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Articles

Mercury-Sensitive Residues and Pore Site in AQP3 Water Channel[†]

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ABSTRACT: Water channel function of all aquaporins (AQPs) but AQP4 can be inhibited by mercurial reagents. Mercurial reagents are believed to bind specifically to cysteine residues and block the aqueous pore of AQPs. Because of the low homology of AQP3 to other AQPs, it is not certain whether the pore structure of AQP3 is similar to that of the others. Determination of mercury-sensitive cysteine residues in AQP3 and comparison with those in other AQPs will help to resolve this question. When AQP3 was expressed in *Xenopus* oocytes, incubation with 0.3 mM $HgCl_2$ decreased its osmotic water permeability (P_f) by ~30%. To identify the mercury-sensitive site, six individual cysteine residues in human AQP3 (at positions 11, 29, 40, 91, 174, and 267) were altered by site-directed mutagenesis. Mutants of C11S and C11A had a similar basal P_f to wild-type but acquired mercury resistance. Replacement of Cys-11 with Trp, which possesses a large side chain, did not change P_f. Mercurial inhibition of P_f was still observed in five other Cys-to-Ser mutants. These results suggest that Cys-11 is the mercury-sensitive residue in AQP3 and that this residue might be independent of water channel function. Mutation of Tyr-212, a position corresponding to the mercury-sensitive residues in AQP1 and AQP2, to cysteine enhanced the mercurial inhibition of P_f. Y212W had no water channel activity. Expression of AQP3 increased glycerol permeability (P_{gly}) 3.1-fold, whereas P_{gly} of Y212W-expressing oocytes was similar to P_{gly} of control oocytes. Cysteine mutation at Tyr-212 increased the inhibitory effect of mercury on P_{gly}. These results suggest that the structure of the aqueous pore of AQP3 resembles those of AQP1 and AQP2 and support the hypothesis that water and small molecules share a common pore in AQP3.

Water movement across the plasma cell membrane is a fundamental process for the maintenance of the intracellular environment. Aquaporins (AQPs)[1] are a family of integral membrane proteins which transport water selectively. Five mammalian AQPs (AQP1−AQP5) have been identified so far, and all of them have been shown to be widely distributed in water-transporting epithelia and endothelia of a variety of tissues (*1*−*7*). Mercury reagents are known to block water channel function, presumably by inducing conformational change of the protein, and the blockade is reversed by reducing agents such as 2-mercaptoethanol (*8*, *9*). In fact, reversible mercurial inhibition has been observed in all mammalian AQPs but AQP4, a mercury-resistant water channel (*1*−*7*). It is believed that cysteine residue is the target site for the mercury effect (*10*, *11*). Recent studies demonstrated that the mercury-sensitive residues in AQP1 and AQP2 are Cys-189 (*10*, *11*) and Cys-181 (*12*), respectively. In amino acid sequence alignment, these cysteines are located at the identical position near the conserved Asn-Pro-Ala (NPA) motif, suggesting the structural similarity in

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[1] Abbreviations: AQP, aquaporin; MIP, eye lens major intrinsic protein; P_f, osmotic water permeability; P_{gly}, glycerol permeability; E_a, activation energy; pCMBS, *p*-chloromercuriphenylsulfonate.

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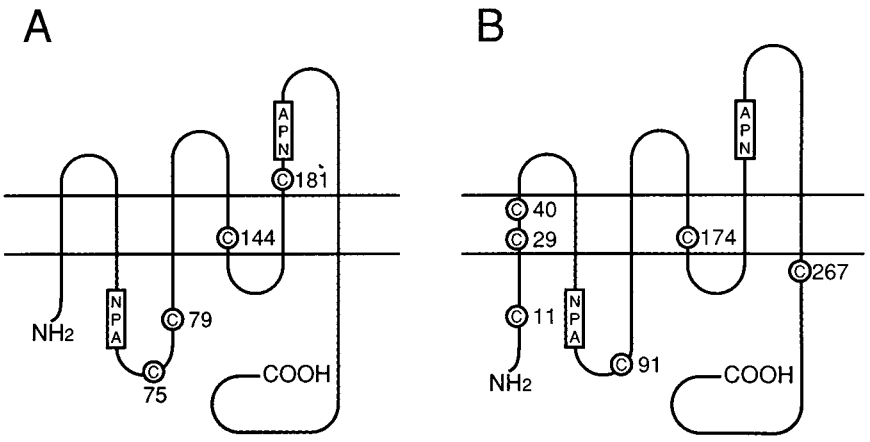

FIGURE 1: Predicted membrane topology of human AQP2 (A) and AQP3 (B). Both AQP2 and AQP3 possess six presumed transmembrane segments and five connecting loops. The location of cysteine residues is shown, and two conserved Asn-Pro-Ala (NPA) motifs are boxed.

AQP1 and AQP2. Moreover, tryptophan replacement of the mercury-sensitive cysteine completely inhibited water channel function of AQP1 and AQP2 (*10*−*12*). These observations raise the hypothesis that the mercury-sensitive cysteine participates in the formation of the aqueous pore and that the large side chain of tryptophan causes physical occlusion of the pore (*10*−*12*).

AQP3 is present predominantly in the basolateral membrane of the kidney collecting duct and the colon villas epithelium (*3*, *4*, *13*). A unique characteristic of AQP3 is that it transports nonionic small molecules such as glycerol and urea in addition to water (*3*, *4*). Figure 1 shows deduced membrane topologies of human AQP2 and AQP3 which are consistent with the general rule of the major intrinsic protein (MIP) family: two tandem sequence repeats, two conserved NPA motifs, and six predicted membrane-spanning domains. AQP3 contains six cysteine residues, but the position equivalent to the mercury-sensitive site in AQP2 is tyrosine (Tyr-212), not cysteine. To determine the mercury inhibitory site in AQP3, individual cysteine residues were mutated in the present study. In addition, AQP3 mutants at Tyr-212 were constructed to examine the significance of this site.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis and in Vitro cRNA Synthesis. Mutants of human AQP3 were made with the polymerase chain reaction technique using the AQP3 expression vector as a template (*12*, *14*). A fragment between the *Cla*I site and *Bgl*II site (nucleotide 932) in pAQP3/ev1 was replaced by a polymerase chain reaction coding for mutants. Table 1 lists the AQP3 mutants constructed in this study. Mutations were confirmed by a fluorescence DNA sequencer (Applied Biosystems 373A). Capped RNA transcripts of mutated AQP3 and wild-type AQP2 and AQP3 were synthesized in vitro with T3 RNA polymerase using *Not*I-digested AQP cDNA.

Osmotic Water Permeability and Glycerol Permeability of Oocyte. Oocytes at stages V−VI were obtained from *Xenopus laevis* (*2*). Each oocyte was injected with 40 nL of water, 3 ng of wild-type AQP2 cRNA or 5 ng of wild-type of mutated AQP3 cRNA, and incubated for 48 h at 20 °C in Barth's buffer. The osmotic water permeability (P_f) of oocyte was measured at 20 °C from the time course of osmotic cell swelling. Oocytes were transferred from 200 mOsm Barth's buffer to 70 mOsm buffer in a temperature jacketed chamber (Kitazato MD-10F) and then imaged on a CCD camera connected to an area analyzer (Hamamatsu Photonics C3160). Serial images at 0.5-s intervals were stored in a computer. P_f was calculated from the initial 15-s response of cell swelling as reported (*14*). To examine the effect of mercury on oocyte P_f, oocytes were incubated in Barth's buffer containing 0.3 mM $HgCl_2$ for 5 min before the assay. To determine whether the mercurial effect on oocyte P_f was reversible, oocytes were exposed to a reducing agent, 2-mercaptoethanol, at 5 mM for 15 min after the $HgCl_2$ treatment.

Table 1: Site-Specific Mutations in the Human AQP3 Water Channel

wild-type		mutant	mutant	
amino acid	codon	name	amino acid	codon
cysteine 11	TGC	C11S	serine 11	TCC
		C11A	alanine 11	GCC
		C11W	tryptophan 11	TGG
cysteine 29	TGC	C29S	serine 29	TCC
cysteine 40	TGT	C40S	serine 40	TCT
cysteine 91	TGC	C91S	serine 91	TCC
cysteine 174	TGT	C174S	serine 174	TCT
cysteine 267	TGC	C267S	serine 267	TCC
tyrosine 212	TAC	Y212C	cysteine 212	TGC
		Y212S	serine 212	TCC
		Y212W	tryptophan 212	TGG
alanine 213	GCC	A213C	cysteine 213	TGC
		A213S	serine 213	TCC
		A213W	tryptophan 213	TGG

The glycerol permeability (P_{gly}) was measured from the initial rate of glycerol uptake into oocytes (*3*, *15*). Oocytes were incubated for 2 min at 20 °C in Barth's buffer containing 92 kBq/mL of [U-^{14}C]glycerol (specific activity, 5.59 GBq/mmol; Amersham). After the incubation, the oocytes were rapidly rinsed 3 times in ice-cold Barth's buffer. The individual oocytes were lysed im 0.2 mL of 10% SDS, followed by liquid scintillation counting. To test the mercurial effect on P_{gly}, oocytes were incubated for 5 min in the presence of 0.3 mM $HgCl_2$. P_{gly} was also measured at 4 and 30 °C to obtain E_a from Arrhenius plot.

Western Blot Analysis. Oocyte membranes were isolated as previously described (*10*). The membrane fraction was resuspended in a loading buffer containing 3% SDS, 65 mM Tris-HCl, 10% glycerol, and 5% 2-mercaptoethanol. After being heated at 70 °C for 10 min, solubilized proteins were separated by SDS−PAGE. Membrane proteins from 3

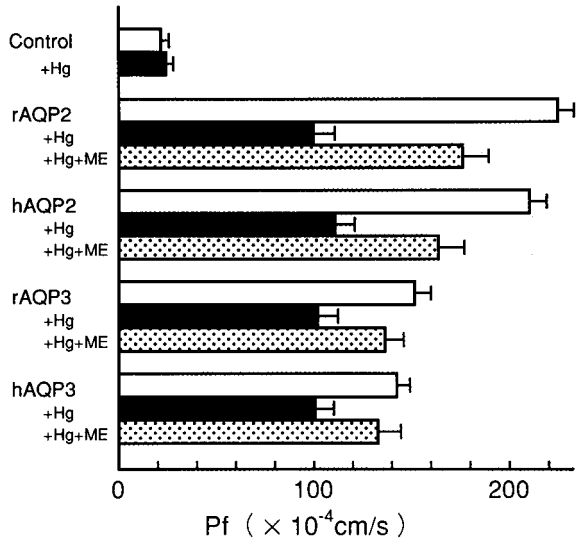

FIGURE 2: Osmotic water permeability (P_f) and $HgCl_2$ inhibition of *Xenopus* oocytes expressing rat (r) or human (h) AQP. Oocytes were injected with 40 nL of water (Control), 3 ng of AQP2 cRNA, or 5 ng of AQP3 cRNA 48 h prior to experiments. P_f was calculated from the time course of osmotic cell swelling of the oocytes. Where indicated, the oocytes were incubated for 5 min in the presence of 0.3 mM $HgCl_2$ (+Hg) or were further incubated for 15 min in the presence of 5 mM 2-mercaptoethanol (+Hg+ME). Each bar represents the mean ± SE of 30−37 measurements.

oocytes were applied in each lane. The proteins were transferred to Immobilon-P filter (Millipore) using a semidry system. The filters were incubated for 1 h with an affinity-purified antibody against 15 COOH terminal amino acids of AQP3 (3). The filters were further incubated for 1 h with [^{125}I]protein A solution, followed by autoradiography.

RESULTS

AQP3 was expressed in oocytes, and the effect of $HgCl_2$ on P_f was examined. AQP2 was also expressed as a positive control. Figure 2 summarizes results of P_f measurement in oocytes injected with water or AQP cRNA. P_f of water-injected oocytes (Control) was 23 ± 2 (SE) × 10^{-4} cm/s. Five-minute incubation with 0.3 mM $HgCl_2$ had no effects on P_f of Control oocytes. Injection of rat AQP2 cRNA increased P_f to (214 ± 8) × 10^{-4} cm/s. P_f of rat AQP2-expressing oocytes was decreased to (101 ± 10) × 10^{-4} cm/s after treatment with 0.3 mM $HgCl_2$. Mercurial inhibition of P_f was reversed by 68% after subsequent incubation with 2-mercaptoethanol, indicating that the inhibition was a specific rather than a nonspecific effect. In oocytes injected with human AQP2 cRNA, the P_f value, the effect of mercury (−48%), and the recovery by 2-mercaptoethanol (+54%) were similar to those observed in rat AQP2. Expression of cRNA encoding rat and human AQP3 increased P_f to (152 ± 8) × 10^{-4} and (143 ± 7) × 10^{-4} cm/s, respectively. Treatment with 0.3 mM $HgCl_2$ inhibited P_f by 30−33%, and further treatment with 2-mercaptoethanol reversed P_f by 70−74%. These results suggested that AQP3 is relatively resistant to mercury compared to AQP2.

To identify the site of P_f inhibition by $HgCl_2$ in human AQP3, each of the six cysteine residues, namely, the cysteines at 11, 29, 40, 91, 174, and 267 (Figure 1), were mutated to serine (the nucleotide sequence of human AQP3 cDNA has been deposited in the GenBank data base under

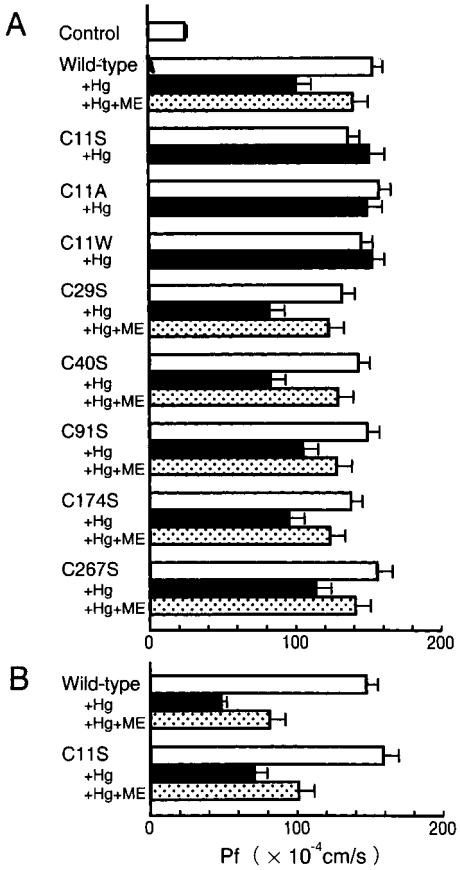

FIGURE 3: Mutation of cysteine residues in human AQP3 and the effect on oocyte P_f of $HgCl_2$ at 0.3 mM (A) and 1 mM (B). Six individual cysteine residues in the amino acid sequence of AQP3 were mutated to serine. In addition, cysteine 11 was mutated to alanine or tryptophan. Oocytes were injected with water (Control), 5 ng of cRNA encoding wild-type or mutated human AQP3. Where indicated, oocyte P_f was measured after 5-min treatment in 0.3 mM (A) or 1 mM (B) of $HgCl_2$ (+Hg), or after subsequent 15-min incubation in 5 mM 2-mercaptoethanol following $HgCl_2$ treatment (+Hg+ME). Each bar represents the mean ± SE of 25−33 measurements.

accession number AB001325). As shown in Figure 3A, oocyte P_f of the Cys-to-Ser mutants [(132−157) × 10^{-4} cm/s] was comparable to P_f of wild-type type AQP3. Mercury sensitivity differed among these mutants. P_f of C11S was not inhibited by 0.3 mM $HgCl_2$, whereas the P_fs of the 5 other mutants were reversibly blocked by 27−41% after incubation with $HgCl_2$, suggesting that Cys-11 is the mercury-binding site. When C11S-expressing oocytes were treated with 1 mM $HgCl_2$ for 5 min, a 55% decrease was observed in P_f (Figure 3B). Subsequent incubation with 2-mercaptoethanol resulted in only a 34% reversal of P_f, suggesting that 1 mM $HgCl_2$ exerts some nonspecific effect on oocyte P_f. Similar results were obtained in P_f of oocytes expressing wild-type AQP3 (66% inhibition and 32% reversal). Thus we did not use 1 mM $HgCl_2$ as a test dose in the present study. To characterize the specificity of the Cys-11 residue, mutants of C11A and C11W were examined. These mutants also exhibited water channel function and resistance to 0.3 mM $HgCl_2$ (Figure 3A).

The residue Tyr-212 in human AQP3 is at the position equivalent to the mercury-sensitive cysteine in AQP1 and AQP2. This residue was mutated to cysteine, serine, or tryptophan. P_f of Y212C oocytes [(135 ± 11) × 10^{-4} cm/s] was similar to P_f of wild-type AQP3 oocytes (Figure 4).

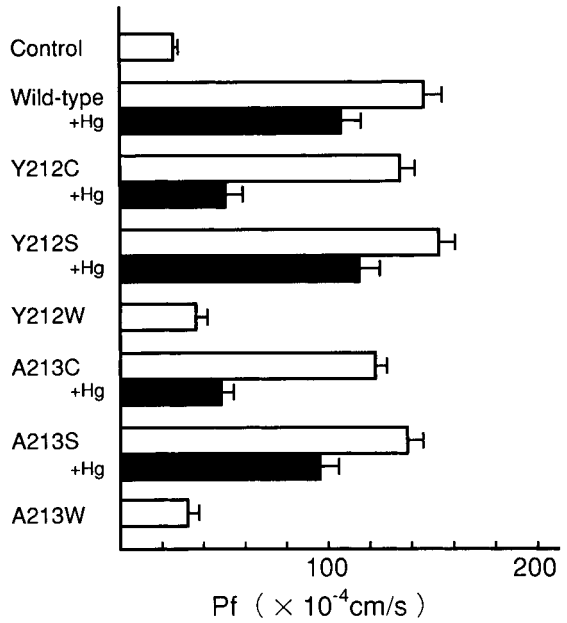

FIGURE 4: Effect of $HgCl_2$ on osmotic water permeability of oocytes expressing wild-type and mutants at residues 212 and 213. Residues of human AQP3 at Tyr-212 or at Ala-213 were replaced by cysteine, serine, or tryptophan. Oocytes were injected with water (Control) or with 5 ng of wild-type or the mutated human AQP3 cRNA. P_f was determined without incubation or after incubation with 0.3 mM $HgCl_2$ for 5 min (+Hg). Each bar represents the mean ± SE of 23−38 measurements.

Incubation with 0.3 mM $HgCl_2$ decreased P_f by 63% in Y212C, indicating that Tyr-to-Cys mutation made AQP3 more sensitive to mercury. Y212S showed a similar P_f value, but the mercury sensitivity was not altered. P_f of Y212W oocytes was $(36 \pm 6) \times 10^{-4}$ cm/s, a value comparable to P_f of water-injected oocytes, suggesting that Tyr-to-Trp mutation abolished water channel function. Ala-213, next to Tyr-212, was also mutated to cysteine, serine, or tryptophan. In A213C-expressing oocytes, P_f was unchanged, whereas the mercury sensitivity was increased (Figure 4). The P_f value and mercury sensitivity of A213S were not different from those of wild-type AQP3. Just as in the case of Y212W, A213W did not exhibit water channel function.

We examined whether the difference in oocyte P_f of Tyr-212 and Ala-213 mutants was derived from the difference in the expression of AQP3 protein in the oocyte membranes. Wild-type and mutant AQP proteins were analyzed by Western blot using an affinity-purified antibody against human AQP3 (Figure 5). The immunoblot of wild-type AQP3 detected an apparent molecular mass of a 26-kDa and a broad 33−45-kDa band. Similar bands were demonstrated in all of the Tyr-212 and Ala-213 mutants, suggesting that all wild-type and mutants expressed similar quantities of AQP3 proteins in the oocyte.

Figure 6A summarizes measurements of oocyte P_{gly}. The P_{gly} of Control oocytes was $(3.1 \pm 0.5) \times 10^{-6}$ cm/s. Injection of wild-type AQP3 cRNA increased P_{gly} 3.1-fold. P_{gly} of Y212C-expressing oocytes [$(10.3 \pm 0.9) \times 10^{-6}$ cm/s] was similar to P_{gly} of wild-type AQP3. Treatment with $HgCl_2$ inhibited P_{gly} by 33% and 61% in wild-type AQP3 and Y212C, respectively, indicating that replacement of Tyr-212 with cysteine increased mercury sensitivity to P_{gly}. P_{gly} values of Y212W and A213W were $(3.6 \pm 0.6) \times 10^{-6}$ and $(3.4 \pm 0.4) \times 10^{-6}$ cm/s, respectively, showing no difference from control P_{gly}. P_{gly} did not change after incubation with

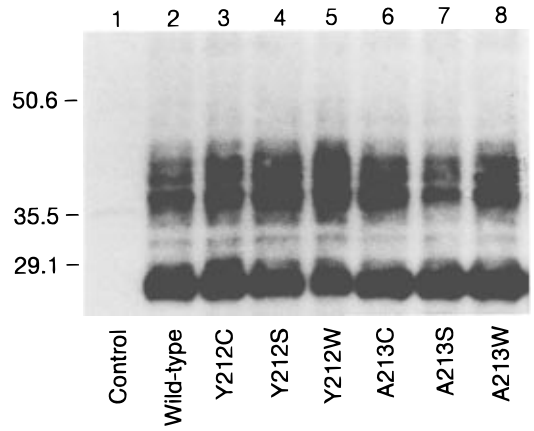

FIGURE 5: Western blot analysis of oocyte membrane proteins probed with an affinity-purified antibody against human AQP3. Oocytes were injected with water (Control, lane 1), 5 ng of cRNA of wild-type human AQP3 (lane 2), or mutated human AQP3 (lanes 3−8), where Tyr-212 or Ala-213 was altered to cysteine, serine, or tryptophan. Membranes prepared from three oocytes were loaded in each lane.

$HgCl_2$ in Control, Y212W, and A213W oocytes. To estimate E_a for P_{gly} of AQP3 from the Arrhenius plot, P_{gly} was measured at 4, 20, and 30 °C in Control and wild-type AQP3-expressing oocytes. AQP3-dependent P_{gly} was obtained by subtracting the Control P_{gly} from the P_{gly} of oocytes expressing AQP3 at each temperature (Figure 6B). AQP3-dependent P_{gly} showed an E_a of 4.5 kcal/mol.

Echevarria et al. have recently examined the effect of another mercurial reagent, *p*-chloromercuriphenylsulfonate (pCMBS), on P_f and P_{gly} of AQP3-expressing oocytes (15). As some differences in the mercurial effects were found between their resuls and ours, we repeated the experiments under the same conditions used in their study. Incubation with 1 mM pCMBS for 30 min decreased P_f of AQP3-expressing oocytes by 71% (Figure 7A). P_f was not reversed after subsequent 15-min incubation with 5 mM 2-mercaptoethanol nor 25-min incubation with 5 mM dithiothreitol. P_f decreased by 40% after incubation with 0.3 mM pCMBS and reversed by 78% after incubation with 2-mercaptoethanol. Control P_{gly} was not affected by 30-min treatment with 1 mM pCMBS (Figure 7B). The same treatment resulted in a 44% inhibition of P_{gly} in AQP3, and the recovery of P_{gly} was not observed. By contrast, the inhibition of P_{gly} by 0.3 mM pCMBS was reversed by 87%.

DISCUSSION

It is well-known that mercurial reagents cause a reversible inhibition of water channel function (*1*−*4*, *7*−*12*). In previous studies where the mercurial effect was examined in AQP-expressing oocytes, P_f was measured after the incubation with 0.3 mM $HgCl_2$ for 5 min, and its reversibility was determined after subsequent incubation with 5−10 mM of 2-mercaptoethanol for 5−30 min (*1*−*3*, *10*, *11*, *14*). In the preliminary experiments of the present study, we carefully compared the effect of 0.3 and 1 mM $HgCl_2$ on P_f and its reversibility. The effect of 0.3 mM $HgCl_2$ on P_f was recovered by 54−74%. We found that 1 mM $HgCl_2$ caused a larger inhibition than 0.3 mM $HgCl_2$ in AQP3 (Figure 3B). However, the recovery of P_f was 32%, suggesting that it included some nonspecific effect on AQP protein and/or oocytes. A possible nonspecific effect of high-dose $HgCl_2$

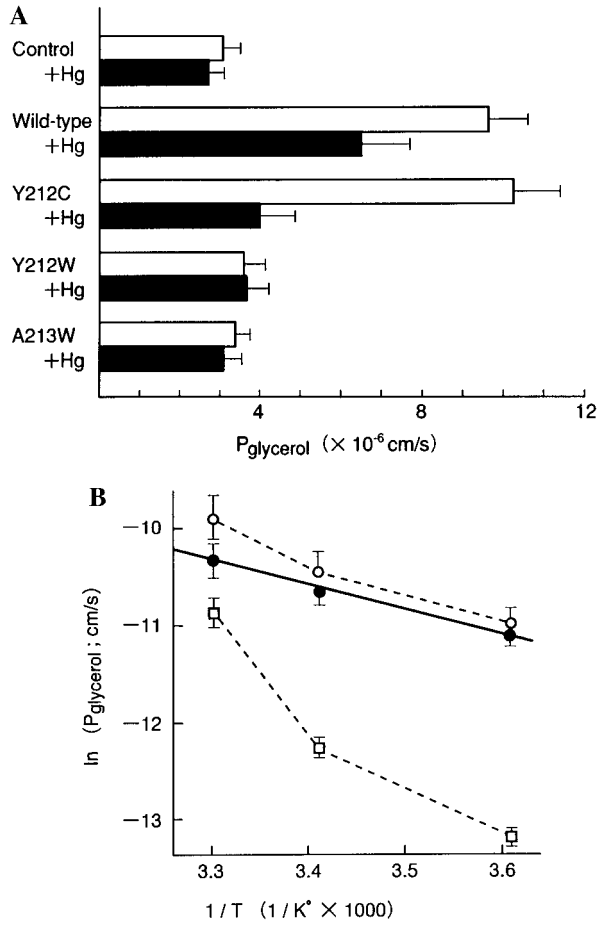

FIGURE 6: Glycerol permeability (P_{gly}) of oocytes expressing wild-type and mutant AQP3 (A) and estimation of AQP3-dependent E_a for P_{gly} (B). (A) Oocytes were incubated for 2 min in Barth's buffer containing [^{14}C]glycerol. After three rinses, the oocyte was lysed to measure intracellular ^{14}C activity by a liquid scintillation counter. Oocytes were incubated for 5 min with 0.3 mM $HgCl_2$ to test the mercurial effect on P_{gly}. Each bar represents the mean ± SE of 10−12 measurements. (B) P_{gly} was measured in control (□) and wild-type AQP3-expressing oocytes (○) at 4, 20, and 30 °C. To estimate AQP3-dependent P_{gly} (●), P_{gly} of Control oocytes was subtracted from the measured P_{gly} of AQP3 oocytes at each temperature. AQP3-dependent E_a was calculated as 4.5 kcal/mol from the fitted line. Each point represents the mean ± SE of 11−14 measurements.

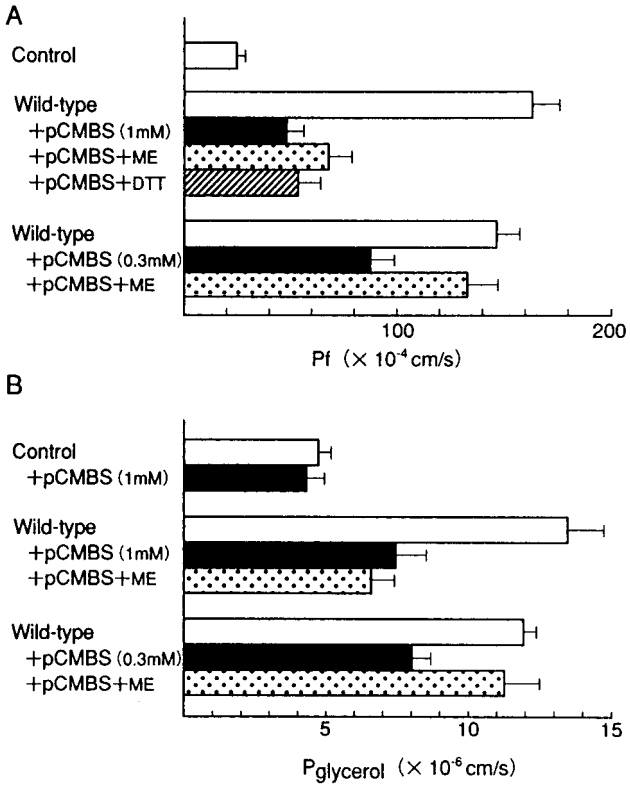

FIGURE 7: Effect of pCMBS on P_f (A) and P_{gly} (B). Oocytes were injected with 5 ng of wild-type AQP3 cRNA. The oocytes were incubated for 30 min with 1 or 0.3 mM pCMBS (+pCMBS), or further incubated either for 15 min with 5 mM 2-mercaptoethanol (+pCMBS+ME), or for 25 min with 5 mM dithiothreitol (+pCMBS+DTT). Each bar represents means ± SE of 7−15 measurements.

has been observed in AQP4- and AQP5-expressing oocytes (*5*, *7*, *16*). We used the term "mercury-sensitive" in this paper when an initial decrease of P_f by 5-min incubation with 0.3 mM of $HgCl_2$ was reversed by >50% after subsequent 15-min incubation with 5 mM 2-mercaptoethanol.

Following such a rigid criteria for mercurial sensitivity, we found here that incubation with 0.3 mM $HgCl_2$ decreased P_f by 48−53% in AQP2 and 30−33% in AQP3, indicating that AQP3 was relatively resistant to mercury compared to AQP2 (Figure 2). Taking these data and previous reported values together, the sensitivities of AQPs to 0.3 mM $HgCl_2$ may be arranged in the following order: AQP1 (62−79% inhibition) > AQP2 (47−53%) > AQP3 (30−33%) > AQP4 (0%) (*1*, *2*, *5*, *6*, *10*). We previously reported a 65% inhibition of P_f in rat AQP3 after 5-min treatment with 0.3 mM $HgCl_2$ (3), an apparently larger inhibition than the present one. The reason for this difference is not readily clear, but the viability of the oocytes may be an important factor. In the present study, we used oocytes in which mercury-induced inhibition of P_f was mostly reversed by mercaptoethanol. If the viability of the oocytes is not adequate, the magnitude of inhibition by 0.3 mM $HgCl_2$ may be large, and the recovery by mercaptoethanol may be poor. Our exclusion of such oocytes in the present study may have been the cause of the relatively small inhibition.

Our results showed that Cys-11 is a mercury-sensitive residue in AQP3 (Figure 3A). Mercury-sensitive cysteine was first demonstrated in AQP1, in which the substitution with serine or alanine at Cys-189 acquired mercury resistance (*10*, *11*). The Cys-189 is located at 3 residues prior to the second NPA sequence, and substitution with tryptophan has been shown to block the channel. Subsequent study in AQP2 showed similar results (*12*), suggesting that the highly conserved NPA motifs and mercury-sensitive cysteine play important roles in the expression of channel function in AQP1 and AQP2. In AQP3, substitution of Cys-11 with tryptophan did not abolish water channel function. Hydropathy analysis of AQP3 predicts that Cys-11 is located in the cytoplasm near the NH_2 terminal (Figure 1). Thus, mercury-sensitive cysteine might not be directly associated with the aqueous pore formation in AQP3. Alternately, the side chain of Cys-11 might not extended into the pore, although Cys-11 is located at the pore site. The significance of the mercury-sensitive Cys-11 in AQP3 remains unclear at present.

Lack of cysteine at position 212, the position equivalent to mercury-inhibitable residue in AQP1 and AQP2, may be responsible for the relative resistance of AQP3 to mercurial inhibition. This speculation was confirmed by mutation to Tyr-212; Y212C increased mercurial sensitivity, and Y212W

did not function as a water channel (Figure 4). In the mercury-insensitive AQP4 water channel, Ala-210 is a residue corresponding to the mercury-sensitive sites in AQP1 and AQP2. Cysteine mutation at this position failed to confer mercury sensitivity (*6*, *16*), but cysteine mutation at the next residue, Ser-211, made AQP4 mercury-sensitive (*16*). Mutation at Ala-213 in AQP3 gave essentially the same results as Tyr-212 mutation (Figure 4). Our findings in AQP3 mutants together with the results of mutation studies in AQP1, AQP2, and AQP4 suggest that the basic structure of the aqueous pore is similar in AQP1−4 and that residues just prior to the second NPA box contribute to the pore formation.

Expression of wild-type human AQP3 increased P_{gly} 3.1-fold (Figure 6A). P_{gly} values for Y212W- and A213W-expressing oocytes were not different from the value of the control P_{gly}. Treatment with $HgCl_2$ significantly decreased P_{gly} in AQP3-expressing oocytes but not in control oocytes. Y212C showed an enhanced inhibitory effect of mercury on P_{gly}, just as it did on P_f. Our estimated E_a for P_{gly} (4.5 kcal/mol) was within the range predicted for glycerol movement through a pore filled with water (*17*, *18*). Taken together, our findings are consistent with the hypothesis that water and glycerol share a common channel pore in AQP3. By contrast, Echevarria et al. expressed rat AQP3 in oocytes and observed that 30-min treatment with 1 mM pCMBS inhibited P_f but had no effects on P_{gly} (15). They also calculated E_a for P_{gly} to be 12.2 kcal/mol and concluded that AQP3 possesses an independent transport mechanism for small molecules in addition to the aqueous pore for water transport. We tested the effect of 1 mM pCMBS on P_f and P_{gly} under the same conditions as used in their study (Figure 7). pCMBS inhibited not only P_f, but also P_{gly} on AQP3. We failed to observe the recovery of P_f by 2-mercaptoethanol. The P_f recovery was still absent after a 25-min treatment with 5 mM dithiothreitol, the procedure which brought about the recovery of P_f after pCMBS treatment when it was performed by Echevarria et al. (*4*). By contrast, 0.3 mM pCMBS reversibly inhibited P_f and P_{gly}. Our results suggest that 1 mM pCMBS, as well as 1 mM $HgCl_2$, has some nonspecific effect on AQP protein and/or oocytes. At present, we are not able to explain the difference between their results and our own.

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