Effects of Trifluoperazine on Function and Structure of Toad Urinary Bladder

ROLE OF CALMODULIN IN VASOPRESSIN-STIMULATION OF WATER PERMEABILITY

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ABSTRACT Calcium ion plays a major regulatory role in many hormone-stimulated systems. To determine the site of calcium's action in the toad urinary bladder, we examined the effect of trifluoperazine, a compound that binds specifically to the calcium binding protein, calmodulin, and thereby prevents activation of enzymes by the calcium- calmodulin complex. 10 μM trifluoperazine inhibited vasopressin stimulation of water flow, but did not alter vasopressin's effects on urea permeability or short-circuit current. Trifluoperazine also blocked stimulation of water flow by cyclic AMP and methylisobutylxanthine, implying a "postcyclic AMP" site of action. Consistent with these results, trifluoperazine did not decrease epithelial cyclic AMP content or the cyclic AMP-dependent protein kinase activity ratio. Assay of bladder epithelial supernate demonstrated calmodulin-like activity of 1.5 U/μg protein. Morphologic studies of vasopressin-treated bladders revealed that trifluoperazine did not alter the volume density of cytoplasmic microtubules or significantly decrease the number of fusions between cytoplasmic, aggregate-containing, elongated vesicles and the luminal membrane. Nonetheless, the frequency of luminal membrane aggregates, structures that correlate well with luminal membrane water permeability, was decreased by >50%. Thus, trifluoperazine appears to inhibit the movement of intramembranous particle aggregates from the fused intracellular membranes to the luminal membrane, perhaps by blocking an effect of calcium on microfilament function.

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INTRODUCTION

Cyclic AMP and calcium ion have been established as major classes of intracellular regulators, which mediate and modulate the actions of neurotransmitters and several hormones, including vasopressin. The interactions of cyclic AMP and calcium may be important in both coarse and fine control of cellular responses to stimuli. In the case of cyclic AMP, activation of cyclic AMP-dependent protein kinases, which in turn leads to phosphorylation of various substrates, appears to be the only well-documented biochemical action described to date. Over the past decade, work from several laboratories has shown that many of the regulatory actions of calcium, in a wide variety of systems, are mediated by a single calcium-binding protein, calmodulin. (This protein has also been called calcium-dependent regulator, phosphodiesterase activator, modulator protein, etc.) Calmodulin is a calcium-dependent activator for a variety of enzymes, including adenylate cyclase (in some systems), cyclic nucleotide phosphodiesterase, Ca-Mg-ATPase, myosin light-chain kinase, muscle phosphorylase kinase, and Ca-dependent protein kinase of synaptosomes (1). It is apparent that the effects of cyclic nucleotides and calcium are closely intertwined, and they may be both synergistic and antagonistic at many intracellular steps (2).

A role for cyclic AMP as second messenger for vasopressin-stimulated water transport is well established in toad urinary bladder (3). We recently demonstrated that vasopressin causes a dose-dependent *in situ* activation of cyclic AMP-dependent protein kinase in toad bladder epithelial cells (4), similar to its effect in the mammalian renal collecting tubule. Furthermore, agents that selectively enhance (hydrazine) or inhibit (methohexital) vasopressin's effect on osmotic water

flow lead to a similar directional changes in the activation of cyclic AMP-dependent kinase (5). Thus, a general correlation between vasopressin's physiological effect on transport and activation of kinase is apparent, though the substrate(s) for this kinase remain unknown. Whatever the intervening steps may be, the final effect of vasopressin is an increase in the hydraulic permeability of the luminal membrane of the bladder's granular cells, a phenomenon closely associated with the occurrence of aggregates of particles within this membrane (5–7).

The role of calcium in vasopressin's action is, however, less clearly defined. Calcium ionophores have been shown to increase both basal (vasopressin-independent) water permeability and water permeability stimulated by low concentrations of vasopressin (8), whereas these ionophores inhibit the action of maximally stimulating concentrations of vasopressin (8, 9). Although the mechanism for this is unknown, calcium could lead to alterations in the function of microtubules and microfilaments, both of which are important for the occurrence of aggregates in the luminal membrane as well as in the onset and/or maintenance of vasopressin-stimulated osmotic water flow (10–12). Furthermore, vasopressin increases efflux of labeled calcium from the bladder into the tissue's mucosal bathing medium (13), though its effect on cytoplasmic calcium concentration is uncertain.

Although all these studies indicate some role for calcium in modifying vasopressin's action, they provide little direct insight into possible mechanisms and sites of action. Therefore, we examined the role of the calcium-calmodulin complex and its interaction with the cyclic AMP system as a modulator of vasopressin's action on the toad bladder epithelium. Our results show that the bladder's epithelial cells contain calmodulin-like activity. Blockage of calmodulin's action by trifluoperazine (Stelazine), a specific inhibitor, leads to a selective decrease in stimulation of osmotic water flow by vasopressin, cyclic AMP, 8-bromo-cyclic AMP, and methylisobutylxanthine, suggesting a "postcyclic AMP" site of action. Accordingly, we could demonstrate no decrease in either cyclic AMP content or *in situ* cyclic AMP-dependent kinase activity in trifluoperazine-treated tissues. Because we observed that the number of vasopressin-induced intramembranous particle aggregates but not fusion events was markedly reduced in the presence of trifluoroperazine, it seems probable that the most significant site of calcium-calmodulin interaction occurs somewhere between kinase activation and particle insertion, and may indeed be at a site where microfilament function in aggregate translocation is critical.

METHODS

Permeability studies. Female Dominican toads (National Reagents, Bridgeport, Conn.) were doubly pithed and glass tubes were tied into both urinary bladders *in situ*. The bladders were removed and filled to capacity with 6–8 ml of phosphate-buffered Ringer's solution. (120 mM Na, 4 mM K, 0.5 mM Ca, 116 mM Cl, 5 mM phosphate, pH 7.4, and 230 mosmol/kg H_2O). Bladders were washed inside and outside with Ringer's solution to remove any endogenous vasopressin, and were finally refilled with Ringer's solution diluted 1:2 with distilled water to provide an osmotic gradient for water flow. Bladders were then suspended in aerated Ringer's solution. In many of the experiments, [^{14}C]urea or [^{14}C]sucrose (New England Nuclear, Boston, Mass.) was added to the mucosal baths for measurement of permeability. Magnetic stirrers were used inside and outside to minimize the effects of unstirred layers on measured isotope permeability (14). Water movement was determined by carefully blotting the bags and weighing them (15). Short-circuit current was determined in plastic chambers with a central dividing partition.

Trifluoperazine HCl and chlorpromazine HCl (SK & F Co., Carolina, P. R.) were stored as 5-mM solutions and added to the serosal or mucosal bath of the experimental hemibladders as required. Equivalent volumes of Ringer's solution were added to the baths of the control tissues. Arginine vasopressin (Sigma Chemical Co., St. Louis, Mo.) was added to the serosal baths as required. When the effects of cyclic AMP (Sigma Chemical Co.), 8-bromo cyclic AMP (Plenum Scientific Research, Hackensack, N. J.) or the phosphodiesterase inhibitor 1-methyl, 3-isobutyl xanthine (MIX)[1] (Aldrich Chemical Co., Inc., Milwaukee, Wis.) were examined, all serosal baths (experimental and control) were replaced with fresh Ringer's solution containing the stimulatory agent with or without trifluoperazine as needed. The prostaglandin synthesis inhibitor, naproxen, which was kindly provided by Syntex Laboratories, Inc., Palo Alto, Calif., was included in serosal baths as required. Isotope counting was performed in a liquid scintillation counter.

Measurement of phosphodiesterase activity. Epithelial cells were gently scraped with a glass coverslip off bladders from Ringer's-perfused toads into 1–2 ml of Ringer's solution. After a 2-min centrifugation in a clinical centrifuge, the cells were suspended in 5 mM Tris, 5 mM NaCl, pH 7.4, and homogenized by 18–20 strokes with a tight Dounce homogenizer. After an additional 2 min centrifugation to remove unhomogenized tissue, the supernate was collected and cyclic AMP phosphodiesterase activity measured according to the method of Rangel-Aldao et al. (16) as reported (17). The reaction mixture consisted of 40 mM Tris, pH 8.0, 10 mM $MgCl_2$, 2 mM dithiothreitol, 1 mg/ml bovine serum albumin, 0.5 μM [^{3}H]cyclic AMP (New England Nuclear) (40,000–60,000 cpm/assay). The assay was performed at 30°C for 15 min. Additional unlabeled cyclic AMP was added to achieve the final concentration needed. Hydrolysis of cyclic AMP proceeded linearly over the period of time and protein concentrations used. Phosphodiesterase activity in the homogenate follows Michaelis-Menten kinetics with a single K_m of about 3 μM over a cyclic AMP concentration of 0.5 to 100 μM (17). All determinations were performed in duplicate. Assays performed in the absence of enzyme and with heat-denatured enzyme were identical and served as blanks.

Determination of prostaglandin synthesis rates. In those experiments where prostaglandin synthesis rates were determined, serosal baths were replaced at 15-min intervals with fresh solutions containing vasopressin and trifluoperazine as needed. At the end of each period, an aliquot of the serosal bath was removed, immediately frozen, and stored at −20°C

[1] *Abbreviations used in this paper*: cAMP, cyclic AMP; −cAMP, without exogenous cAMP; +cAMP, with saturating levels of cAMP; MIX, 1-methyl, 3-isobutyl xanthine.

until the day of assay. Immunoreactive prostaglandin E_2 (PGE_2) content was determined in duplicate by the immunoassay method of Dray et al. (18) using specific antibody supplied by Boehringer Mannheim Biochemicals (Indianapolis, Ind). We have used this method previously (19).

Measurement of protein kinase activity. In the toad urinary bladder, the *in situ* activity of cyclic AMP-dependent protein kinase has been estimated from the ratio of kinase activities measured in the absence of exogenous cyclic AMP (−cAMP) and in the presence of saturating levels of exogenous cyclic AMP (+cAMP) (4). Excised bladders were washed in Ringer's. Each hemibladder was divided into two approximately equal parts, for a total of four tissue segments per toad. After a 45-min incubation in aerated Ringer's solution at room temperature, the segments were transferred to individual beakers containing Ringer's solution with trifluoperazine and/or vasopressin as needed. At the end of the incubation, the tissues were placed on a plastic plate and the epithelial cells rapidly scraped with a glass cover slip into 2–4 ml of ice-cold homogenization buffer (10 mM K phosphate, pH 6.8, 2 mM Na-EDTA, 100 mM KCl). This buffer maintains the kinase activity constant throughout the remainder of the preparatory steps (4). The cells were immediately disrupted by a 5-s burst with a Polytron sonicator (Polytron Corp., Elkhart, Ind.). When cyclic AMP content was to be determined (see below), a 0.5 ml aliquot was immediately transferred into 1 ml of boiling Na-acetate buffer, pH 6.8 for 3 min, and then stored frozen until assay. The remainder of the homogenate was centrifuged at 30,000 *g* for 20 min. The supernate was removed and saved, and the pellet was washed with 0.5 ml of homogenization buffer and recentrifuged, with the second supernate added to the first and used for measurement of kinase activity on the day of preparation.

In experiments where calmodulin-like activity was determined, aliquots of this supernatant solution were heat-inactivated for 3 min at 95°C. After centrifugation at 2,000 rpm for 10 min, the supernate was stored at −70°C until assay. Protein kinase activity was determined by the method we reported previously. The kinase reaction mixture contained 20 mM K phosphate, pH 6.8, 10 mM Mg acetate, 0.05 mM gamma-32P ATP ($1–2 \times 10^6$ cpm), (New England Nuclear), 80 μg histone F2b (Worthington Biochemical Corp., Freehold, N. J.) and, for the +cAMP assay, a saturating concentration of 2 μM cyclic AMP in a final vol of 0.1 ml. The reaction mixture was preincubated at 30°C for 2 min. Samples incubated without enzyme and with heat-inactivated enzyme served as blanks and accounted for <20% of the lowest experimental counts. The kinase activities were linear for incubation times up to 3 min and for the protein concentrations used (4).

Measurement of cell cyclic AMP content. The heat-inactivated aliquots of the original homogenate prepared as outlined above were centrifuged at 900 *g* for 30 min. The supernate was removed and cyclic AMP levels measured directly in duplicate by radioimmunoassay (Collaborative Research Inc., Waltham, Mass.) as reported from this laboratory (4, 5, 20). The pellet was resuspended in 0.1 N NaOH for determination of protein (21).

Measurement of Calmodulin-like activity. Calmodulin-like activity in bladder epithelial supernates was determined by the activation of calmodulin-deficient phosphodiesterase prepared from bovine brain as described by Cheung et al. (22). Aliquots of heat-inactivated 30,000-*g* supernate (0.5–2.0 μg protein-assay) from epithelial cell homogenates (see above) were tested for activation of calmodulin-deficient phosphodiesterase. A standard curve was prepared using purified calmodulin, which was prepared from bovine brain by the method of Watterson et al. (23). The assay mixture for these experiments contained 100 μM [^{3}H]cyclic AMP (200,000 cpm/assay), 2 mM dithiothreitol, 10 mM $MgCl_2$, 50 μM $CaCl_2$, 40 mM Tris, pH 8.0, and 2.5 μg of calmodulin-deficient brain phosphodiesterase. Various amounts of purified calmodulin were added for determination of a standard curve. Maximal activation occurred with 0.1–0.2 μg of added calmodulin. Duplicate determinations were made in a final vol of 0.1 ml at 30°C for 5 min. No more than 10% hydrolysis of cyclic AMP occurred during the assay. 1 U of calmodulin was defined as the amount of calmodulin that resulted in 50% (of maximal) stimulation of calmodulin-deficient phosphodiesterase activity under these conditions. Activity is expressed as units of calmodulin per microgram of protein in the heat-inactivated supernate.

Morphologic studies. At the end of one set of water flow measurements, (100 mU/ml vasopressin ±10 μM trifluoperazine), paired bladders were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer and processed for both freeze-fracture and thin-section electron microscopy as described. (10). Tissue thin sections were used to quantitate microtubule volume density in granular cells. For this, 8–10 micrographs were obtained from one minimally obstructed section of each bladder according to a predetermined pattern. These were printed at a final magnification of ×55,000 and analyzed by the stereologic point-counting method of Weibel (24). A 2.54-mm lattice was used to estimate microtubule volume and a 25.4 mm lattice was used to estimate cytoplasmic volume. Freeze-fracture replicas were used to quantitate both the frequency of vasopressin-induced aggregates of intramembranous particles in granular cell luminal membrane, and the number of fusion events associated with their delivery from the cytoplasm. The procedure followed is described in detail (6, 11). In all cases, morphologic assessment, including final quantitative measurements on printed micrographs, was accomplished without knowledge of tissue status (experimental vs. control). In all experiments, statistical analysis was by Student's *t* test for paired data (25).

RESULTS

Trifluoperazine's effect on osmotic water flow. Trifluoperazine's effect on vasopressin-stimulated osmotic water flow depended on the trifluoperazine concentration, the vasopressin concentration, and the site of trifluoperazine addition. 100 μM serosal trifluoperazine almost totally blocked the bladder's response to 100 mU/ml vasopressin, a maximally-stimulating concentration (Table I). Inhibition of water flow by trifluoperazine was significant, though far less than total, at serosal bath trifluoperazine concentrations of 30 and 10 μM. At lower concentrations of trifluoperazine (2 μM) we could detect no inhibition of water flow stimulated by either 100 mU/ml (supramaximal) or 1.5 mU/ml (submaximal) vasopressin, though the response to 0.5 mU/ml vasopressin was significantly attenuated. In contrast to the results with serosal trifluoperazine, mucosal trifluoperazine did not inhibit water flow significantly at vasopressin concentrations of either 10 or 100 mU/ml.

Similar results were obtained with chlorpromazine, another phenothiazine derivative. 20 μM chlorpromazine inhibited the water flow response to 100 mU/ml vasopressin by a mean of 34% ($n = 4$; $P < 0.05$), whereas 100 μM chlorpromazine inhibited the response by 89% in two tissues tested.

TABLE I

Effect of Trifluoperazine on Stimulation of Osmotic Water Flow by Vasopressin

Trifluoperazine	Vasopressin	Vasopressin increment in water flow: Vasopressin	Trifluoperazine + vasopressin	Decrease
μM	*mU/ml*	*µl/min*		%
Trifluoperazine added to serosal bath				
100 (12)	100	26.3±2.5	1.9±1.9*	93
30 (15)	100	33.4±1.2	9.4±0.9*	72
30 (6)	5	34.5±2.9	5.9±1.6*	83
10 (12)	100	41.9±3.3	22.5±3.3*	46
10 (6)	5	29.1±3.0	16.3±2.6*	44
2 (3)	100	30.1±3.3	31.1±3.3	—
2 (6)	1.5	14.1±3.6	15.5±3.7	—
2 (5)	0.5	9.7±2.5	4.5±1.0‡	54
Trifluoperazine added to mucosal bath				
10 (5)	100	48.7±3.3	38.6±5.7	21
10 (4)	10	30.1±3.0	30.3±3.1	—

Data shown as mean±SE. Numbers in parentheses are number of experiments.
* $P < 0.01$.
‡ $P < 0.05$.

The inhibitory effects of 10 μM trifluoperazine were not the result of a diminution in the bulk osmotic gradient. Measurement of mucosal bath sodium concentration from four pairs of bladders studied during vasopressin stimulation of water flow revealed that mucosal bath sodium concentration increased gradually in both sets of tissues to the extent expected from the osmotic removal of water from the mucosal baths. By 30 min after addition of vasopressin, mucosal bath sodium concentration was 1.3 mM higher in the control tissues than in the trifluoperazine-treated tissues ($P < 0.05$), as anticipated from the larger water flow in the former group. Thus the bulk osmotic gradient was if anything higher in the trifluoperazine-treated tissues, and thus is not the cause of the decreased water flow.

Trifluoperazine's inhibitory effect on water flow was partially reversible after removal of the drug from the serosal bath. Two types of studies were used to evaluate reversibility: In the first set, paired bladders were both incubated with 10 μM trifluoperazine for a 60-min period in the absence of vasopressin, and then for an additional 30 min with 100 mU/ml vasopressin added. The serosal baths were then replaced with either trifluoperazine-free Ringer's solution (control) or Ringer's solution containing 10 μM trifluoperazine. Water flow rates in the trifluoperazine-free control tissues exceeded those in the tissues that were maintained in trifluoperazine (control 25.3±3.3 μl/min, trifluoperazine 18.0±2.3; $P < 0.02$, $n = 4$). The second set of studies was designed to determine whether reversibility was complete. First, bladders were incubated in the absence of an osmotic gradient to eliminate any complicating effects of water flow on permeability. Ringer's solution was used for the serosal bath of control tissues and Ringer's solution with 10 μM trifluoperazine for the experimental tissues. Undiluted Ringer's solution was used for both mucosal baths. After a 30 min incubation in the absence of vasopressin, 100 mU/ml vasopressin was added to both control and experimental serosal baths, and incubation continued for an additional 30 min. Both tissues were then washed, and solutions replaced with identical fresh diluted (mucosal) or undiluted (serosal) Ringer's solution. After 30-min incubation in the absence of vasopressin, the hormone was then added to both serosal baths, and water flow measurements performed for an additional 30 min. Water flows in the control tissues were significantly greater than in the trifluoperazine tissues (control 38.0±2.7 μl/min, experimental 28.5±1.6; $P < 0.05$, $n = 4$). Thus, reversibility after removal of trifluoperazine was partial, but not total.

Trifluoperazine was effective whether added before, or at the same time as vasopressin (Fig. 1). The inhibitory effect of trifluoperazine did not become apparent for ~10 min after addition of vasopressin, both when the bladders were preincubated with trifluoperazine for 40 min before addition of vasopressin and when the bladders were exposed to vasopressin and trifluoperazine simultaneously (the differences shown in Fig. 1B are statistically significant, but very small). A total of 15 paired tissues have been studied using the preincubation protocol of Fig. 1A, with water flows measured every 5 min after addition of vasopressin with confirmation of these results (Table II). Trifluoperazine inhibited water flow when added after vasopressin. 100

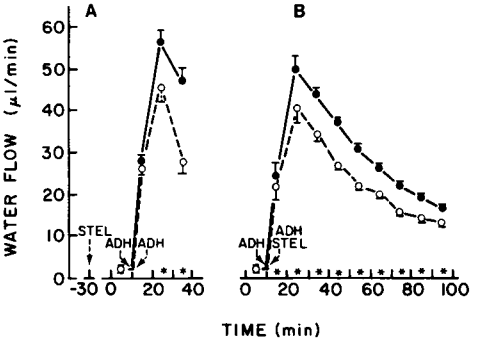

FIGURE 1 Effect of 10 μM trifluoperazine (STEL) on water flow when added to serosal bath either 40 min before 100 mU/ml vasopressin (ADH) (a; six toads) or at same time as vasopressin (*B*; nine toads). In both sets of experiments, vasopressin added at 10 min. *$P < 0.05$ for 10 min water flow measurement.

TABLE II

Response of Bladders (n = 15) Preincubated with 10 μM Trifluoperazine for 40 Min to Addition of 100 mU/ml Vasopressin

	Water flow	
	Control	Trifluoperazine
min	*μl/min*	
Basal 10	2.3±0.8	1.7±0.4 NS
Postvasopressin		
0–5	10.4±1.1	9.2±0.7 NS
5–10	44.8±1.0	43.0±1.6 NS
10–15	52.6±2.2	45.2±1.8*
15–20	52.4±2.2	40.6±2.2*
20–30	45.6±2.1	27.5±1.4*

* $P < 0.01$.

μM trifluoperazine rapidly decreased water flow to levels below those of the control tissues when added 30 min after 100 mU/ml vasopressin (control 34.7±1.6 μl/min, trifluoperazine 4.0±1.6; $P < 0.02$, $n = 6$). With 10 μM trifluoperazine, the inhibition of water flow was smaller than that seen with trifluoperazine pretreatment. We observed an inhibition of water flow stimulated by 0.5 mU/ml vasopressin when 10 μM trifluoperazine was added after hormone (control 21.0±3.9 μl/min, trifluoperazine 15.8±3.9; $P < 0.05$, $n = 8$), but not when higher concentrations of vasopressin were used.

To examine whether trifluoperazine might influence water transport through alterations in prostaglandin synthesis, we determined PGE_2 production by radioimmunoassay during paired transport experiments. PGE_2 synthesis before addition of vasopressin was 0.83±0.07 pmol/min per hemibladder in control tissues, and 0.62±0.19 in tissues that had been pretreated with 10 μM serosal trifluoperazine for 30 min ($n = 4$). After addition of 100 mU/ml vasopressin, synthesis rates were 0.51±0.08 and 0.47±0.14, respectively. Thus, trifluoperazine did not alter PGE_2 synthesis. Water flow studies were also consistent with this observation: Inhibition of vasopressin-stimulated water flow by trifluoperazine persisted even when prostaglandin synthesis was blocked by incubation of the tissues for 60 min with 10 μM naproxen. (Control 32.5 ±1.7 μl/min, trifluoperazine 23.0±2.8; six paired bladders, $P < 0.02$.)

To determine whether trifluoperazine interfered with cyclic AMP generation, we examined its effect on stimulation of water flow by exogenous cyclic AMP, 8-bromo-cyclic AMP, and the phosphodiesterase inhibitor MIX. In all of these cases, stimulated levels of water flow were reduced by trifluoperazine (Table III). Similar to the pattern seen with vasopressin-stimulated water flow, 2 μM trifluoperazine did not inhibit the response to 0.3 mM 8-bromo-cyclic AMP, but did inhibit water flow stimulated by 0.03 mM 8-bromo-cyclic AMP.

TABLE III

Effect of Serosal Trifluroperazine on Stimulation of Osmotic Water Flow by cAMP, 8-Bromo cAMP and MIX

		Stimulated increment in water flow		
Trifluoperazine	Stimulant	Stimulant	Trifluoperazine + stimulant	Decrease
μM	*mM*	*μl/min*		*%*
100 (6)	8-bromo cAMP, 1	15.6±5.0	0.1±0.4*	100
10 (3)	8-bromo cAMP, 0.3	32.3±2.2	21.4±3.3‡	34
10 (6)	MIX, 4	11.9±2.8	2.1±1.4*	82
2 (6)	8-bromo cAMP, 0.3	26.1±2.8	25.2±1.5	3
2 (10)	8-bromo cAMP, 0.03	10.8±2.0	7.7±1.3‡	29
2 (8)	cAMP, 10	15.8±2.3	7.8±2.3*	51

* $P < 0.01$.
‡ $P < 0.02$.

Trifluoperazine's effect on nonelectrolyte solute permeability. 100 μM trifluoperazine increased both basal and (100 mU/ml) vasopressin-stimulated urea permeability compared to control bladders (Table IV). This concentration of trifluoperazine also led to an extremely high sucrose permeability of 123±27 × 10^{-7} cm/s. 10 μM trifluoperazine caused no increase in either urea permeability (Table IV) or sucrose permeability (control 7.6±3.8, trifluoperazine 10.8±5.7 × 10^{-7} cm/s; five tissues). This indicates that the inhibi-

TABLE IV

Effect of Serosal Trifluoperazine on Isotopic Urea Permeability

		Urea permeability	
Trifluoperazine	Vasopressin	Vasopressin	Trifluoperazine + vasopressin
μM	*mU/ml*	*×10⁻⁷ cm/s*	
100 (6)	0	13±3	377±143*
100 (6)	100	155±36	1368±239*
30 (3)	100	235±39	211±43
10 (6)	100	299±50	271±80
2 (5)	0.5	206±56	200±42

* $P < 0.01$.

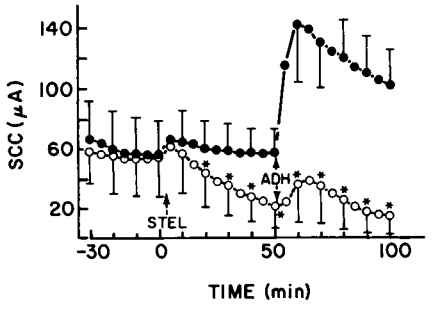

FIGURE 2 Effect of 100 μM trifluoperazine on short-circuit current (SCC). Trifluoperazine (STEL) added to tissues (○) at 0 min. 100 mU/ml vasopressin (ADH) was added to both experimental and control (●) tissues at 50 min. four paired tissues. *$P < 0.05$.

tory effect of 10 μM trifluoperazine was selective for water flow.

Effect of trifluoperazine on short-circuit current. Both short-circuit current (an estimate of active sodium transport) and open-circuit potential decreased rapidly after addition of 100 μM trifluoperazine to the serosal bath (Fig. 2). The response to vasopressin was also markedly diminished. The open-circuit potential difference fell to 11±5% of the zero-time value in the trifluoperazine-treated tissues, whereas it showed the expected increase above zero-time values in the controls (165±28%; $P < 0.02$). In contrast, 10 μM trifluoperazine did not alter short circuit current either in the unstimulated bladder or after addition of vasopressin to concentrations of either 10 mU/ml (Fig. 3) or 100 mU/ml (not shown). The potential difference was also unaffected by this concentration of trifluoperazine: Potentials at 100 min were 160±18% and 145±29% of zero time experimental and control values, respectively, for the tissues shown in Fig. 3.

Effect of trifluoperazine on phosphodiesterase activity for cyclic AMP. Addition of 100 μM trifluoperazine to the assay mixture decreased phosphodiesterase activity in crude bladder epithelial homogenates from

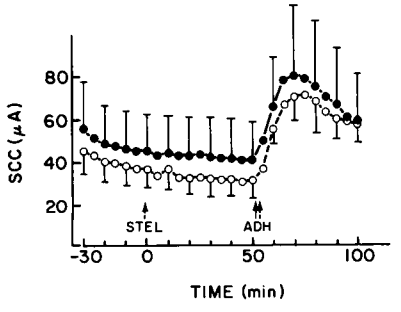

FIGURE 3 Effect of 10 μM trifluoperazine on short-circuit current (SCC). Trifluoperazine (STEL) added to tissues (○) at 0 min. 10 mU/ml Vasopressin (ADH) was added to both experimental and control (●) tissues at 50 min. four paired tissues.

11.5±0.3 to 8.5±0.4 pmol/min per mg protein (three experiments, $P < 0.02$).

Protein kinase activity and cyclic AMP content in tissues incubated with trifluoperazine. To localize further the steps involved in trifluoperazine's inhibitory action, we determined both intracellular cyclic AMP content and the degree of activation of epithelial cell cyclic AMP (cAMP)-dependent protein kinase in response to vasopressin treatment of the intact tissues (Table V). In control bladders, 100 mU/ml vasopressin increased cAMP content from a basal value from 21.0±3.5 to 27.2±3.1 pmol/mg protein ($n = 12; P < 0.05$), and increased the protein kinase activity ratio −cAMP/+cAMP from 0.22±0.02 to 0.27 ±0.03 ($n = 12; P < 0.05$). (It should be noted that these experiments were carried out in the absence of phosphodiesterase inhibitor [e.g., MIX], so that the values for both AMP content and kinase activity are much lower than those we reported previously in tissues assayed with added MIX [4].) The increase in kinase ratio was due to *in situ* activation of cAMP kinase, whereas cAMP-independent kinase activity, as determined from assays performed in the presence of specific protein kinase inhibitor (26), was unchanged by vasopressin.

Treatment of bladders with either 10 or 100 μM trifluoperazine did not significantly alter cAMP content in response to vasopressin, (though the values are slightly higher) and even increased the degree of *in situ* activation of cAMP-dependent kinase, both with and without vasopressin. These findings suggest that trifluoperazine does not interfere with cAMP generation, and are consistent with a decrease in cAMP breakdown secondary to the inhibition of cAMP phosphodiesterase by trifluoperazine described above.

Measurement of calmodulin-like activity in heat-inactivated bladder epithelial cell supernate. To measure directly the amount of calmodulin-like activity in the bladder epithelial cells, we employed the activation assay of calmodulin-deficient phosphodiesterase described by Cheung et al. (22). Under standard conditions, calmodulin-deficient phosphodiesterase was activated in a dose-dependent manner by purified calmodulin (Fig. 4). Addition of the heat-inactivated 30,000 *g* supernate of the epithelial cell homogenates also resulted in a concentration-dependent activation of the phosphodiesterase (Fig. 4), with a pattern of activation similar to that of purified calmodulin. The calmodulin-like activity in the supernates was 1.5±0.4 U/μg protein ($n = 8$).

Activation of either purified calmodulin or heat-inactivated supernate was fully inhibited by 10 μM trifluoperazine or 2 mM EGTA. Pretreatment of aliquots of heat-inactivated supernate with trypsin (2 h with 10 μg/ml trypsin in 25 mM Tris, pH 8.0, 0.1 mM $CaCl_2$ at 30°C followed by inactivation of trypsin at

TABLE V

Effect of Incubation of Bladder Segments with Trifluoperazine and Vasopressin on Protein Kinase Activity and cAMP Content in Scraped Epithelial Cells

	10 μM Trifluoperazine experiments (n = 6)				100 μM Trifluoperazine experiments (n = 6)			
	No vasopressin		100 mU/ml Vasopressin		No vasopressin		100 mU/ml Vasopressin	
	Control	Trifluoperazine	Control	Trifluoperazine	Control	Trifluoperazine	Control	Trifluoperazine
	pmol/mg protein/2 min							
Kinase activity								
−cAMP, +Inhibitor	185±30	187±24	198±25	200±45	152±53	225±25	193±27	252±39
−cAMP	407±56*	481±64	447±60	492±64	398±28‡	587±73	494±53‡	871±189
+cAMP	1705±161	1893±203	1632±170	1600±148	2039±138‡	2694±451	1972±234	2517±552
−cAMP/+cAMP	0.23±0.02	0.24±0.02	0.28±0.03‡	0.31±0.03	0.21±0.04	0.24±0.05	0.26±0.04‡	0.37±0.07
	pmol/mg protein							
cAMP content	22.0±6.9	21.8±4.5	27.8±6.4	32.7±8.8	20.0±2.2	21.6±2.5	26.6±1.2	32.0±5.2

* $P < 0.01$ between segments with and without trifluoperazine receiving same concentration of vasopressin (i.e., 0 or 100 mU/ml).
‡ $P < 0.05$.

95°C for 3 min) resulted in the loss of calmodulin-like activity (Fig. 4). This is the same as the effect of trypsin on calmodulin (22), and further supports the identity of calmodulin and the phosphodiesterase-stimulating component of the epithelial supernates.

Trifluoperazine and luminal membrane particle aggregates. Vasopressin's effect on bladder permeability to water is associated with the occurrence within the luminal membrane of particle aggregates. These are derived preformed from the membranes of long tubular structures, which fuse with the luminal membrane as a consequence of vasopressin treatment (11). Whereas the fusion between cytoplasmic aggregate-containing membranes and the luminal membrane involves the function of microtubules, the process by which aggregates gain final appearance at functionally significant portions of the luminal membrane involves a translocation that is dependent on microfilaments (10, 11). Consistent with its inhibitory action on vasopressin-stimu-

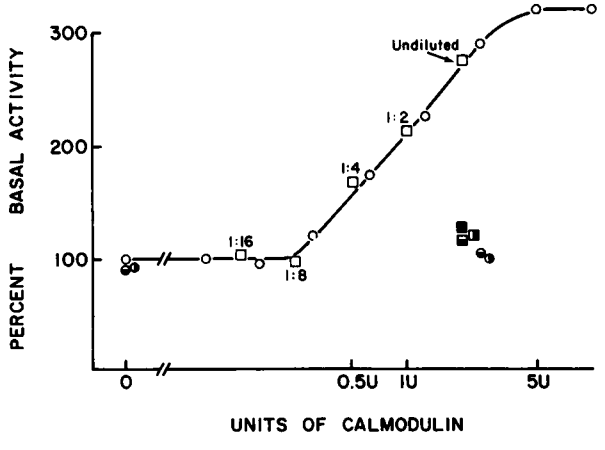

FIGURE 4 Activation of calmodulin-deficient phosphodiesterase by serial dilutions of purified calmodulin (○) or heat-inactivated toad bladder epithelial supernates (□). Half-filled symbols show phosphodiesterase activity measured in presence of 10 μM trifluoperazine (◑—calmodulin, ◨—undiluted supernate) or 2 mM EGTA (◒—calmodulin, ◪—undiluted supernate). Effect of trypsin-pretreatment on activation by undiluted bladder supernate is shown as ■.

TABLE VI

Comparison of Effects of Trifluoperazine and Low-Dose Vasopressin on Luminal Membrane Freeze-Fracture Morphology

	100 mU/ml Vasopressin		Vasopressin	
	No trifluoperazine	10 μM Trifluoperazine	20 mU/ml	0.06–1 mU/ml
	No./235 μm²			
Aggregate frequency	244±16	89±20*	229±16	84±13*
Fusion events	29±3	21±3	31±2	20±2‡

Two groups of paired bladders are shown. The left-hand group (n = 5) is a comparison of tissues receiving 100 mU/ml vasopressin with tissues receiving 100 mU/ml vasopressin and 10 μM trifluoperazine. Data on the right (n = 7) are selected from a large set of tissues studied previously (37), in which the effects of low and high concentrations of vasopressin on water flow were compared. Trifluoperazine was not used in these experiments. The seven pairs shown were chosen to match the aggregate frequencies obtained in the 100 mU/ml vasopressin group for optimum comparison of fusion events.
* $P < 0.01$ for paired tissues; ‡ $P < 0.02$.

lated water flow, trifluoperazine caused a marked decrease in the frequency of granular cell intramembranous particle aggregates (Table VI). On the other hand, trifluoperazine did not decrease the frequency of fusion events to a statistically-significant degree ($0.2 < P < 0.3$).

Table IV also shows the results of stimulation of bladders with concentrations of vasopressin that lead to submaximal stimulation of water flow, compared to paired tissues receiving maximally stimulating concentrations of vasopressin. Trifluoperazine was not used in these experiments. The data demonstrate that the alterations in aggregate frequency and fusion events in the tissues receiving low-dose vasopressin were quite comparable to the effects of trifluoperazine on tissues receiving maximally stimulatory concentrations of vasopressin. Trifluoperazine did not alter the cytoplasmic volume density of microtubules (control 0.40 ±0.04%, trifluoperazine 0.41±0.03%; $n = 5$).

DISCUSSION

Our present results suggest that vasopressin's hydroosmotic effect is mediated by two second-messenger systems: cAMP and calcium-calmodulin. These two systems may interdigitate at several points, with the final effect of their interaction being the occurrence of particle aggregates within the granular cell luminal membrane. This hypothesis is supported by the following experimental evidence.

First, the phenothiazines trifluoperazine and chlorpromazine inhibit the hydroosmotic effect of vasopressin in a dose-dependent manner. (Mamelak et al. have examined the effects of chlorpromazine in more detail [27].) Phenothiazines, and particularly trifluoperazine, have been shown to bind specifically to calmodulin (28) and thereby prevent activation of several enzymes by the calcium-calmodulin complex. In our studies, 10 μM trifluoperazine specifically inhibits the vasopressin-induced increase in water permeability. At this concentration it does not alter the effect of vasopressin on either urea permeability or sodium transport (as estimated from sodium gradient measurements as well as from open-circuit potential and short-circuit current). Moreover, both the low sucrose permeability and high transepithelial electrical resistance suggest that the paracellular barriers to solute transport remain intact. In accord with our previous work, this demonstrates the independence of the water, sodium, and urea pathways (5, 7, 17).

The inhibitory effect of trifluoperazine was partially, but not completely reversible when the bladders' bathing media were replaced by trifluoperazine-free solutions. We cannot distinguish at this time between persistent binding of the drug within the tissue and an inhibition that persists despite complete removal of the drug.

Of particular interest are the observations that 10 μM trifluoperazine did not alter water flow when added subsequent to vasopressin in concentrations greater than 0.5 mU/ml, and that in bladders pretreated with 10 μM trifluoperazine, inhibition of water flow does not occur until 10 min after addition of vasopressin. These observations suggest to us that there is a critical interaction of the vasopressin-responsive and calcium-responsive systems, which occurs only at the time of vasopressin addition and takes ~10 min to effect inhibition of water flow. Once this interaction occurs, it is only partially reversible by removal of trifluoperazine, despite washout and readdition of vasopressin. Our results with 10 μM trifluoperazine stand in contrast to those obtained at 100 μM trifluoperazine. At the higher concentration, both sucrose and urea permeabilities are increased to supranormal levels, consistent with opening intercellular junctions, and both short-circuit current and transepithelial potential difference fall. The transport data are similar to those recently reported by Ausiello and Hall with 100 μM trifluoperazine (29), and may well indicate damage to the bladder at this concentration of the drug.

In several tissues, though not in all (1), Ca-calmodulin-dependent adenylate cyclases have been described. Although we have not assayed directly for an inhibitory effect of trifluoperazine on adenylate cyclase in the toad bladder, such an effect would be relatively unimportant in this setting. The inhibitory effect of trifluoperazine is not overcome by exogenous cAMP or its analog 8-bromo-cAMP, or by increasing endogenous cAMP levels using the phosphodiesterase inhibitor MIX. This pattern suggests that trifluoperazine's effect on water flow is exerted primarily at a site distal to cAMP in the intracellular cascade. Further support for this hypothesis was obtained from direct measurements of cAMP content and of the extent of *in situ* activation of cAMP-dependent protein kinase as estimated from the ratios of its activities (−cAMP/+cAMP). The post-cAMP site of action is the same as that shown by Hardy (30) to be the site of inhibition of hypertonicity-induced water flow by calcium-free serosal bathing medium.

Trifluoperazine's pattern of action stands apart from those of other inhibitory and stimulatory agents which we have examined in the past: methohexital, for example, inhibits vasopressin stimulation of both water permeability and adenylate cyclase and decreases the protein kinase activity ratio. Hydrazine, on the other hand, enhances vasopressin-stimulation of water flow, stimulates cyclase, and increases the protein kinase activity ratio. Neither of these agents alters the water flow response to exogenous cAMP (5, 17). In contrast, trifluoperazine inhibited vasopressin-stimulated water flow but did not alter the intracellular cAMP increment associated with vasopressin administration. If anything, the vasopressin-stimulated cAMP levels tended

to be even higher in the tissues receiving trifluoperazine. Furthermore, trifluoperazine significantly increased the protein kinase activity ratio. This effect was totally abolished in the presence of specific cAMP-dependent kinase inhibitor (26), and therefore is not the result of an increase in cAMP-independent kinase. This increased *in situ* activation of cAMP-dependent kinase is consistent with our demonstration that trifluoperazine inhibits (presumably calmodulin-dependent) phosphodiesterase. One should note however that even at 100 μM trifluoperazine, inhibition of phosphodiesterase activity was only about 25% (see above), so that the effects of 10 μM trifluoperazine on cAMP breakdown *in situ* are almost certainly small, compared with trifluoperazine's effects which are exerted at sites beyond kinase. Furthermore, inhibition of phosphodiesterase by trifluoperazine would tend to stimulate, not inhibit water flow.

Prostaglandins are known to inhibit vasopressin's action through mechanisms that are not yet fully defined. Direct determination of PGE_2 synthesis by intact bladders showed no effect of 10 μM trifluoperazine under either basal or vasopressin-stimulated conditions. Furthermore, vasopressin failed to increase PGE_2 synthesis, consistent with out previous report (19). Trifluoperazine's inhibitory effect persisted in the presence of the prostaglandin synthesis inhibitor naproxen, offering further evidence against a prostaglandin-mediated effect of trifluoperazine.

Employing the activation assay of calmodulin-deficient phosphodiesterase described by Cheung (22), we were able to demonstrate directly that toad bladder epithelial cells contain calmodulin-like activity. Support for the identity of calmodulin and the phosphodiesterase activator in our heat-treated epithelial supernates derives from the observations that (*a*) the activator has the same concentration dependence as calmodulin, (*b*) both could be blocked by either trifluoperazine or EGTA, and (*c*) both could be destroyed by prior incubation with trypsin (22).

It is likely that vasopressin, in addition to its effect on cAMP generation, also brings about a graded increase in cytosolic calcium concentration. This could be caused by either an increased influx of extracellular calcium or by release of intracellularly bound calcium into the cytosol. The mechanism of such an effect of vasopressin could well be via an alteration in membrane phospholipid. It is well-documented that in rat hepatocytes, vasopressin both increases phosphatidylinositol turnover and promotes glycogenolysis by a calcium-dependent, but AMP-independent mechanism that involves protein phosphorylation (31–33). Alternatively, it is possible that a certain basal level of calcium-calmodulin interaction is required within the cell for vasopressin to exert its full effect on water flow, however the importance of calcium as a regulator in other systems makes this a less likely possibility.

Turning now to the morphologic aspects of this study, both the pattern of transport inhibition and direct measurement of cAMP content and kinase activity suggest that the predominant effect of trifluoperazine lies somewhere beyond activation of kinase by cAMP, and eventually leads to a diminution in the number of luminal membrane particle aggregates that are closely and specifically associated with luminal membrane water permeability (5–7). We do not know precisely where between kinase and aggregates trifluoperazine (and presumably calcium-calmodulin) exerts major regulatory effects. However, several possibilities deserve evaluation.

Disruption of microtubules by drugs such as colchicine inhibits vasopressin's action at a site distal to cAMP (34). In particular, colchicine blocks initiation of fusion of cytoplasmic, aggregate-containing structures with the luminal membrane, and hence decreases vasopressin stimulation of both luminal membrane aggregate frequency and osmotic water permeability (10, 11). However, electron microscopic comparison of control and trifluoperazine-treated tissues in the present study indicates that 10 μM trifluoperazine has no effect on microtubule integrity in the toad bladder granular cell. An effect of trifluoperazine at the microfilament level seems more likely. Experiments with cytochalasin B, an agent which disrupts microfilaments, imply an important role for microfilaments in the final luminal membrane occurrence of aggregates, in maintenance of their organized structure, and in the hydroosmotic response to vasopressin (10). Microfilaments are contractile elements, and their function may well be calcium-calmodulin dependent. Pearl and Taylor (35) have demonstrated actinlike proteins and actin filaments in microfilament regions of toad bladder epithelial cells using the heavy meromyosin arrowhead labeling technique (35), and it is possible that calcium-calmodulin plays a regulatory role at this level.

As one potential mechanism for regulation, calcium-calmodulin activation of myosin light-chain kinase is known to be necessary for actin-induced activation of myosin ATPase activity (1). Actomyosin's ability to generate contractile force has been implicated as critical to mediation of cell secretion in a number of nonmuscle systems (12). Thus, it becomes attractive to speculate that vasopressin's action in toad bladder also requires Ca-calmodulin interaction with myosin light-chain kinase. This, in turn, would lead to activation of actomyosin and eventually to an effect on the translocation of particle aggregates from the membrane of the long, fused vesicle (which extends deep within the cell and adds little to permeability because of rapid dissipation of the transmembrane osmotic gradient at the

mouth of the vesicle due to unstirred layer effects) to the flat portion of the luminal cell membrane.

The decreased number of aggregates seen in trifluoperazine-treated tissues without significant reduction in fusion sites is consistent with a site of trifluoperazine action at the microfilament level. However, in view of the observation that fusion frequency with trifluoperazine is similar to that seen with submaximally stimulating concentrations of vasopressin, it is clear that localization of the site of action of calcium-calmodulin to microfilaments alone must be considered cautiously. One might postulate, for example, that trifluoperazine could, in addition, block a step between kinase and fusion in the vasopressin response, and therefore cause the same pattern of structural response as is seen with a submaximal concentration of vasopressin. Certainly we would not rule out the possibility that calcium-calmodulin might play an important role in mediating the fusion process, a phenomenon that has recently been demonstrated in lipid bilayers by Cohen and co-workers (36).

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