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Comparative effect of metals on antidiuretic hormone induced transport in toad bladder: specificity of mercuric inhibition of water channels

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We previously reported that $HgCl_2$ inhibits water and urea flux in tissues fixed with glutaraldehyde after antidiuretic hormone (ADH) stimulation and suggested that the ADH-induced water channel may share characteristics of the red blood cell and proximal tubule water transport pathway. To determine the specificity of mercury's action, we examined the effect of numerous other metals. In tissues fixed after ADH stimulation, water flow and urea and sucrose permeabilities are maintained from mucosal bath pH 2.5 through pH 12. Several metals including Ba, Co, Fe, Sr and Zn did not alter flux. Al, Cd, La, Li, Pb and U inhibited urea permeability but not water flow. At pH 2.8, Cu inhibited water flow by 30% and urea permeability by 50%. At pH 4.9–7.4, Cu inhibited urea permeability but not water flow. At pH ≤ 3.0, Pt inhibited flow in ADH-pretreated tissues. The inhibitory effect was not present at pH > 3.0. At pH < 3.0, Au inhibited flow by 90% in tissues fixed after pretreatment with ADH but increased the permeability of tissues fixed in the absence of ADH. Ag inhibited flow by 70% but also increased sucrose, urea, and basal permeabilities. This suggests that Ag and Au disrupt epithelial integrity. These results indicate that at physiologic pH, the ADH-induced water channel is specifically blocked by Hg but not by other metals. This specificity may reflect the presence of a large number of sulfhydryl groups in the water channel.

Keywords: vasopressin, mercurial reagents, copper, gold, platinum

Introduction

Vasopressin activates adenylate cyclase and elicits an increase in water and urea permeability in the toad urinary bladder, an amphibian analog of mammalian collecting duct (Finkelstein 1987, Hays 1991). These two transport pathways are believed to be separate. Water and urea transport are passive and preserved without modification by glutaraldehyde fixation (Chevalier *et al.* 1981, Eggena 1983, Hardy 1985, Parisi *et al.* 1985, Kondo and Imai 1987). An agent that blocks transport in the glutaraldehyde-fixed bladder is potentially a direct inhibitor of the water or urea channel.

We previously reported that mercurial reagents inhibit water flow and urea permeability in bladders which had been fixed with glutaraldehyde after pretreatment with vasopressin (Hoch *et al.* 1989). Others (Ibarra *et al.* 1989, Shi & Verkman 1989, Harris *et al.* 1990, Paredes *et al.* 1990) have extended these observations in the course of experiments aimed ultimately at isolating the water channel.

Mercurials are known to block the water channels present in erythrocyte, proximal renal tubule, and internodal cells of *Nitellopsis obtusa* (Macey & Farmer 1970, Pratz *et al.* 1986, Wayne & Tazawa 1990). It is commonly believed that Hg blocks water channels by interaction with sulfhydryl groups (Macey 1984, Finkelstein 1987). However, other sulfhydryl reagents such as 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), iodoacetamide (IAM) and *N*-ethylmaleimide (NEM) do not block water channels (Finkelstein 1987). Metals other than the mercurials have not been shown to block the water channels constantly present in erythrocyte and proximal renal tubule. Levitt & Mlekoday (1983) noted that $CuCl_2$ inhibited ethylene glycol but not water permeability

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in erythrocyte. We are unaware of any investigation of a large series of metals on the water channels in erythrocyte and proximal renal tubule.

To determine the specificity of mercury's action, we studied the effect of numerous other metals. The metals studied were selected as potential inhibitors of either water or urea transport. Ag, Au, Cd and Cu are known to bind avidly to sulfhydryl groups. Ag, Ba, Cd, Cu, Fe, Li, Pb, Pt and U all have known nephrotoxicity (Heidbreder *et al.* 1988; Kone *et al.* 1990, Lee *et al.* 1990, Bennett *et al.* 1991). Ag, Au, Cd, Cu and Zn are transition metals near Hg in the periodic table. Cd, Cu, Sr and Zn inhibit the hydrosmotic response to vasopressin in unfixed tissues (Bentley 1966, Parisi & Picini 1972, Bentley *et al.* 1975, Carvounis *et al.* 1985). Hardy (1985) demonstrated that La inhibits urea flux in glutaraldehye-fixed tissues but does not inhibit water flow.

We examined the metals' effect at a wide range of pH, with particular attention to acid pH. We and others have observed that mercurials are more potent inhibitors of water flow at acid pH (Ibarra *et al.* 1990). Sucrose permeability, which is not increased by antidiuretic hormone (ADH), was used as a measure of epithelial integrity.

Failure of metals other than Hg to block the ADH-induced water channel in toad urinary bladder would suggest important functional and perhaps structural homology with the water channels in erythrocyte and proximal renal tubule.

Materials and methods

Dominican female toads (*Bufo marinus*, National Reagents, Bridgeport, CT) were double pithed. Their bladders were tied on glass tubing so that they were fully distended at a volume of 6–8 ml and washed with phosphate-buffered Ringer (PBR; 120 mM Na, 4 mM K, 0.5 mM Ca, 116 mM Cl, 5 mM phosphate, pH 7.4 and 230 mosmol kg^{-1} H_2O) as previously reported in detail (Levine *et al.* 1981, Schlondorff *et al.* 1981). The tissues were maintained at room temperature and transferred to beakers containing 35 ml of PBR bubbled with air. Arginine vasopressin (Sigma, V5501) was added to the serosal bath of all tissues at time 0 at concentration 24 mU/ml in all experiments using ADH. Tissues were exposed to ADH for 20 min in the absence of an osmotic gradient and fixed on the mucosal surface for 10 min in 1% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) in the continued presence of ADH.

The tissues were then immersed in fresh mucosal and serosal bathing media. The serosal bath was 35 ml PBR bubbled with air. The test metal was present at the indicated concentration and pH in the mucosal bath of experimental tissues (Table 1). The mucosal bath of control tissues contained the equivalent sodium salt at identical concentration and pH. Water flows were measured gravimetrically at 15 min intervals. To investigate the effect of pH on glutaraldehyde-fixed tissues, 30 mM NaCl solutions pH 0.7 through pH 13 were studied. In these experiments, the control solution was pH 7.4.

Table 1. Concentration and pH of metals studied

$AlCl_3$	1.0 mM, pH 3.6
$AgNO_3$	0.1–1.0 mM, pH 5.4
$HAuCl_4$	0.1–1.0 mM, pH 2.7–6.0
$BaCl_2$	1.0 mM in 1/5th PR, pH 7.2
$CdCl_2$	10.0 mM, pH 2.8–5.9
$CdCl_2$	1.0 mM in 20 mM Tris–HCl, pH 8.6
$CoCl_2$	5.0 mM in 1/5th PR, pH 6.9
$CuCl_2$	10.0 mM, pH 2.8
$CuCl_2$	1.0 mM, pH 4.9–7.4
$CuCl_2$	1.0 mM in 20 mM Tris–HCl, pH 8.3
$FeCl_3$	1.0 mM in 1/5th PR, pH 2.8
$LaCl_3$	1.0 mM, pH 5.3
$LaCl_3$	1.0 mM in 20 mM Tris–HCl, pH 8.6
LiCl	5.0 mM in 1/5th PR, pH 7.2
$Pb(NO_3)_2$	1.0 mM, pH 4.9
$SrCl_2$	1.0 mM, pH 5.4
$ZnCl_2$	1.0 mM in 1/5th PR, pH 6.2
Chloroplatinic acid	1.0 mM in 1/5th PR, pH 2.9
Chloroplatinic acid	5.0 mM in 1/5th PR, pH 3.0–7.2
Uranyl acetate	1.0 mM, pH 4.7
Uranyl acetate	1.0 mM in 20 mM sodium acetate, pH 5.8

To analyze sucrose permeability or urea transport, tracer quantities of [^{14}C]sucrose or [^{14}C]urea were added to the mucosal bath 45 min after the start of the experiment. Samples were pipetted into scintillant and counted. Data were analyzed for significance with Student's *t*-test for paired data (Snedecor & Cochran 1967).

Results

Experiments with varying pH

In glutaraldehyde-fixed tissues, ADH-stimulated water flow and and urea permeability are maintained over the pH range 2.5–12 (Table 2). Sucrose permeability is also not significantly altered. At pH 2.0, water flow is immediately inhibited by 20% (32 ± 2 versus 40 ± 3 $\mu l\ min^{-1}$, $n = 24$, $P < 0.001$). Urea and sucrose permeabilities are unaffected. At pH 0.7 water flow is immediately inhibited by 75% (10 ± 2 versus 40 ± 4 $\mu l\ min^{-1}$, $n = 9$, $P < 0.001$) and urea and sucrose permeabilities increased.

At pH 13, water flow is initially unaffected but after 30 min significantly inhibited (24 ± 2 versus 38 ± 2 $\mu l\ min^{-1}$, $n = 8$, $P < 0.001$). Urea and sucrose permeabilities are increased. The stability of

Table 2. Experiments with varying pH

pH (mucosal)	Water flow (μl min^{-1})			KTrans sucrose (cm [s × 10^7]$^{-1}$)			KTrans urea (cm [s × 10^7]$^{-1}$)		
	pH 7.4	Test pH		pH 7.4	Test pH		pH 7.4	Test pH	
0.7	34 ± 3	8 ± 1***	(9)	11 ± 7	83 ± 30*	(5)	227 ± 75	505 ± 62**	(4)
1.5	32 ± 2	21 ± 2***	(8)	34 ± 7	22 ± 5	(4)	234 ± 91	221 ± 51	(4)
2.0	36 ± 2	30 ± 2**	(24)	15 ± 4	29 ± 12	(7)	256 ± 16	281 ± 21	(13)
2.5	35 ± 2	38 ± 3	(10)	17 ± 4	19 ± 6	(5)	225 ± 24	246 ± 61	(4)
3.0	39 ± 3	41 ± 3	(11)	54 ± 15	47 ± 14	(5)	223 ± 50	268 ± 57	(6)
9.0	37 ± 3	38 ± 3	(10)	39 ± 9	34 ± 11	(6)	179 ± 37	285 ± 89	(4)
10.0	40 ± 4	43 ± 4	(9)	34 ± 9	35 ± 5	(4)	293 ± 72	302 ± 67	(5)
11.0	28 ± 3	27 ± 4	(8)	14 ± 3	27 ± 5	(4)	129 ± 41	139 ± 38	(4)
12.0	32 ± 5	32 ± 4	(9)	28 ± 8	49 ± 10	(5)	315 ± 62	368 ± 52	(4)
13.0	43 ± 3	35 ± 2**	(8)	145 ± 48	383 ± 33*	(4)	412 ± 31	1046 ± 158*	(4)

Values are means ± SE. Data represent average values for 60 min of water flow and 45 min of tracer flux. The number of paired experiments is given in parentheses. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

the glutaraldehyde-fixed preparation permits the study of the effects of numerous metals over the pH range 2.5–12.

Metals with no apparent effect on water flow or urea permeability

$BaCl_2$, $CoCl_2$, $FeCl_3$, $SrCl_2$ and $ZnCl_2$ do not alter water flow or urea permeability in glutaraldehyde-fixed tissues (Figure 1). Sucrose permeability is slightly but significantly decreased in Ba treated tissues (12 ± 2 versus 17 ± 2, $P < 0.05$) but not significantly altered by Co, Fe, Sr or Zn.

Metals which inhibit urea permeability but not water flow

LiCl does not alter water flow but does significantly inhibit urea permeability (Table 3). Al, Cd, La, Pb and U all elicit a significant increase in water flow. These metals significantly inhibit urea and sucrose permeability.

Experiments with Cu

At pH 2.8, 10.0 mM $CuCl_2$ inhibits water flow by 30% and urea permeability by 50% (Figure 2). Sucrose permeability is not significantly altered. At pH 4.9 and 7.4, 1.0 mM Cu increases water flow while inhibiting urea permeability. Sucrose permeability is also significantly inhibited (pH 4.9, 4 ± 2 versus 89 ± 18 cm [s × 10^7]$^{-1}$, $n = 6$, $P < 001$; pH 7.4, 67 ± 20 versus 100 ± 18 cm [s × 10^7]$^{-1}$, $n = 6$, $P < 0.05$). At pH 8.3, 1.0 mM Cu inhibits water flow by 10% and does not alter urea or sucrose permeability. In tissues fixed without prior exposure to

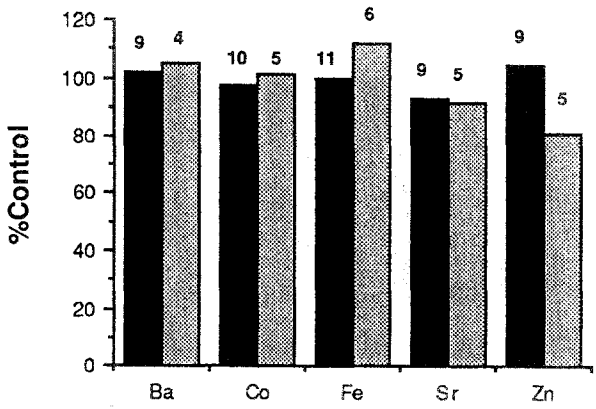

Figure 1. Metals with no effect on water or urea flux. Data represent % control values for 60 min of water flow and 45 min of urea flux. The number of paired experiments is given above the bars. Flow, ■; urea, □.

ADH, Cu does not affect water or sucrose permeability.

Experiments with Pt

At pH ≤ 3.0, 1.0–5.0 mM chloroplatinic acid inhibits water flow (Figure 3). Sucrose and urea permeabilities are not altered. No inhibitory effect is apparent at pH > 3.0. In tissues fixed with glutaraldehyde without prior exposure to ADH, Pt does not alter water, urea, or sucrose permeability.

Experiments with Au

At pH 2.7, 1.0 mM chloroauric acid markedly inhibits water flow without apparent effect on urea or sucrose permeability (Table 4). Au (1.0 mM) does not inhibit flow in ADH-treated tissues at pH ≥ 3.0.

Table 3. Metals which inhibit urea but not water flux

Metal	pH	Water flow (μl min^{-1})			Ktrans sucrose (cm [s × 10^7]$^{-1}$)			Ktrans urea (cm [s × 10^7]$^{-1}$)		
		Control	Test metal		Control	Test metal		Control	Test metal	
Al	3.6	60 ± 5	68 ± 6*	(22)	50 ± 10	8 ± 1**	(10)	427 ± 30	285 ± 46**	(12)
Cd	2.8	57 ± 5	55 ± 7	(10)	51 ± 25	23 ± 7	(4)	468 ± 36	353 ± 33*	(6)
Cd	5.9	65 ± 5	74 ± 6**	(14)	97 ± 1	4 ± 1***	(4)	566 ± 51	349 ± 54**	(10)
Cd	8.6	66 ± 5	72 ± 6*	(12)	82 ± 21	23 ± 8*	(6)	479 ± 44	220 ± 37**	(6)
La	5.3	50 ± 7	57 ± 7	(13)	31 ± 6	2 ± 1*	(4)	272 ± 26	182 ± 33**	(9)
La	8.6	68 ± 5	80 ± 3**	(10)	96 ± 28	1 ± 1*	(6)	528 ± 77	216 ± 61*	(4)
Li	7.2	48 ± 5	47 ± 4	(8)	66 ± 19	66 ± 21	(4)	333 ± 36	256 ± 41*	(4)
Pb	4.9	73 ± 5	85 ± 6**	(17)	48 ± 10	2 ± 1**	(8)	176 ± 27	62 ± 7**	(8)
U	4.7	83 ± 5	114 ± 3***	(14)	68 ± 16	9 ± 4*	(4)	491 ± 47	367 ± 49**	(10)
U	5.8	52 ± 3	63 ± 2**	(12)	47 ± 5	1 ± 0**	(6)	286 ± 62	143 ± 42**	(6)

Values are means ± SE. Data represent average values for 60 min of water flow and 45 min of tracer flux. The number of paired experiments is given in parentheses. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

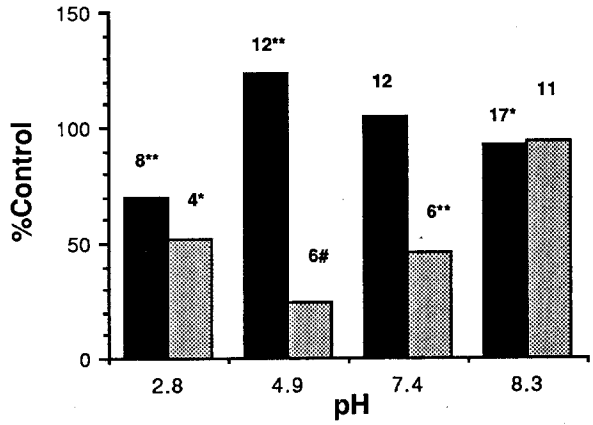

Figure 2. Experiments with Cu. Data represent % control values for 60 min of water flow and 45 min of tracer flux. The number of paired experiments is given above the bars. Flow, ■; urea, ▨. $^*P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.001$.

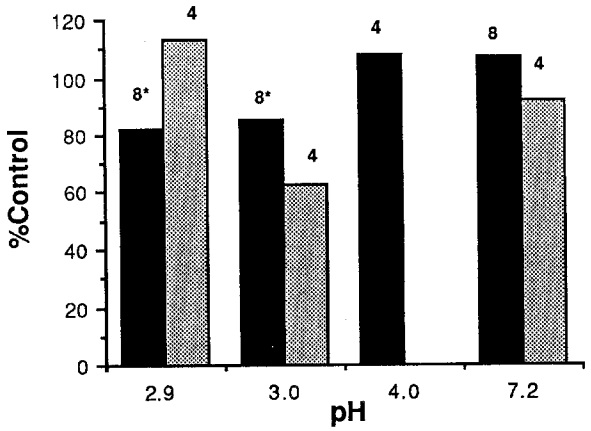

Figure 3. Experiments with Pt. Data represent % control values for 60 min of water flow and 45 min of urea flux. The number of paired experiments is given above the bars. Flow, ■; urea, ▨. $^*P < 0.05$.

Urea permeability is significantly inhibited at pH 3.5–4.0. In tissues fixed without prior exposure to ADH, water flow, urea and sucrose permeabilities are significantly increased.

Experiments with Ag

At pH 5.4, 1.0 mM $AgNO_3$ inhibits flow (Table 5). However, urea and sucrose permeabilities are markedly increased. In tissues fixed without prior exposure to ADH, Ag significantly increases water flow, urea and sucrose permeabilities.

Discussion

The glutaraldehyde-fixed preparation allows direct study of potential inhibitors of the water and urea channels. In unfixed tissues, vasopressin's effects can be altered at multiple sites proximal and distal to adenylate cyclase. In contrast, in fixed tissues, in the absence of disruption of epithelial integrity, an inhibitory effect on transport implies direct blockade of the water or urea channels.

Our results demonstrate that at physiologic pH, the vasopressin-induced water channel is specifically blocked by Hg. Cd, Sr and Zn which were previously reported to be water flow inhibitors in unfixed tissues do not directly inhibit the water channel. The results of experiments with Cu, Pt, Au and Ag are in sharp contrast to those with $HgCl_2$. $HgCl_2$ inhibits water flow in tissues fixed with glutaraldehyde after

Table 4. Experiments with Au

[Au] (mM)	pH	Water flow (μl min^{-1})			KTrans sucrose (cm [s × 10^7]$^{-1}$)			KTrans urea (cm [s × 10^7]$^{-1}$)		
		Control	Au		Control	Au		Control	Au	
Tissues fixed after pretreatment with vasopressin										
0.1	3.5	82 ± 8	81 ± 9	(6)	18 ± 4	22 ± 3	(6)			
1.0	2.7	53 ± 5	6 ± 1***	(14)	13 ± 2	21 ± 5	(6)	174 ± 23	171 ± 24	(8)
1.0	3.0	71 ± 8	65 ± 9	(8)	34 ± 6	33 ± 4	(4)	415 ± 31	283 ± 47	(4)
1.0	3.5	43 ± 10	39 ± 7	(7)	46 ± 10	33 ± 9	(3)	307 ± 28	217 ± 18*	(3)
1.0	4.0	66 ± 6	59 ± 8	(8)	42 ± 10	71 ± 20	(4)	412 ± 59	220 ± 27*	(4)
1.0	6.0	68 ± 9	66 ± 8	(8)	84 ± 5	65 ± 15	(4)	440 ± 99	370 ± 30	(4)
Tissues fixed in the absence of vasopressin										
0.1	3.5	2 ± 0	2 ± 1	(6)	3 ± 1	6 ± 2	(6)			
1.0	2.7	2 ± 0	5 ± 1***	(18)	3 ± 0	15 ± 5*	(10)	38 ± 6	161 ± 16***	(6)
1.0	4.0	3 ± 1	7 ± 1***	(12)	6 ± 2	13 ± 3*	(8)	50 ± 8	199 ± 13**	(4)
1.0	5.0	4 ± 1	6 ± 1*	(8)	8 ± 3	14 ± 4*	(4)	91 ± 34	217 ± 103	(4)
1.0	6.0	4 ± 0	6 ± 0***	(8)	6 ± 2	11 ± 1	(4)	42 ± 14	119 ± 17**	(3)

Values are means ± SE. Data represent values for 60 min of water flow and 45 min of tracer flux. The number of paired experiments is given in parentheses. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 5. Experiments with Ag (pH 5.4)

[Ag] (mM)	Water flow (μl min^{-1})			KTrans sucrose (cm [s × 10^7]$^{-1}$)			KTrans urea (cm [s × 10^7]$^{-1}$)		
	Control	Ag		Control	Ag		Control	Ag	
Tissues fixed after pretreatment with vasopressin									
1.0	87 ± 7	28 ± 2***	(8)	87 ± 23	140 ± 23*	(4)	414 ± 81	640 ± 71*	(4)
0.5	81 ± 7	80 ± 5	(8)						
0.1	101 ± 7	95 ± 7	(4)	93 ± 30	133 ± 32*	(4)			
Tissues fixed in the absence of vasopressin									
1.0	4 ± 0	16 ± 1***	(8)	12 ± 4	80 ± 12*	(4)	36 ± 6	282 ± 45**	(4)
0.5	6 ± 1	12 ± 1**	(8)	17 ± 4	50 ± 12*	(4)	106 ± 37	187 ± 29	(4)
0.1	6 ± 1	8 ± 1*	(8)	14 ± 3	26 ± 10	(4)	47 ± 7	114 ± 15*	(4)

Values are means ± SE. Data represent values for 60 min of water flow and 45 min of tracer flux. The number of paired experiments is given in parentheses. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

treatment with vasopressin over a wide range of pH including pH 7.4. Sucrose permeability is not increased. In tissues fixed with glutaraldehyde without pretreatment with vasopressin, $HgCl_2$ does not alter water or sucrose permeability (Hoch *et al.* 1989). Thus, if Cu, Pt and Au do indeed block the water channel, they do so at a much more narrow range of pH than Hg. The rise in basal water, urea and sucrose permeabilities caused by Ag and Au further suggests that these metals are disrupting epithelial integrity and that they may not be specifically interacting with the ADH-induced water channel. This issue might be resolved by measuring P_f and P_d in the presence of Ag and Au using the methods of Harris *et al.* (1990) or Verkman *et al.* (1988).

In red blood cells, the water and urea channels are thought to be separate (Macey 1984). Indeed, Gargus & Mitas (1988) and Heaton & McLoughlin (1982) have associated the urea channel with the kidd antigen which is not felt to have a role in water flux. Many investigators have demonstrated dissociation of ADH stimulated water and urea transport in toad urinary bladder and collecting duct as well (Hardy 1985, Finkelstein 1987, Knepper *et al.* 1989; Knepper & Star 1990). Phloretin inhibits urea but not water transport in toad bladder, collecting duct and red blood cells. Our data provide further strong evidence for this dissociation. Li inhibits urea

permeability but does not alter water flow. Al, Cd, La, Pb and U all inhibit urea flux while simultaneously mediating a slight increase in water flow. The mechanism of this increased flow is not clear but is associated with attenuation of the increased sucrose permeability normally observed in fixed tissues. Cu inhibits urea flux but not water permeability at pH 4.9–7.4. In contrast to the water channel, the urea channel is non-specifically blocked by numerous metals at pH > 3.0.

The observation that Au, Cu and Pt block ADH induced water flow at pH < 3.0 may result from titration of SH groups. At higher pH the S–S form predominates while as the pH decreases, these groups may be titrated to SH and more susceptible to attack. The protein identified by Harris as the putative aqueous channel contains 10% cysteine (H. W. Harris Jr, personal communication). Hill *et al.* (1991) have observed that disulfide bonds appear crucial to the function of the membrane permeabilizing defensins.

It is also possible that Hg blocks water channels by a mechanism other than binding to sulfhydryl groups. Of the metals we tested, Ag, Au, Cd and Cu are known to avidly bind sulfhydryl groups. Cd does not block the water channel and, as noted, Ag and Au may have non-specific toxicity. Even at pH 2.8, 10 mM Cu inhibits water flow by only 30%. DTNB, IAM and NEM do not block water channels (Finkelstein 1987). De Kruijff (1987) has suggested that non-bilayer lipids found in every biologic membrane may form aqueous channels under physiologic conditions. $HgCl_2$ is known to alter membrane lipids (Morrison *et al.* 1984) although this may be secondary to phospholipid hydrolysis. Means & Feeney (1971) have noted that mercurials do not interact exclusively with sulfhydryl groups.

In conclusion, the metals studied herein do not block the ADH-induced water channel at physiologic pH. Al, Cd, La, Li, Pb and U all block urea permeability but not water flow. The ADH-induced water and urea channels are therefore distinct. As in red blood cells and proximal renal tubule, only the mercurials have been demonstrated to block the ADH-induced water channel. The specificity of inhibition of the ADH-induced water channel by Hg suggests important functional and perhaps structural homology between the vasopressin-induced water channel and the water channels constantly present in erythrocyte and proximal renal tubule. ^{203}Hg might, therefore, be used as an aid in isolating the water channel. Structural analysis of the purified water channel will explain its specific blockade by the mercurials.

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