma membrane and that AQP2 mutant proteins are retarded in the endoplasmic reticulum. In conclusion, water transport through AQP2 is less sensitive to mercury inhibition than through AQP1. Furthermore, substitution of the mercury-sensitive cysteine for a serine results in an impaired routing of human and rat AQP2. Similar mutations have no effect on AQP1 function, which is indicative of structural differences between AQP1 and AQP2.

THE CLONING of the first discovered water channel, aquaporin-1 (AQP1), opened an exciting new field of research, and, at present, six different mammalian aquaporins are known (AQP0–AQP5) (5–7, 9, 10, 13, 16, 18). Aquaporins are members of the major intrinsic protein (MIP) family, traverse the membrane six times, and have intracellular amino and carboxyl termini (19). A characteristic amino acid stretch present in every member of the MIP family is the NPA box, found in the first intracellular loop (loop B) and in the third extracellular loop (loop E) (Fig. 1).

So far, the best-studied water channels are AQP1 and AQP2. AQP1 is constitutively expressed in erythrocytes, renal proximal tubules, descending limb of Henle, and in several other epithelia (15). AQP2 has been shown to be the vasopressin-regulated water channel that is exclusively expressed in renal collecting duct principal cells and inner medullary collecting duct cells (6).

Water permeation through AQP1 can be inhibited by binding of mercury to cysteine 189 (17, 22). When this cysteine is replaced by a serine, the water permeability (Pf) remains unaffected, but mercury sensitivity is lost. Recent reports show that mercury binding to cysteine 181 in AQP2 also results in inhibition of the Pf and substitution of cysteine 181 for serine or alanine results in loss of mercury sensitivity, together with a 20–50% reduction of Pf compared with wild-type (wt) AQP2 (1, 2).

Recently, we have reported mutations in the AQP2 gene, which are the cause of the autosomal recessive form of nephrogenic diabetes insipidus (NDI) (4, 20). All missense AQP2 proteins in NDI were found to be impaired in their routing to the oolemma (3). Jung et al. (11) reported that coexpression of AQP1 missense mutants and an AQP1 truncation mutant (D237Z) in oocytes overcomes the impaired routing to the plasma membrane (11). To apply a similar strategy as used by Jung et al. in the study of AQP2 missense mutants, we must be able to discriminate between water movement through the missense mutant and through a truncated AQP2 protein. Coexpression in oocytes of mercury-insensitive AQP2 missense proteins together with a truncated wild-type AQP2 should result in mercury-insensitive water flow when the missense mutant is still a functional water channel. Therefore, the usefulness of mercury (ins)ensitivity of AQP2 proteins as a tool to discriminate between water permeation conferred by the truncated or the mutant AQP2 proteins was investigated.

MATERIALS AND METHODS

Expression constructs. The human AQP2 C181S clone was obtained by introducing a C-to-G transition at position 634 and a G-to-C transition at position 586 in the human AQP2 cDNA using the Altered Sites II in vitro mutagenesis kit (Promega, Madison, WI). The clone that was identical to wt-AQP2, except for the above-mentioned transitions, was selected by sequence analysis (8). After digestion with BamHI and Kpn I, a 282-bp fragment was isolated by gel electrophoresis and inserted into the corresponding sites of pT7AQP2 (4). In this vector, an Xba I-Nde I fragment had been removed from the polylinker to have a unique BamHI site in the AQP2 cDNA. The rat AQP2 C181S and C181A cDNAs (2) in the pXBGev1 expression vector were kindly provided by Drs. K. Fushimi and S. Sasaki (Tokyo, Japan). These constructs were checked by restriction analysis. The human AQP1 and the AQP1 C189S cDNAs (17) in the PBGev1 expression vector were kindly provided by Drs. G. M. Preston and P. Agre (Baltimore, MD).

Transcription. The constructs were linearized with Sal I (pT7Ts) or Xba I (pXBGev1) and in vitro transcribed using T7 RNA polymerase (pT7Ts) or T3 RNA polymerase (pXBGev1), according to Promega’s (1991) Protocols and Principles Guide, except that nucleotide triporphosphates and 7-methyl-diguano­sine triphosphate were used at a final concentration of 1 mM. The cRNAs were purified and dissolved in diethyl pyrocarbonate-treated milliQ water. The integrity of the cRNA was
Fig. 1. Proposed topology model of aquaporin-1 and -2 (AQP1 and AQP2). Highly conserved NPA boxes in loops B and E and the mercury-sensitive cysteine (C189 in AQP1, C181 in AQP2) in loop E are indicated.

checked by agarose gel electrophoresis, and the concentration was determined spectrophotometrically.

Water permeability. Stage V and VI oocytes of Xenopus laevis were isolated and injected with water or 10 ng of cRNA. After incubation for 3 days in modified Barth solution (MBS) at 18°C, oocytes were analyzed in a swelling assay as described previously (4). Oocyte swelling was performed at 22°C following transfer from 200 to 70 mosmol. For the mercury inhibition studies, oocytes that exhibited a high \( P_f \) were selected and incubated for 5 min in MBS containing 1 or 3 mM HgCl\(_2\). During the swelling assay, the same concentration of HgCl\(_2\) was present in the diluted buffer. After this assay, the same oocytes were incubated for 15 min in buffer containing 5 mM \( \beta \)-mercaptoethanol and assayed again in diluted buffer containing 5 mM \( \beta \)-mercaptoethanol.

Oocyte lysis and membrane isolation. To determine the stability and size of the AQP1 and AQP2 proteins, eight oocytes were homogenized in 160 \( \mu \)l homogenization buffer A ([in mM]: 20 tris(hydroxymethyl)aminomethane (Tris, pH 7.4), 5 MgCl\(_2\), 5 Na\(_2\)HPO\(_4\), 80 sucrose, 1 M EDTA, 1 diithiothreitol (DTT), 1 phenylmethylsulfonyl fluoride, and 5 \( \mu \)g/ml leupeptin and pepstatin) at 4°C at 1, 2 and 3 days after injection. Subsequently, the lysates were centrifuged twice for 10 min at 125 \( g \) to remove yolk proteins. On the 3rd day after injection, plasma membranes were isolated from 25 oocytes according to Wall and Patel (21). Oocyte lysates of an equivalent of one oocyte were digested with recombinant endoglycosidase H (endo H) (New England Biolabs, Beverly, MA) according to the manufacturer, except that protein samples were digested for 18 h after denaturation for 30 min at 37°C.

Immunoblotting. Lysates or plasma membranes equivalent to 0.1 oocyte or 8 oocytes, respectively, were denatured for 30 min at 37°C in sample buffer (2% (wt/vol) sodium dodecyl sulfate (SDS), 50 mM Tris (pH 6.8), 12% (vol/vol) glycerol, 0.01% (wt/vol) Comassie brilliant blue, 100 mM DTT), electrophoresed through a 13% SDS-polyacrylamide gel (12), and transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) as described previously (3). Efficiency of protein transfer was checked by reversible staining of the membrane with Ponceau red. For immunodetection, the membrane was incubated with a 1:10,000 dilution of affinity-purified rabbit polyclonal antibodies directed against the 15 COOH-terminal amino acids of rat AQP2 (3) or a 1:200 dilution of a mouse monoclonal antibody directed against dog AQP1 (gift from M. L. Jennings, Galveston, TX). As a secondary antibody, a 1:5,000 dilution of affinity-purified anti-rabbit or anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (Sigma, St. Louis, MO) was used. Proteins were visualized using enhanced chemiluminescence (Boehringer, Mannheim, Germany).

Immunocytochemistry. Three days after injection, oocytes were stripped from remaining vitelline membranes and were incubated for 1 h in 1% wt/vol paraformaldehyde fixative (14), dehydrated, and embedded in paraffin. After blocking with 10% (vol/vol) goat serum in Tris-buffered saline (TBS), sections of oocytes expressing AQP2 proteins were incubated overnight at 4°C with affinity-purified polyclonal AQP2 antibodies diluted 1:500 in 10% (vol/vol) goat serum in TBS. The sections of oocytes expressing AQP1 proteins were incubated with a 1:100 dilution of a rabbit polyclonal AQP1 antibody. This antibody was prepared by immunization of rabbits with a synthetic peptide representing the last 15 COOH-terminal amino acids of rat AQP1 coupled to rabbit serum albumin. After three washes for 10 min in TBS, the sections were incubated for 1 h in a 1:100 dilution of anti-rabbit IgG coupled to fluorescein isothiocyanate (FITC) (Sigma). The sections were again washed three times for 10 min, dehydrated by washing in 70–100% (vol/vol) ethanol, and mounted in mowiol 4–88, 2.5% (wt/vol) Na\(_2\)MoO\(_4\). Photographs were taken with a Zeiss Axioskop microscope equipped with epifluorescent illumination, and Kodak EPH P1600X films were used.

RESULTS

to determine the water permeability of wt-human (h)AQP2 and hAQP2 C181S, in comparison with wt-AQP1 and hAQP1 C189S, cRNAs encoding these proteins were injected into Xenopus oocytes. \( P_f \) measurements revealed that the water transport mediated by hAQP2 and hAQP1 was comparable (Fig. 2). After incubation of the same oocytes in 1 mM HgCl\(_2\), the percentage inhibition of water transport was 40 ± 8% (mean ± SE) for AQP2, whereas the inhibition of water transport was 75 ± 5% for AQP1. Incubation of the same oocytes in 5 mM \( \beta \)-mercaptoethanol fully restored the \( P_f \) of hAQP2 and hAQP1. Stronger inhibition was

Fig. 2. Osmotic water permeability (\( P_f \)) of oocytes 3 days after injection of water or 10 ng of the following cRNAs: hAQP1, hAQP2, hAQP1 C189S, hAQP2 C181S, rAQP2 C181S, or rAQP2 C181A. Identical oocytes were subjected to the standard osmotic swelling assay (hatched bars), after incubation with 1 mM HgCl\(_2\) (closed bars), and after subsequent incubation with 5 mM \( \beta \)-mercaptoethanol (open bars). Values are means ± SE of 15–40 oocytes.
MERCURY-SENSITIVE CYSTEINE IN AQP1 AND AQP2

The absence of $P_f$ in oocytes injected with cRNA encoding the AQP2 C181 mutants could be caused by the absence of the protein or a disturbed trafficking to the plasma membrane. To confirm the presence of AQP2 and to determine the stability, oocyte lysates were prepared at 1, 2, and 3 days after injection and subjected to immunoblotting. Reversible Ponceau red staining showed that comparable amounts of oocyte lysates were loaded (data not shown). Chemiluminescence revealed a band of ~29 kDa in all lanes of oocytes injected with cRNA encoding AQP2, representing the native, unglycosylated form of AQP2 (Fig. 3A). The hAQP2 C181S mutant protein showed, besides the native 29-kDa band, a strong additional band of ~32 kDa. Oocytes expressing rAQP2 C181S or rAQP2 C181A mutants also showed unglycosylated AQP2 and a larger AQP2-specific band, but these bands migrated somewhat faster than the human AQP2 protein (~27 and ~31 kDa) (Fig. 3A).

Immunoblots of oocytes expressing hAQPl revealed one band of 28 kDa representing the native unglycosylated form of AQPl. Oocytes expressing hAQPl C189S showed the same native band and a minor additional band observed after incubation for 5 min in 3 mM HgCl$_2$, with the same relative difference in $P_f$ between AQP2 and AQPl (data not shown). Expression of hAQPl C189S resulted in a high $P_f$, which was unchanged in the presence of 1 mM HgCl$_2$. In contrast, oocytes injected with cRNA coding for hAQP2 C181S revealed a $P_f$ that was not different from water-injected control oocytes. Because the latter result is totally in contrast to similar studies with rat AQP2 (1, 2), cRNAs encoding hAQP2 C181S or AQP2 C181A were also injected into oocytes. Swelling tests on these oocytes also revealed $P_f$ values that were not different from water-injected control oocytes (Fig. 2).

The absence of $P_f$ in oocytes injected with cRNA encoding the AQP2 C181 mutants could be caused by the absence of the protein or a disturbed trafficking to the plasma membrane. To confirm the presence of AQP2 and to determine the stability, oocyte lysates were prepared at 1, 2, and 3 days after injection and subjected to immunoblotting. Reversible Ponceau red staining showed that comparable amounts of oocyte lysates were loaded (data not shown). Chemiluminescence revealed a band of ~29 kDa in all lanes of oocytes injected with cRNA encoding AQP2, representing the native, unglycosylated form of AQP2 (Fig. 3A). The hAQP2 C181S mutant protein showed, besides the native 29-kDa band, a strong additional band of ~32 kDa. Oocytes expressing rAQP2 C181S or rAQP2 C181A mutants also showed unglycosylated AQP2 and a larger AQP2-specific band, but these bands migrated somewhat faster than the human AQP2 protein (~27 and ~31 kDa) (Fig. 3A).

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band of ~31 kDa (Fig. 3B). Endoglycosidase H (endo H) digestion of oocyte lysates expressing AQP2 proteins and subsequent immunoblotting revealed that the additional bands of hAQP1 C189S (not shown), hAQP2 C181S, rAQP2 C181S, and rAQP2 C181A were not detectable anymore (Fig. 4). No AQP1 or AQP2 signals were obtained in lanes loaded with lysates from water-injected oocytes.

To determine the plasma membrane expression of wild-type and mutant aquaporins, a fraction enriched for plasma membranes was subjected to immunoblotting (Fig. 5). Chemiluminescence revealed that wt-hAQP2 was clearly present in the plasma membrane, whereas hC181S, rC181S, or rC181A mutant proteins could not be detected in this fraction (Fig. 5A). Wild-type AQP1 and AQP1 C189S were expressed in the plasma membrane to the same extent (Fig. 5B). In the plasma membrane fraction of oocytes expressing AQP1 C189S, a relatively higher amount of glycosylated AQP1 (40–45 kDa) was present than in the plasma membrane fraction of wt-AQP1.

Immunocytochemical analysis of oocytes expressing wt-hAQP2 (Fig. 6A), wt-AQP1 (data not shown), or AQP1 C189S (not shown) showed a clear, intense staining of the plasma membrane with a weak staining of the cytoplasm. In contrast, oocytes expressing hAQP2 C181S, rAQP2 C181S, or rAQP2 C181A showed an intense staining of the cytoplasm and only a very faint staining of the plasma membrane (Fig. 6, B–D). No AQP2 or AQP1 labeling was found in water-injected control oocytes (not shown).

To check the expression system, the Pf measurements and immunoblots were repeated with oocytes isolated from Xenopus laevis from an unrelated source. These experiments yielded identical results (data not shown).

**DISCUSSION**

To drag NDI-related AQP2 mutants to the plasma membrane of Xenopus oocytes to obtain information on the structure-function relationship of AQP2, two requirements had to be fulfilled: 1) oocytes expressing wt-AQP2 should reveal a large decrease in \( P_f \) on incubation with mercurials, and 2) the mutation of cysteine 181 to serine in hAQP2 should not affect the expression and function of the protein.

To address the first issue, mercury sensitivity of AQP2 was compared with that of AQP1. \( P_f \) studies revealed that the \( P_f \) values of oocytes expressing hAQP1 or hAQP2 were comparable (Fig. 2) but that the mer-
In conclusion, our results show that water transport in oocytes, human AQP2 C181S, rat AQ2 P2 C181s, and rat AQ2 P2 C181A are not functional because they are severely disturbed in their routing to the oolemma. The misrouting of AQ2 P2 C181S precluded our goal to use this mutation in AQ2 P2 to discriminate between water permeability conferred by a truncation mutant and NDI-related AQ2 P2 mutants. In addition, the water permeability obtained for rat C181S and C181A by Bai et al. (1, 2) was of critical importance for their conclusion that the water pore in AQ2 P2 is different from the one in AQ2 P1. They concluded that loops C and D are located near the pore in AQ2 P2 and that loops B and E are not of critical importance in AQ2 P2 as in AQ2 P1. Our results with their clones and human AQ2 P2 C181S makes this conclusion at least doubtful.

In conclusion, our results show that water transport through AQ2 P2 is less sensitive to mercury inhibition than through AQ2 P1 and that substitution of the cysteine residue in loop E for a serine completely disturbs proper folding, assembling, and/or routing of human and rat AQ2 P2, whereas the same mutation has no effect on AQ2 P1. This suggests that mutations in AQ2 P1 are better tolerated than in AQ2 P2 and thus that AQ2 P1 and AQ2 P2 might differ in their tertiary structure.

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Address for reprint requests: S. Mulders, 162 Dept. of Cell Physiology, Trigon Bldg., KUN, PO Box 9101, 6500 HB Nijmegen, The Netherlands.

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