Selective Inhibition of Osmotic Water Flow by General Anesthetics in Toad Urinary Bladder

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ABSTRACT Vasopressin increases the permeability of the toad urinary bladder, an analogue of the mammalian renal collecting duct, to water and small solutes, especially the amide urea. We have observed that three general anesthetic agents of clinical importance, the gases methoxyflurane and halothane and the ultrashort-acting barbiturate methohexital, reversibly inhibit vasopressin-stimulated water flow, but do not depress permeability to urea, or to the lipophilic solute diphenylhydantoin.

In contrast to their effects in vasopressin-treated bladders, the anesthetics do not inhibit cyclic AMP-stimulated water flow, consistent with an effect on vasopressin-responsive adenylate cyclase. The selectivity of the anesthetic-induced depression of water flow suggests that separate adenylate cyclases and cyclic AMP pools may exist for control of water and urea permeabilities in the toad bladder. Furthermore, theophylline's usual stimulatory effect on water flow, but not its effect on urea permeability, was entirely abolished in methoxyflurane-treated bladders, suggesting that separate phosphodiesterases that control water and urea permeabilities are present as well.

We would conclude that the majority of water and urea transport takes place via separate pathways across the rate-limiting luminal membrane of the bladder cell, and that separate vasopressin-responsive cellular pools of cyclic AMP appear to control permeability to water and to urea.

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INTRODUCTION

There is evidence in several epithelial tissues that water and small amides such as urea cross the cell membrane via independent pathways. In the toad urinary bladder, for example, vasopressin-stimulated urea transport is blocked by agents such as phloretin, tannic acid, and the oxidizing agents permanganate and chromate, while osmotic water flow is unaltered (1-3). These inhibitory agents appear to act at a luminal membrane site, beyond the generation of cyclic AMP, since cyclic AMP-stimulated urea transport is inhibited as effectively as transport stimulated by vasopressin (3).

The present study describes the action of three anesthetic agents, the gases methoxyflurane and halothane and the short-acting barbiturate methohexital, which have an effect opposite to that of phloretin and the oxidizing agents; the inhibition of osmotic water flow with no alteration in urea transport. In contrast to the selective inhibitors of urea transport, the anesthetics do not inhibit cyclic AMP-stimulated water movement. This suggests that the independent pathway for water movement may involve not only a separate site for penetration at the luminal membrane, but a specific and separate adenylate cyclase-cyclic AMP-mediated control system as well.

METHODS

Female toads (National Reagents, Bridgeport, Conn.) were doubly pithed, and glass bungs tied into both urinary bladders *in situ*. The bladders were removed and filled with 8 ml of phosphate-buffered Ringer's solution (120 mM Na⁺, 4.0 mM K⁺, 0.5 mM Ca⁺⁺, 116 mM Cl⁻, 5 mM phosphate, pH 7.4, and 230 mosmol/kg H₂O). Bladders were washed inside and out three times with fresh Ringer's solution to remove any endogenous vasopressin, and were finally refilled with 8 ml of Ringer's solution or, where osmotic water flow was to be determined, Ringer's solution diluted

1:1 with distilled water. Bladders were then suspended in 30–35 ml of Ringer's solution (control) or Ringer's solution containing the anesthetic agent to be tested (experimental). Stirring was provided by magnetic stirrers inside and out to minimize the effect of unstirred layers on measured permeability (4). Isotope was added to the mucosal bath for measurement of mucosal to serosal permeability, and to the serosal bath for measurement of permeability in the opposite direction.

The anesthetic agents tested were the gases halothane (Fluothane, Ayerst Laboratories, New York) and methoxyflurane (Penthane, Abbott Laboratories, North Chicago, Ill.) and the injectable barbiturate methohexital (Brevital, Eli Lilly and Co., Indianapolis, Ind.). They were obtained as manufactured for clinical use. Halothane and methoxyflurane were bubbled in oxygen through the serosal bath of the test bladders using a Fluomatic (Foregger, Roslyn Heights, N. Y.) or Pentec (Cyprane Ltd., Keighley, England) vaporizer, respectively. Control bladders received oxygen only. Methoxyflurane bubbling did not alter bath osmolality.

The effect of the nonvolatile methohexital was measured by adding it to the serosal bath of the test bladders; both test and control bladders were bubbled with compressed air.

Water movement was determined by carefully blotting the bags and weighing them (5). Short circuit current was determined in plastic chambers with a central dividing partition. Isotope permeability (K_{trans})¹ (6) was measured for a single 15-min period before vasopressin, and for two consecutive 15-min periods after vasopressin. These two latter periods were considered as a single 30-min period for ease of presentation. When effects of cyclic AMP or theophylline were to be measured, all serosal baths (test and control) were replaced after the initial base-line period with fresh Ringer's solution containing cyclic AMP or theophylline. Test bladder serosal baths contained the anesthetic agent as well. Serosal bath volume for the cyclic AMP studies was 18 ml to minimize the quantity of nucleotide necessary. Isotope counting was performed in Aquasol (New England Nuclear, Boston, Mass.) or Triton-Toluene-Omnifluor in a Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). [¹⁴C]-Urea and diphenylhydantoin were obtained from New England Nuclear. Vasopressin (Pitressin) was obtained from Parke, Davis & Co., Detroit, Mich. and 3'5'-cyclic AMP (cAMP) from Sigma Chemical Co., St. Louis, Mo. In all experiments, results obtained in test hemibladders were compared with those from control hemibladders by the method of pair analysis (7).

RESULTS

The effect of methohexital, methoxyflurane, and halothane on vasopressin-stimulated water and urea permeabilities. Methohexital, methoxyflurane, and halothane selectively inhibited vasopressin-stimulated water flow, without altering base-line water flow or base-line and vasopressin-stimulated K_{trans} urea (Table I, Fig. 1). Methohexital, at concentrations of 0.1 and 0.3 mM, inhibited water flow by 24% and 48%, respectively, but

FIGURE 1 Effect of methohexital concentration on osmotic water flow (○---○) and K_{trans} urea (●—●) in paired bladders after addition of 86 mU/ml vasopressin. Data taken from Table I and shown as mean $\pm$ SEM.

had no effect on K_{trans} urea. At 1 mM methohexital, water flow was inhibited by 75%, while K_{trans} urea showed a 20% inhibition, not significant in the series of four experiments. In view of its strong and selective inhibitory action, 0.3 mM methohexital was used in all subsequent experiments.

The effect of the anesthetic gases was similar to that of methohexital. 0.4% and 0.7% methoxyflurane inhibited ADH-stimulated water flow by 27% and 50%, respectively, without altering K_{trans} urea. 1.5% halothane caused a similar selective depression.

To ensure the independence of water flow and K_{trans} urea in the anesthetic-treated bladders, the effect of methohexital was determined in the absence of an osmotic gradient, and the effect of methoxyflurane was determined on both mucosal $\rightarrow$ serosal (M $\rightarrow$ S) and S $\rightarrow$ M movement of urea (Table I). The selective effect of the anesthetics proved to be independent of the presence of osmotic flow or the direction of measured K_{trans} urea.

The effect of varying vasopressin concentration in anesthetic-treated bladders. The effect of 2.9–290 mU/ml of vasopressin in bladders treated with 0.3 mM methohexital or 0.7% methoxyflurane is shown in Figs. 2 and 3. (Different toads were used at each vasopressin level, accounting for the large variation in control permeabilities seen in Fig. 2). At the lowest vasopressin concentrations used, methohexital inhibited both water flow and urea permeability compared to control. At higher vasopressin concentrations, the inhibitory effect on urea was overcome, while the effect on water persisted. The inhibitory effect on water was significant by pair analysis even at 290 mU/ml vasopressin, although the extent of depression was diminished.

The pattern of response to 0.7% methoxyflurane was similar to the response to methohexital. At very low vasopressin concentrations, both urea and water were inhibited, while at higher concentrations only water was

¹ Abbreviations used in this paper: ADH, vasopressin or antidiuretic hormone; cAMP, 3'5'-cyclic AMP; I_{sc} , short circuit current; K_{trans} , isotope permeability; M, mucosal; S, serosal.

TABLE I
Effect of Anesthetic Agents on K_{trans} Urea and Water Flow

	K_{trans} [^{14}C] urea, $\times 10^{-7}$ cm/s						Water flow, $\mu\text{l}/\text{min}$					
	Control		Experimental		$\Delta(\text{C-E})$		Control		Experimental		$\Delta(\text{C-E})$	
	I	II*	I	II	I	II	I	II	I	II	I	II
Methohexital												
0.1 mM (M $\rightarrow$ S) (6)	156 $\pm$ 47	484 $\pm$ 55	155 $\pm$ 43	473 $\pm$ 50	1 $\pm$ 10	12 $\pm$ 24	1.7 $\pm$ 0.3	39.9 $\pm$ 3.4	1.8 $\pm$ 0.3	30.5 $\pm$ 4.2	-0.1 $\pm$ 3.0	9.5 $\pm$ 3.2§
0.3 mM (M $\rightarrow$ S) (6)	210 $\pm$ 26	476 $\pm$ 23	201 $\pm$ 33	447 $\pm$ 37	9 $\pm$ 8	29 $\pm$ 27	2.8 $\pm$ 0.5	35.2 $\pm$ 1.4	2.9 $\pm$ 0.4	18.3 $\pm$ 3.5	0.0 $\pm$ 0.4	17.0 $\pm$ 3.7
1 mM (M $\rightarrow$ S) (4)	102 $\pm$ 28	337 $\pm$ 59	98 $\pm$ 37	271 $\pm$ 55	4 $\pm$ 18	66 $\pm$ 29	2.5 $\pm$ 0.3	31.7 $\pm$ 2.7	2.3 $\pm$ 0.3	8.0 $\pm$ 1.2	0.3 $\pm$ 0.5	23.8 $\pm$ 3.8
0.3 mM (M $\rightarrow$ S) (8)†	20 $\pm$ 6	213 $\pm$ 54	24 $\pm$ 10	180 $\pm$ 40	-4 $\pm$ 6	33 $\pm$ 19	—	—	—	—	—	—
Methoxyflurane												
0.7% (M $\rightarrow$ S) (7)¶	123 $\pm$ 30	384 $\pm$ 57	135 $\pm$ 29	380 $\pm$ 43	-12 $\pm$ 13	5 $\pm$ 43	3.0 $\pm$ 0.6	34.2 $\pm$ 5.7	1.6 $\pm$ 0.2	16.3 $\pm$ 3.7	1.5 $\pm$ 0.6§	17.9 $\pm$ 2.7**
0.7% (S $\rightarrow$ M) (7)¶	68 $\pm$ 29	383 $\pm$ 49	83 $\pm$ 34	332 $\pm$ 50	-15 $\pm$ 8	51 $\pm$ 24	1.8 $\pm$ 0.3	41.7 $\pm$ 4.7	1.7 $\pm$ 0.3	21.6 $\pm$ 3.1	0.1 $\pm$ 0.4	20.1 $\pm$ 2.5**
0.7% both (14)¶	95 $\pm$ 21	383 $\pm$ 36	109 $\pm$ 23	356 $\pm$ 33	-14 $\pm$ 7	28 $\pm$ 24	2.5 $\pm$ 0.4	38.0 $\pm$ 3.7	1.7 $\pm$ 0.2	19.0 $\pm$ 2.4	0.8 $\pm$ 0.4	19.0 $\pm$ 1.8**
0.4% (M $\rightarrow$ S) (10)	78 $\pm$ 22	288 $\pm$ 52	62 $\pm$ 14	249 $\pm$ 42	16 $\pm$ 10	39 $\pm$ 20	1.9 $\pm$ 0.2	33.6 $\pm$ 3.7	1.3 $\pm$ 0.3	24.5 $\pm$ 2.6	0.6 $\pm$ 0.5	9.1 $\pm$ 3.4§
Halothane												
1.0% (M $\rightarrow$ S) (6)	128 $\pm$ 51	240 $\pm$ 65	102 $\pm$ 45	212 $\pm$ 62	26 $\pm$ 23	28 $\pm$ 24	3.8 $\pm$ 0.6	19.0 $\pm$ 1.9	3.3 $\pm$ 0.9	12.5 $\pm$ 1.3	0.5 $\pm$ 0.4	6.4 $\pm$ 1.2

* ADH, 86 mU/ml added at start of period II.

† No osmotic gradient.

§ $P < 0.05$.

|| $P < 0.01$.

¶ 30-min period I. All other experiments 15-min period I. The 14 experiments labeled "both" are a summary of the M $\rightarrow$ S and S $\rightarrow$ M experiments immediately above.

** $P < 0.001$.

inhibited. The inhibition of water flow did not diminish at very high vasopressin concentrations, however, in contrast to the methohexital-treated bladders. The pattern of response to 0.4% methoxyflurane, not shown, followed closely that to 0.7% methoxyflurane, except that the degree of inhibition of water flow was greater with 0.7% than with 0.4% methoxyflurane.

Reversibility. The reversibility of methohexital's effect on water permeability was studied after measuring

bladder permeability for a 15-min base-line period, and for 30-45 min after addition of 86 mU/ml vasopressin. The usual inhibition of water permeability was observed in the methohexital-treated bladders. S baths were then replaced with fresh Ringer's containing vasopressin only, and permeabilities measured for an additional 30 min. Methohexital's effect was entirely reversible (Fig. 4); in fact, permeability to both urea and water was significantly higher in the test bladders than in the controls after washout. An additional series of experiments

FIGURE 2 Effect of varying vasopressin concentration on osmotic water flow and K_{trans} urea in control bladders (●—●) and bladders receiving 0.3 mM methohexital (MH) (○---○). Separate bladder pairs were used at each vasopressin concentration. ($n = 4-6$, * $P < 0.05$, ** $P < 0.01$).

FIGURE 3 Effect of varying vasopressin concentration on osmotic water flow and K_{trans} urea in control bladders (●—●) and bladders receiving 0.7% methoxyflurane (MOF) (○---○). Separate bladder pairs were used at each vasopressin concentration ($n = 6-8$, * $P < 0.05$, ** $P < 0.01$).

FIGURE 4 Reversibility of methohexital's (MH) effect on osmotic water flow and K_{trans} urea in control bladders (●—●) and bladders treated with 0.3 mM methohexital (○---○). During base-line period, experimental bladders treated with methohexital; no ADH present. ADH 86 mU/ml added to both experimental and control S baths at start of ADH period. At start of washout period, control and experimental S baths replaced with fresh Ringer's solution containing ADH but no methohexital. (* $P < 0.05$, $n = 4$).

was performed in which both M and S baths were replaced at the start of the washout period to ensure equality of the osmotic gradients in control and experimental bladders. Identical results were obtained. The reversibility was rapid, and was complete within 5–10 min after removal of methohexital. Halothane's effect, not shown, was reversible as well.

Effect of methohexital in bladders pretreated with vasopressin. In the experiments described so far, anesthetic was added to test bladders before addition of vasopressin. To determine methohexital's effect in bladders pretreated with vasopressin, water flow and urea permeability were measured in paired bladders in the absence of anesthetic for 15 min before (Fig. 5, period I) and 15 min after addition of vasopressin (period II). All S baths were then replaced with fresh Ringer's solution containing vasopressin and, for the experimental bladders, 0.3 mM methohexital. Permeabilities were then measured for an additional 30 min (periods III and IV). Water flow was rapidly attenuated in the methohexital-treated bladder compared to control, while urea permeability remained unaffected by the anesthetic. Thus, methohexital selectively depresses water flow even in bladders already stimulated by vasopressin.

The effect of anesthetics on nonamide solute permeabilities. The permeability of lipophilic nonamides such as diphenylhydantoin is enhanced by vasopressin and unaltered by phloretin (8) suggesting that the fluidity of the lipid phase of the luminal membrane may be al-

TABLE II
Effect of Anesthetic Agents on K_{trans} [^{14}C]Diphenylhydantoin before and after Vasopressin

Agent	$K_{trans} \times 10^7$ cm/s		
	Control	Experimental	$\Delta(C-E)$
Methohexital, 0.3 mM (6)			
Base line	37 $\pm$ 7	52 $\pm$ 6	-15 $\pm$ 5*
Vasopressin (86 mU/ml)	63 $\pm$ 9	63 $\pm$ 7	0 $\pm$ 7
Increment			15 $\pm$ 3*
Methoxyflurane, 0.7% (4)			
Base line	36 $\pm$ 6	73 $\pm$ 8	-37 $\pm$ 6†
Vasopressin	72 $\pm$ 10	120 $\pm$ 11	-48 $\pm$ 11†
Increment			-11 $\pm$ 7

* $P < 0.01$.

† $P < 0.05$.

tered by vasopressin. This increased membrane lipid fluidity has been suggested as the direct cause of vasopressin-stimulated water flow (9). Both methohexital and methoxyflurane enhanced K_{trans} diphenylhydantoin in the absence of vasopressin (Table II). In the methoxyflurane-treated bladder, postvasopressin K_{trans} diphenylhydantoin was increased as well, and the vasopressin-induced permeability increment was well maintained. In contrast, the vasopressin-induced permeability increment in the methohexital-treated bladders was significantly attenuated, although postvasopressin permeabilities were equal in control and test bladders. Thus, the anesthetic effects on diphenylhydantoin permeability do not parallel those for either water or urea.

The effect of anesthetics on bladder electrical parameters. To demonstrate bladder integrity during the ex-

FIGURE 5 Effect of 0.3 mM methohexital (MH) in bladders pretreated with vasopressin. Control (●—●) and test (○---○) bladders identical before (period I) and after (period II) addition of 86 mU/ml vasopressin. Test bladders received methohexital at start of period III. $P < 0.05$ for water flow in periods III and IV. ($n = 4$).

FIGURE 6 Effect of 0.3 mM methohexital on I_{sc} . Experimental bladder halves (○--○) received methohexital at 0 min. Both experimental and control (●—●) bladder halves received 80 mU/ml vasopressin at 30 min. (* $P < 0.05$, $n = 6$).

perimental conditions examined, short circuit current (I_{sc}) and transmembrane resistance (calculated as open circuit potential/ I_{sc}) were measured in six bladders (Fig. 6). After a stable base line was achieved, methohexital was added to the S bath of the experimental segment to a concentration of 0.3 mM. A second portion of the same bladder served as an untreated control. After 30 min, there was a slight, but significant decrease in the base-line I_{sc} of the methohexital-treated bladder. After vasopressin, there was a sharp increase in I_{sc} in both bladders. I_{sc} in the methohexital-treated bladders increased slowly after vasopressin, but reached at its peak 84% of the peak levels in the control bladders. In addition, mean transmembrane resistance in experimental and control tissues differed by less than 7% throughout the studies. Thus, bladder integrity was well maintained after methohexital. Although I_{sc} was not measured in the methoxyflurane-treated bladders, open circuit potentials were routinely measured at the end of

each experiment. A significant decrease in potential averaging $50 \pm 12\%$ was observed with methoxyflurane.

Site of methohexital inhibition. At least four steps in the cAMP-mediated control pathway may be subject to alteration, including (a) unstimulated adenylate cyclase activity, (b) activation of adenylate cyclase by hormone, (c) the rate of destruction of cAMP by phosphodiesterase, and (d) the steps beyond cAMP leading to a change in membrane permeability. While this single sequence is certainly an oversimplification, at least a preliminary definition of the site of action of a given inhibitor may be established by examining its effect in the presence of antidiuretic hormone (ADH), exogenous cAMP and the phosphodiesterase inhibitor theophylline (10). For example, an agent which alters the response to ADH, but not to cAMP or theophylline would be considered to affect the interaction between hormone and receptor or the ability of the hormone-receptor complex to activate cyclase; one which alters the response to ADH and theophylline, but not to cAMP would act on both stimulated and unstimulated adenylate cyclase, while an agent which alters the response to ADH and to cAMP could act on either the cAMP-mediated steps leading to the membrane permeability increase or on phosphodiesterase. This formulation is undoubtedly incomplete (see Discussion); however, its usefulness has been well established.

Although methohexital selectively inhibited ADH-stimulated water permeability, it did not diminish either K_{trans} urea or water flow in the presence of 6 or 12 mM cAMP (Table III) consistent with an effect at adenylate cyclase or at the hormone-receptor-cyclase interaction. Methohexital also produced a moderate inhibition of water flow, but not K_{trans} urea, in response to 20 mM theophylline.

The sites of methoxyflurane inhibition. Methoxyflurane's action was similar to that of methohexital. Like methohexital, methoxyflurane altered neither water nor urea permeabilities in cAMP-stimulated bladders (Table IV). In contrast to the barbiturate's moderate inhibition of theophylline-stimulated water flow, however,

TABLE III
Effect of 0.3 mM Methohexital on K_{trans} Urea and Water Flow in the Presence of cAMP and Theophylline

	K_{trans} urea $\times 10^{-7}$ cm/s			Water flow, μ l/min		
	Control	Exp	Δ (C-E)	Control	Exp	Δ (C-E)
Methohexital, 0.3 mM						
cAMP, 6 mM (4)	203 $\pm$ 76	178 $\pm$ 73	25 $\pm$ 19	5.4 $\pm$ 1.2	3.3 $\pm$ 0.7	2.1 $\pm$ 0.9
cAMP, 12 mM (7)	158 $\pm$ 37	152 $\pm$ 25	6 $\pm$ 41	16.2 $\pm$ 3.9	18.2 $\pm$ 2.4	-2.0 $\pm$ 5.2
Theophylline, 20 mM (14)	127 $\pm$ 19	124 $\pm$ 18	3 $\pm$ 7	10.0 $\pm$ 1.9	6.5 $\pm$ 1.9	3.5 $\pm$ 1.3*

* $P < 0.05$.

TABLE IV
Effect of 0.7% Methoxyflurane on K_{trans} Urea and Water Flow in the Presence of cAMP and Theophylline

	K_{trans} urea $\times 10^{-7}$ cm/s			Water flow μ /min		
	Control	Exp	Δ (C-E)	Control	Exp	Δ (C-E)
Methoxyflurane, 0.7%						
cAMP, 6 mM (8)	111 $\pm$ 30	125 $\pm$ 43	-14 $\pm$ 27	7.7 $\pm$ 1.3	13.1 $\pm$ 3.0	-5.5 $\pm$ 2.5
cAMP, 12 mM (8)	165 $\pm$ 58	131 $\pm$ 43	34 $\pm$ 16	22.1 $\pm$ 3.5	22.8 $\pm$ 3.0	-0.6 $\pm$ 4.6
Theophylline, 20 mM (6)	233 $\pm$ 41	198 $\pm$ 38	35 $\pm$ 9*	15.2 $\pm$ 3.7	1.2 $\pm$ 0.2	14.0 $\pm$ 3.8†

* $P < 0.05$.

† $P < 0.001$.

methoxyflurane entirely inhibited theophylline-stimulated water flow at two theophylline concentrations (Table IV and Fig. 7). The response of K_{trans} urea to theophylline was again unaltered. Although these results initially suggested that methoxyflurane blocked both unstimulated and vasopressin-stimulated adenylate cyclase, a subsequent experiment showed that this was not necessarily so.

The following experiment suggests that methoxyflurane may block theophylline's action on a phosphodiesterase associated with water, but not urea transport. Paired bladder halves were both bubbled with 0.7% methoxyflurane. After a 15-min control period, S baths were changed to Ringer's solution with methoxyflurane and 12 mM cAMP, or to Ringer's solution with methoxyflurane and cAMP plus 3 mM theophylline. Ordinarily, in the absence of methoxyflurane, theophylline further enhances both cAMP-stimulated water flow and cAMP-stimulated K_{trans} urea. In the present experiment, however, a different pattern of response was observed (Fig. 8): theophylline enhanced the cAMP ef-

fect on K_{trans} urea, but produced no enhancement of cAMP-stimulated water flow. Thus, methoxyflurane prevented theophylline from increasing either base-line or cAMP-stimulated water flow, while theophylline's stimulatory effect on K_{trans} urea persisted unaltered by methoxyflurane.

The role of fluoride in the methoxyflurane-mediated depression of water permeability. An elevated concentration of fluoride, a product of the hepatic metabolism of methoxyflurane, has been implicated as the etiology of the vasopressin-resistant polyuria observed in humans and animals receiving methoxyflurane anesthesia (11, 12). To determine the role of fluoride in the present study, K_{trans} urea and osmotic water flow were measured in bladders treated with 1-5 mM NaF. Although inhibitions of water flow to levels as low as 20% of control were observed in the fluoride-treated bladders after vasopressin, the responses of K_{trans} urea and water to vasopressin were inhibited proportionally, and no selective effect was seen. In view of these results, it seems unlikely that the selective depressive effect of methoxyflurane on water in the toad bladder is mediated by an increased level of fluoride, which acts nonselectively on both water and urea permeabilities.

FIGURE 7 Effect of 3 mM theophylline in control bladders (●—●) and bladders treated with 0.7% methoxyflurane (MOF) (○--○). $P < 0.05$ for water flow in theophylline period. Other differences insignificant. ($n = 5$).

FIGURE 8 Water flow and K_{trans} urea in bladders receiving methoxyflurane (MOF) 0.7% and cAMP 12 mM compared with bladders receiving methoxyflurane, cAMP, and 3 mM theophylline (THEO). No significant difference in water flows is seen. Urea permeabilities differ significantly. ($P < 0.01$, $n = 6$).

DISCUSSION

The effect of vasopressin on both water and urea transport in the toad bladder is believed to occur via a sequence of events in which the hormone attaches to receptors on the basolateral cell surface, stimulates the production of cAMP, and finally, through a cAMP-dependent series of steps, leads to an increase in the permeability of the luminal membrane (13). It was initially believed that water and solutes crossed the luminal cell membrane together via aqueous pores (14). Recent studies, however, have demonstrated that vasopressin-stimulated urea transport can be selectively blocked by as much as 80% at the rate-limiting luminal membrane by several compounds including phloretin, tannic acid, and the oxidizing agents chromate, permanganate, and periodate, without altering water flow, suggesting the existence of physically distinct pathways for transport of urea and water (1-3, 15). One possible interpretation of these findings, however, has been that the compounds used might in some way deform a common pathway for urea and water so that water transport was unimpaired, while the slightly larger urea molecule could not cross the membrane. The present study demonstrates inhibition of vasopressin-stimulated water flow but not urea permeability and strengthens the separate pathway concept. It is now apparent that most of the transepithelial water flow takes place through sites inaccessible to as small a molecule as urea. We have presented data elsewhere (16) demonstrating that the water pathway inhibited by methohexital is associated with aggregation of particles within the granular cell luminal membrane. We cannot, at this time, localize the urea transport pathway.

Furthermore, the anesthetics' ability to depress vasopressin-stimulated water flow without decreasing the permeability of the lipophilic solute diphenylhydantoin suggests that vasopressin-stimulated water flow is not directly related to the postulated vasopressin-induced increase in the fluidity of luminal membrane lipid described above.

The selective inhibitory effect on water flow is shared by three anesthetic agents, including the volatile halogenated hydrocarbons methoxyflurane and halothane and the lipid-soluble, ultrashort-acting barbiturate methohexital, structurally unrelated to the anesthetic gases. The response is easily reversible and is, in the case of methohexital at least, also observed in bladders pretreated with vasopressin.

The patterns of inhibition of methohexital and methoxyflurane have been studied as a function of vasopressin concentration (Figs. 2 and 3). Both agents inhibit water and urea transport at low vasopressin concentrations. In the case of urea, the inhibition is overcome at vasopressin concentrations of 86 and 29 mU/ml, respectively. Inhibition of water flow persists beyond

these concentrations of vasopressin, becoming less pronounced in the case of methohexital, but remaining unchanged in the case of methoxyflurane. Interpretation of this inhibitory pattern cannot be made with certainty because of the many steps linking vasopressin binding to the eventual luminal membrane permeability increase. For example, intracellular cAMP levels have been shown to increase monotonically with increasing vasopressin concentration, far beyond the levels required for maximal water flow (17). It is possible that the inhibitory effect of methohexital on water flow is overcome at extremely high vasopressin concentrations because saturating levels of cAMP are eventually achieved in the methohexital-treated bladder. cAMP levels could still, however, have been considerably lower in the methohexital-treated bladder than in the control.

At what site do these anesthetics act to block vasopressin's effect on water? As noted above, we have examined the responses of bladders to cyclic nucleotides and theophylline to obtain an operational definition of the anesthetics' sites of action. The urea transport inhibitors phloretin and chromate, for example, inhibit both vasopressin- and cAMP-stimulated urea permeabilities and therefore appear to act at the luminal membrane. In contrast, neither methohexital nor methoxyflurane depressed cyclic nucleotide-stimulated water flow, suggesting that the anesthetics alter intracellular cAMP levels and adenylate cyclase activity, rather than modifying cAMPs' effect at the luminal membrane. Grey and Ullmann have recently demonstrated inhibition of both vasopressin and cAMP-stimulated water flow by the barbiturate anesthetics pentobarbital and thiopental (18). Their cAMP studies were, however, performed at very high anesthetic concentrations (2.5-6.5 mM) and recovery of permeability after removal of anesthetic was incomplete, so that the effects they observed may well have been more widespread than in our study because of the higher anesthetic concentrations used.

Although the pattern of response to vasopressin and to cAMP reported here suggests that the anesthetics inhibit vasopressin-stimulated adenylate cyclase activity, their effects on unstimulated cyclase could not be established, since the theophylline used in these experiments did not enhance water flow in the presence of methoxyflurane and cAMP (Fig. 8). We conclude, therefore, that the anesthetics depress vasopressin-stimulated water flow by inhibiting vasopressin-stimulated (and perhaps unstimulated) adenylate cyclase activity and cAMP formation, but do not alter cAMP's rate of breakdown or its effect on water flow.

Even more significant than the anesthetic-mediated depression of ADH-stimulated water flow is the selectivity of this effect. If, as we have suggested, the anesthetics act by blocking ADH-responsive adenylate cyclase with inhibition of cAMP formation, the mainte-

nance of a normal urea permeability response to ADH, cAMP, and theophylline implies the existence of a cAMP pool controlling urea permeability distinct from the cAMP pool determining water flow. The cAMP pool for urea appears to be controlled by a vasopressin-responsive but anesthetic-resistant adenylate cyclase.

It is possible that the inhibitory effects of methoxyflurane and methohexital on urea transport seen with low concentrations of vasopressin (Figs. 2 and 3) are overcome as high enough levels of cAMP are generated by high vasopressin concentrations. Nevertheless, at levels of vasopressin which cause maximum water flows, both methoxyflurane and methohexital sharply inhibit water flow with no effect on urea transport. Furthermore, the inhibition by methoxyflurane of theophylline's action on the phosphodiesterase that controls water flow with maintenance of a normal pattern of response for urea permeability implies that separate phosphodiesterases for urea and water transport may be present as well.

The hypothesis that separate pools of intracellular cAMP may subserve two different ADH-responsive functions is not without precedent in the toad bladder. Peterson and Edelman, Argy et al., and Flores et al. have shown that separate ADH-responsive cyclases and cAMP pools exist for control of sodium and water permeabilities, distinguishable by their responses to calcium and to prostaglandin E_1 (19-21). These "separate pools" in our experiments and in the others cited may coexist within the same cell or in different cells.

A second, but less likely, possibility to explain the selective effect of the anesthetics is that the classical hormone-cyclase-cAMP pathway is not utilized for control of urea permeability *in vivo*, but that a parallel control system which is ADH- and cAMP-responsive, but is not necessarily cAMP-modulated *in vivo* exists. Pietras and Wright have suggested that such a system might explain the differences between ADH-stimulated and cAMP-stimulated permeability increments for transport of large solutes in toad bladder (22).

Anesthetic alteration of cAMP-dependent systems is not unique to the toad bladder. Sprague et al. have suggested that halothane inhibits phenylephrine-induced aortic contraction by stimulating cAMP formation (23). The myocardial depressive effects of halothane and the barbiturate thiamylal on isolated dog myocardium can be reversed by exogenous dibutyryl cAMP, further suggesting that the site of action of these agents occurs before cAMP's effect (24). Triner et al. (25) have recently demonstrated halothane-induced activation of adenylate cyclase in bronchial and uterine smooth muscle. Halothane has also been shown to inhibit glucose-stimulated insulin secretion in isolated pieces of rat pancreas (26).

The relationship of the anesthetic-induced effects seen in toad bladder to clinical disorders of water metabolism

is difficult to ascertain. Halothane, despite its effect in toad bladder, does not impair mammalian renal concentrating ability at levels used clinically. Methoxyflurane is well documented as a cause of nephrogenic diabetes insipidus as well as acute renal failure in man and in certain strains of rat (11, 12, 27, 28). These conditions have been attributed to accumulation in the renal parenchyma of fluoride and oxalate, respectively, two end products of methoxyflurane metabolism. The clinical and experimental diseases, however, develop over a period of several days, in contrast to the acute effects seen in the toad bladder. Furthermore, it is doubtful whether the bladder epithelial cells possess the specialized enzyme systems required for metabolism of methoxyflurane to fluoride. Finally, as demonstrated above, fluoride blocks both the urea and water flow effects of ADH proportionally, in contrast to the selective effect of methoxyflurane on water, hence it is doubtful whether accumulation of fluoride plays any role in the phenomenon demonstrated in this paper.

We would conclude that (a) the majority of water and urea transport takes place via separate pathways across the rate-limiting luminal membrane of the bladder cell, (b) several anesthetics selectively inhibit water flow with a pattern consistent with blocking vasopressin-stimulable adenylate cyclase activity and reducing cAMP levels, and (c) there is now substantial evidence for the existence of separate vasopressin-dependent cellular pools of cAMP controlling permeability to water and to urea.

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