

Inhibition of Vasopressin-stimulated Water Flow in Toad Bladder by Phorbol Myristate Acetate, Dioctanoylglycerol, and RHC-80267

Evidence for Modulation of Action of Vasopressin by Protein Kinase C

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Abstract

The action of vasopressin (AVP) in transporting epithelia is mediated by cyclic AMP (cAMP), whereas its effects in hepatocytes are mediated by calcium and phosphoinositides. Based on our recent observation that AVP stimulates phosphoinositide turnover in toad bladder, we examined the role of calcium-phospholipid-dependent kinase (protein kinase C) as a modulator of AVP's hydroosmotic effect. Phorbol myristate acetate (PMA), which can substitute for diglyceride as an activator of protein kinase C, the diglyceride dioctanoylglycerol, and RHC-80267, a glyceride lipase inhibitor that should increase diglyceride levels, inhibited AVP-stimulated water flow, but not water flow stimulated by cAMP, suggesting inhibition of cyclic AMP production. Both the dioctanoylglycerol and RHC-80267, but not PMA, also decreased water flow in response to 8-bromo cAMP indicating a potential inhibition at post-cAMP events as well. PMA increased prostaglandin synthesis; however, inhibition of water flow persisted even when prostaglandin synthesis was completely blocked by incubation with naproxen. Furthermore, water flow was not inhibited by incubation with the inactive diglyceride substitute phorbol didecanoate, supporting the specificity of the PMA inhibition. Consistent with the site of action at adenylate cyclase suggested by the transport experiments, PMA and RHC-80237 decreased both cell cAMP content and the cyclic AMP-dependent kinase ratio ($-cAMP/+cAMP$), an index of intracellular cyclic AMP effect. Assay for protein kinase C activity in toad bladder epithelial cell supernatant demonstrated that the toad bladder indeed contains a kinase stimutable by phospholipid, calcium, and PMA.

As an apparently independent effect, we found that addition of PMA, but not dioctanoylglycerol or RHC-80267, to the mucosal bath increased both water permeability and the frequency of granular cell luminal membrane aggregates in the absence of vasopressin, consistent with stimulation of fusion events at the luminal membrane.

Our data suggest that protein kinase C can modulate AVP-stimulated water flow in toad bladder by inhibiting cAMP generation, and perhaps post-cAMP steps as well, and support the hypothesis that AVP-stimulated turnover of membrane phos-

phoinositides antagonize the effects of AVP via changes in diglyceride, calcium, and protein kinase C.

Introduction

Numerous studies have suggested that calcium modulates the vasopressin (AVP)¹-stimulated, cyclic AMP (cAMP)-mediated increase in hydraulic permeability both in amphibian skin and urinary bladder and in mammalian renal collecting tubule (see Reference 1 for review). This concept is based largely upon examination of the effects of calcium ionophores, altered bath calcium, and calcium channel blockers. Little is known about the mechanism whereby calcium might influence the intracellular events brought about by exposure to AVP. Some experimental data suggest that calcium inhibits early steps in action of AVP (i.e., cAMP generation [2, 3]), whereas other data support a stimulatory role for calcium and the calcium-binding protein calmodulin at steps linking cAMP to the eventual hydroosmotic response (4).

In contrast to its action in the above tissues, AVP-mediated vasoconstriction and hepatic glycogenolysis are not mediated by cAMP, but instead are calcium-dependent and involve interaction of AVP with membrane phospholipids, particularly the phosphatidyl inositides (5, 6). Hydrolysis of polyphosphoinositides to 1,4,5-inositol trisphosphate and diglyceride, for example, can be demonstrated in hepatocytes within 5–30 s after exposure to AVP. The inositol trisphosphate so formed can release calcium from intracellular stores (6), whereas the diglyceride activates phospholipid-calcium-dependent protein kinase (called protein kinase C [5]). The latter in turn has been shown to phosphorylate several proteins, among them phosphorylase (5). Thus, in hepatocytes, AVP leads to both increased cytoplasmic calcium and activation of protein kinase C via cAMP-independent mechanisms (Fig. 1). This pathway has now been recognized as mediating the action of a number of stimuli, among them α -adrenergic agents and carbachol (7).

Elucidation of the role of protein kinase C in hormone action has been considerably facilitated by the demonstration that certain phorbol esters (e.g., phorbol myristate acetate [PMA]) quite specifically mimic the effect of diglycerides both on protein kinase C activation (8) and on the physiologic response to hormone in intact cells (9). Furthermore, cell-permeable diglycerides such as dioctanoylglycerol (DiC8) can also mimic the action of phorbol esters or of diglycerides generated in the cell (10). We have recently shown that AVP can influence phosphatidylinositol

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1. *Abbreviations used in this paper:* AVP, vasopressin; DAG, diacylglycerol; DiC8, dioctanoylglycerol; DMSO, dimethyl sulfoxide; MIX, methylisobutylxanthine; PDD, phorbol didecanoate; PGE₂, prostaglandin E₂; PMA, phorbol myristate acetate; PS, phosphatidylserine.

turnover in toad bladder epithelial cells (11), thereby suggesting a role for the phospholipid-calcium-protein kinase C system in the physiologic response to AVP here as well.

To examine this hypothesis, we report here the effects of phorbol esters and dioctanoylglycerol, which mimic the effects of endogenous diglycerides, and the putative glyceride-lipase inhibitor RHC-80267 (12), which would increase diglyceride concentration, on AVP-induced water permeability in toad urinary bladder. Our results show that phorbol esters, DiC8, and glyceride lipase inhibitor decrease the hydroosmotic effect of AVP with a pattern suggesting predominantly inhibition of cyclic AMP generation. Furthermore, we demonstrate that toad bladder epithelial cells in fact contain protein kinase C.

Methods

Transport studies. Transport studies were performed in bladder sacs from female Dominican toads by use of techniques that we have previously reported in detail (4, 13, 14). Measurement of basal water flow began 15 min after addition of experimental agent to the serosal bath. After a 30-min basal flow period, AVP (Sigma Chemical Co., St Louis, MO), cAMP (Sigma Chemical Co.), 8-bromo cAMP (Plenum Scientific Research, Hackensack NJ), the phosphodiesterase inhibitor methylisobutylxanthine (MIX) (Aldrich Chemical Co., Milwaukee WI), or the adenylyl cyclase stimulator forskolin (Calbiochem-Behring, Corp., LaJolla, CA) were added to the serosal bath, and the incubations continued. In the AVP studies, a low dose (0.125–1 mU/ml) was added first, and flow was measured for 30 min. An additional 20 mU/ml was then added to the same tissues, and flow was measured for 30 min more. In the cyclic nucleotide and MIX studies, only a single post-stimulation period of 30 min was used.

4 β -phorbol 12 β -myristate 13 α -acetate (PMA) and the inactive analogue 4 α -phorbol 12 β ,13 α -didecanoate (PDD) (both from Sigma Chemical Co.) were stored as 0.2 mg/ml and 1.0 mg/ml solutions, respectively, in dimethylsulfoxide (DMSO), and appropriate aliquots added to the bathing media as required. RHC-80267, a kind gift from Dr. C. A. Sutherland (Revlon Health Care Group, Tuckahoe, NY), was prepared as a 50 mM stock solution in 0.2–1.0% DMSO and added similarly. L- α -1,2-dioctanoylglycerol (DiC8) was obtained from Avanti Polar Lipids, Inc. (Birmingham, AL) and was made up as a 20 mM stock solution in DMSO.

In all cases, control and experimental tissues were exposed to the same concentration of DMSO.

When the cyclooxygenase inhibitor naproxen (kindly provided by Syntex Corp., Palo Alto, CA) was used to inhibit prostaglandin production, the drug was added to the serosal bath at a final concentration of

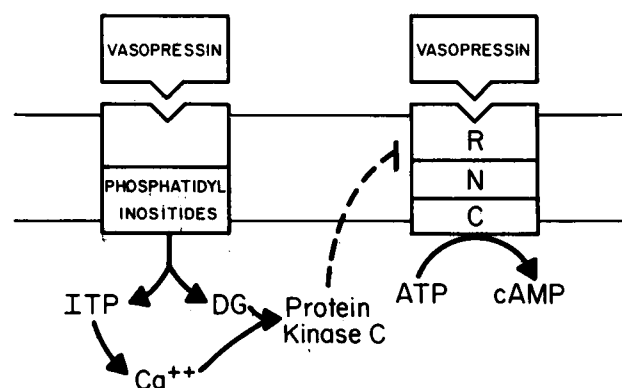


Figure 1. Hypothesis for the interactions of cyclic AMP and protein kinase C in the mechanism of action of AVP. Dash line indicates inhibitory effects.

10^{-5} M at least 45 min before the basal transport period, with the serosal bath replaced several times during that period to remove any residual prostaglandin. Naproxen was maintained in the serosal bath throughout the subsequent experiment.

In several of the experiments, [14 C]urea (New England Nuclear, Boston, MA) was added to the mucosal bath at the start of the basal transport period, with serosal and mucosal samples obtained at 15-min intervals for determination of isotope permeability.

Protein kinase C determinations. For each experiment, epithelial cells were scraped from toad bladders made blood-free by cardiac perfusion. The cells were washed three times with Ringer's solution, and homogenized in 10 ml of ice-cold buffer (7.5 mM Tris, 2 mM EDTA, 0.5 mM EGTA, 2 mM phenylmethylsulfonylfluoride (Sigma Chemical Co.), 2 mM dithiothreitol (Sigma Chemical Co.), and 300 mM sucrose [15]) using 30 strokes of a Dounce homogenizer. The homogenate was centrifuged for 1 min at 400 g to remove unhomogenized cells, and the supernate was centrifuged for 1 h at 100,000 g. The resulting supernate served as the crude enzyme preparation.

In some experiments, the 100,000-g pellet was extracted with the nonionic detergent Nonidet P-40 (NP40) 1% in homogenization buffer and the 100,000-g centrifugation repeated. Kinase C activities in the original 100,000-g supernatant and in the 100,000-g supernatant of the NP40 pellet extract were fractionated using a linear NaCl gradient on a DE52 cellulose column (Whatman Chemical Separation, Inc., Clifton, NJ) which had previously been equilibrated with sucrose-free homogenization buffer (15).

Protein kinase activity was determined as described in Kikkawa et al. (15). The reaction mixture consisted of 20 mM Tris, pH 7.5, 10 mM Mg acetate, 0.75 mM CaCl₂, 50 μ M [32 P]ATP ($\sim 10^6$ cpm/tube) (Amersham Corp., Arlington Heights, IL) and 50 μ g/tube histone type VII (Worthington Biochemical Corp., Freehold, NJ).

The lipid mixture of phosphatidylserine (PS), 10 μ g/assay, and glycerol diolein (DAG), 0.20 μ g/assay (both from Sigma Chemical Co.) was made up fresh each day and added to the reaction mix as described by Kikkawa et al. (14). Reactions were begun by the addition of 50 μ l of the appropriate enzyme preparation and carried out at 30°C in a final volume of 200 μ l. Blanks received 50 μ l of homogenization buffer instead of enzyme. The reactions were terminated by the addition of 2 ml of ice-cold 5% trichloroacetic acid (TCA) followed by 0.1 ml of 0.1% bovine serum albumin (Sigma Chemical Co.). After a 20-min centrifugation at 800 g, the supernate was discarded and the pellet was resuspended in 0.1 ml of 0.1 N NaOH and immediately reprecipitated with 2 ml of cold 5% TCA. The precipitate was trapped on a Whatman GF/B filter paper and the filter washed three times with 10 ml of cold 5% TCA as described (16). The radioactivity remaining on the filter was then determined by liquid scintillation. All assays were carried out in duplicate or triplicate.

Protein kinase activity of the 100,000 g supernate increased linearly with time up to 10 min at protein concentrations of 5–50 μ g/assay both with and without PS/DAG. At higher protein concentrations, enzyme activity tended to level off. All incubations were therefore carried out for 3–5 min using 5–50 μ g of protein/assay tube. Kinase activity in the homogenate and in the 100,000-g pellet could not be determined due to high phosphatase activity in the particulate fraction, as previously observed (15).

cAMP-dependent kinase determinations. At the end of one group of transport determinations, the bladder epithelial cells were scraped into 2–4 ml of ice-cold homogenization buffer (10 mM K phosphate, pH 6.8, 2 mM NaEDTA, 100 mM KCl) and sonicated as described by us previously (16). A 0.5-ml aliquot was immediately transferred into boiling Na-acetate buffer, pH 6.8, and then stored frozen for cAMP assay. The remainder of the homogenate was then centrifuged at 30,000 g for 20 min. The supernate was removed and saved, and the pellet washed with 0.5 ml of homogenization buffer and re-centrifuged, with the second supernate added to the first and used for measurement of kinase activity on the day of preparation. The kinase reaction mixture contained 20 mM K phosphate, pH 6.8, 10 mM Mg acetate, 0.05 mM [γ - 32 P]ATP ($1-2 \times 10^6$ cpm) (New England Nuclear), 80 μ g of histone F2b (Worthington Biochemical Corp.) and, for the +cAMP assay, a saturating

concentration of 2 μM cAMP in a final volume 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 (16).

Measurement of cell cAMP 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 cAMP levels were measured directly in duplicate by radioimmunoassay (Collaborative Research Inc., Waltham MA) as we have previously reported (13, 16). The pellet was resuspended in 0.1 N NaOH for determination of protein.

Determination of prostaglandin synthesis. In those experiments where prostaglandin synthesis was determined, an aliquot of the serosal bath was removed at intervals, immediately frozen, and stored at -20°C until the day of assay. Immunoreactive prostaglandin E₂ (PGE₂) content was determined in duplicate by immunoassay using antibody supplied by the Pasteur Institute, Paris, France, as previously reported (4).

Assay for diglyceride lipase activity. This assay was performed to establish whether or not RHC-80267 in fact diminished diglyceride lipase activity in toad bladder epithelial cells. Epithelial cells were scraped from blood-free perfused toad bladders and washed twice with Ringer's solution. The cells were homogenized with a 5-s burst (position 6) of a Polytron homogenizer (Brinkmann Instruments, Westbury, NY), in 1 ml of ice cold 50 mM Hepes (pH 7.0), 100 mM NaCl, 5 mM CaCl₂, and 10 mM mercaptoethanol. The homogenate was centrifuged for 5 min at 2,000 rpm in a clinical centrifuge to remove unhomogenized tissues. The supernatant was then centrifuged for 60 min at 100,000 g. The 100,000-g supernatant was removed, and the remaining pellet was resuspended in 0.5 ml of the homogenization buffer by a burst with the sonicator. The assay for diglyceride lipase was performed by the method of Majerus and Prescott (17) with minor modifications. In brief, 50 μl of the 100,000-g supernatant or pellet preparation was incubated with an equal volume of homogenization buffer containing 20,000 cpm of 1,2-Di[1-¹⁴C]palmitoylglycerol (115 mCi/mmol), 0.005% Triton X-100, and 15 mM reduced glutathione after a 5-s sonication to resuspend the diglyceride.

Incubations were carried out for 30 min in a shaking water bath at 37°C in the presence of various concentrations of RHC-80267 and stopped by addition of 1.5 ml of chloroform/methanol/heptane (1.25:1.4:1.0 vol/vol/vol) followed by 0.5 ml of water. After separation of phases, the organic phase was dried under nitrogen. The extracted lipids were then separated, together with authentic standards, on silica HL plates (E. Merck & Co., Rahway, NJ) in hexane/diethylether/formic acid (90:60:4). The standards were visualized under iodine, after which the plates were cut into 0.5-cm strips and the radioactivity was determined by liquid scintillation. Because the concentration of labeled diglyceride in the supernatant and pellet fractions is not known, diglyceride lipase activities were expressed as percent conversion of labeled diglyceride to monoglyceride and palmitic acid.

Determination of the effects of RHC 80267 on diglyceride lipase activity in prelabeled epithelial cells. In these experiments we evaluated whether RHC-80267 also inhibited diglyceride lipase in intact toad bladder epithelial cells. Epithelial cells were isolated by incubating the everted hemibladders for 60 min at room temperature in calcium-magnesium-free anuran Ringer's solution containing 2 mg/ml of collagenase type IV (202 U/mg, Worthington Biochemical Corp.), 100 mg% glucose, 5,000 U/ml penicillin, and 5,000 $\mu\text{g}/\text{ml}$ streptomycin (11). The bladders were then transferred to fresh standard Ringer's containing added glucose and antibiotics and incubated for another 30 min. Cells were harvested by gentle scraping, and sheets of cells were broken up by sucking them up and down a Pasteur pipette several times. Isolated cells were then pre-labeled overnight with [³H]palmitic acid (15Ci/mmol; New England Nuclear) (2×10^{-6} cpm/ml) in 10 ml of Ringer's solution containing glucose and antibiotics (as above) under sterile conditions at 25°C with slight agitation (60 cycles/min) under an atmosphere of humidified room air. Cells were then left to settle by gravity, the incubation buffer was removed, and the cells were washed three more times with Ringer's.

Aliquots of prelabeled cells were then incubated for 15 min at 25°C in Ringer's solution containing 20 mU/ml of AVP or 20 mU/ml of AVP and 100 μM RHC-80267. Incubations were terminated by a 30-s centrifugation at 1,000 rpm at 4°C, removal of the supernatant buffer, and addition of 5 ml of cold methanol. After homogenization with a Polytron (30 s at position 6) lipids were extracted twice by the addition of 5 ml of chloroform and 3 ml of water. The chloroform phase was collected and dried, and the extracted neutral lipids were separated by thin-layer chromatography and the radioactivity was determined as described above. Results are expressed as counts per minute/milligram of epithelial cell protein.

Statistics. Statistical analyses were by Student's *t* test for paired data.

Results

Transport studies: effects of phorbol esters in the serosal bath. Addition of PMA to the serosal bathing medium did not alter basal water flow (Table I). Water flow stimulated by low-dose AVP was inhibited by 20 ng/ml PMA, but not by 200 ng/ml PMA; however, both concentrations inhibited water flow during subsequent stimulation by 20 mU/ml AVP. A similar inhibition of flow by PMA was also seen in six tissues where the maximally stimulating 20 mU/ml AVP was added immediately after the basal period instead of after an intervening period of submaximal stimulation. Water flows stimulated by cAMP, 8-bromo cAMP, or MIX were not altered by PMA under these conditions. Water flow in response to the adenylate cyclase stimulator forskolin (18) was slightly inhibited by PMA.

PDD did not alter water flows, even at concentrations of 100 and 1,000 ng/ml. This was true for basal water flows: control, 1.8 ± 0.2 $\mu\text{l}/\text{min}$; PDD (100 ng/ml), 1.9 ± 0.2 $\mu\text{l}/\text{min}$; $n = 5$; control, 1.2 ± 0.2 $\mu\text{l}/\text{min}$; PDD (1,000 ng/ml), 1.2 ± 0.3 $\mu\text{l}/\text{min}$; $n = 4$ —and for low concentration of AVP (0.125–1 mU/ml): control, 14 ± 5 $\mu\text{l}/\text{min}$; PDD (100 ng/ml), 13 ± 4 $\mu\text{l}/\text{min}$; control,

Table I. Effects of Phorbol Esters on Osmotic Water Flow

	Water flow			
	20 ng/ml PMA		200 ng/ml PMA	
	Control	Experimental	Control	Experimental
	$\mu\text{l}/\text{min}$ per hemibladder		$\mu\text{l}/\text{min}$ per hemibladder	
Basal	1.1 ± 0.1 $n = 18$	1.1 ± 0.1	0.9 ± 0.1 $n = 26$	0.8 ± 0.1
AVP (0.125–1 mU/ml)	30 ± 6 $n = 8$	$21 \pm 5^*$	11 ± 4 $n = 7$	10 ± 3
AVP (20 mU/ml)	55 ± 5 $n = 8$	$47 \pm 4^*$	43 ± 2 $n = 10$	$33 \pm 2^*$
cAMP (10 mM)	14 ± 3 $n = 6$	15 ± 3	17 ± 4 $n = 4$	20 ± 4
8-bromo cAMP (0.1–0.2 mM)	22 ± 8 $n = 6$	21 ± 7	18 ± 4 $n = 7$	17 ± 3
MIX (4 mM)	Not done		29 ± 4 $n = 7$	25 ± 3
Forskolin (2 μM)	Not done		18 ± 4 $n = 6$	$14 \pm 3 \ddagger$

* $P < 0.01$.

‡ $P < 0.05$.

33±5 µl/min; PDD (1,000 ng/ml), 34±7 µl/min—as well as for maximal AVP (20 mU/ml): control, 36±6 µl/min; PDD (100 ng/ml), 39 + 6 µl/min; control, 66±10 µl/min; PDD (1,000 ng/ml), 60±10 µl/min.

PMA has been shown to stimulate prostaglandin synthesis in another system (19). We therefore determined the effects of PMA and PDD on PGE₂ concentration in the serosal bath at the end of several of the AVP-stimulated water flow experiments. Serosal bath PGE₂ from bladders receiving PMA (200 ng/ml) and AVP was significantly higher than serosal bath PGE₂ from tissue receiving AVP alone (AVP + PMA, 896±152 pg/hemibladder; AVP, 559±55; *P* < 0.02, *n* = 10). No such increase was observed in tissues which had received PDD (AVP + PDD, 807±135; AVP, 1,089±433; *n* = 4). As shown in Table II, an increase in PGE₂ appearance was observed in the serosal baths of tissues incubated with PMA in the absence of AVP, but not in those which received PDD. Thus PMA, but not PDD, stimulated PGE₂ synthesis in toad bladder.

Because of this stimulatory effect of PMA on PGE₂ synthesis, we performed transport experiments paralleling those shown in Table I using tissues preincubated with the prostaglandin synthesis inhibitor naproxen. Naproxen was also maintained in the serosal bath throughout the transport experiments. Under these conditions, no radioimmunoassayable PGE₂ could be detected in the serosal bath.

As shown in Table III, 200 ng/ml PMA still inhibited water flow stimulated by 20 mU/ml AVP, however, lower concentrations of PMA were no longer inhibitory. In contrast, PDD (1,000 ng/ml) had not effect on basal (control, 0.7±3 µl/min; PDD, 0.6±3 µl/min; *n* = 4), low-dose AVP (0.125 mU/ml) (control, 13±4 µl/min; PDD, 11±3 µl/min), or high-dose AVP (20 mU/ml)-stimulated water flows (control, 48±8 µl/min; PDD, 48±7 µl/min) in the naproxen-pretreated tissues. In these naproxen-pretreated tissues, PMA also inhibited flow stimulation by the phosphodiesterase inhibitor MIX but did not alter water flow stimulated by 8-bromo cAMP.

PMA had slightly inhibited forskolin-induced water flow in the absence of naproxen (Table I) but was not inhibitory when prostaglandin synthesis was suppressed (Table III). This pattern of response would suggest that the inhibitory effect of PMA on forskolin-stimulated flow is not a direct one, but instead is secondary to increased prostaglandin production.

Table II. Effects of PMA and PDD on Serosal Bath PGE₂ Content

Incubation time	Serosal bath PGE ₂ content			
	Group I (<i>n</i> = 6)		Group II (<i>n</i> = 6)	
	Control	PMA (200 ng/ml)	Control	PDD (1,000 ng/ml)
min	pg/hemibladder		pg/hemibladder	
15	611±116	610±141	397±110	357±48
(Diluent, PMA or PDD, added after 15-min sample)				
30	624±103	687±171	337±80	409±57
45	624±101	768±177	378±75	378±67
60	606±122	861±192*	358±60	422±59

* *P* < 0.02 compared to simultaneous control value.

Table III. Effects of Phorbol Esters on Osmotic Water Flow in the Presence of Naproxen

	Water flow (µl/min/hemibladder)			
	20 ng/ml PMA		200 ng/ml PMA	
	Control	Experimental	Control	Experimental
Basal	0.9±0.1 <i>n</i> = 5	0.6±0.1	1.0±0.2 <i>n</i> = 11	1.2±0.2
AVP (0.125 mU/ml)	12±2 <i>n</i> = 5	12±3	12±6 <i>n</i> = 6	8±3
AVP (20 mU/ml)	52±9 <i>n</i> = 5	49±10	53±10 <i>n</i> = 6	33±5*
MIX (4 mM)	Not done		40±7 <i>n</i> = 5	25±4‡
8-bromo cAMP (0.1–0.2 mM)	Not done		15±1 <i>n</i> = 6	17±1
Forskolin (2 µM)			39±2 <i>n</i> = 6	37±3

* *P* < 0.02.

‡ *P* < 0.05.

To eliminate effects of PMA via metabolites of arachidonic acid other than prostaglandins, we performed transport experiments in the presence of mepacrine, a phospholipase inhibitor that prevents the release of arachidonic acid from phospholipids (20). In the presence of mepacrine (0.1 mM), PMA (200 ng/ml) also decreased water flow in response to AVP (1 mU/ml) from 20±3 to 14±2 µl/min; *n* = 12; *P* < 0.05. Those data indicate that the inhibitory effect of PMA is independent not only of prostaglandins but of any arachidonate metabolite.

Taken together, these results suggest that PMA exerts an inhibitory effect on AVP- or MIX-stimulated water flow, which is consistent with suppression of cAMP synthesis. Inhibition is observed both in the absence of prostaglandin synthesis, and during inhibition of arachidonic acid release by mepacrine.

PMA inhibited flow only when it was added either before AVP (Tables I and II) or simultaneously with AVP (AVP [1 mU/ml] 29±6 µl/min; AVP + 20 ng/ml PMA, 17±5 µl/min; *n* = 6, *P* < 0.02). Adding PMA 15 min after AVP did not alter flow, indicating that PMA inhibits a step associated with the initial response to AVP. When PMA (200 ng/ml) was added to unstimulated tissues for 30 min and then removed, inhibition of flow still was observed when AVP was added 30 min after PMA washout (59±6 vs. 37±5 µl/min; *n* = 4, *P* < .01) but not when AVP was added 90 min after washout (control, 64±7 µl/min vs. PMA, 58±5 µl/min, *n* = 6).

Basal open circuit potential and short circuit current were not altered by either PMA or RHC-80267; however, the five bladders examined had very small responses to AVP, precluding definitive measurement of its stimulatory effect.

PMA decreased AVP-stimulated, but not basal urea permeabilities in six paired tissues (basal: control 25±10⁻⁷ cm/s; PMA [200 ng/ml] 25±12 [NS]; AVP [20 mU/ml]: control 281±39; AVP + PMA 222±32 [*P* < 0.01]).

Effects of mucosal phorbol esters on transport. The effects of mucosal PMA on transport in the absence of AVP were quite different from those of serosal PMA: serosal PMA did not alter

either AVP-independent water flow or AVP-independent urea permeability. In contrast, 200 ng/ml mucosal PMA led to a slow increase in both the water and urea permeabilities of the bladder in the absence of AVP (Table IV). Flow increased over the first 45 min after addition of PMA, and then remained stable for at least another 30 min at levels about one-third those typically achievable with maximal AVP. (Mazur et al. [21] have reported a similar stimulatory effect of mucosal PMA.) Examination of these tissues using freeze-fracture electron microscopy (4) revealed the typical luminal membrane particle aggregates that accompany the water permeability increases induced by AVP or cAMP, consistent with a common final pathway for the PMA and cAMP-induced transport increases.

After addition of AVP, only a small additional increase in both urea and water permeabilities was observed in the PMA-treated tissues, whereas permeabilities in the tissues that received AVP, but not PMA, rose to their normal high levels. This pattern was observed both in the presence and the absence of naproxen incubation. Thus mucosal PMA increases basal transport, but blocks further stimulation by AVP.

Mucosal PDD did not alter water or urea transport either in the presence or the absence of AVP.

Effect of DiC8 on transport. We also examined the effect of DiC8 at 50 μ M, a concentration that mimics the action of PMA on protein kinase C and on intact cells in another system (10). Serosal DiC8 had no effect on basal water flows, but significantly inhibited the flow induced by submaximal (1 mU/ml) AVP (AVP, 14.2 \pm 2.6 μ l/min; AVP \pm DiC8, 8.3 \pm 0.8 μ l/min; $n = 8$; $P < 0.05$) and maximal (20 mU/ml) AVP (AVP, 44.4 \pm 6.0 μ l/min; AVP + DiC8, 33.4 \pm 2.8 μ l/min; $n = 7$; $P < 0.05$). This inhibition also persisted in the presence of naproxen (AVP [1 mU/ml], 33.3 \pm 3.0 μ l/min; AVP \pm DiC8, 28.0 \pm 2.5 μ l/min; $n = 10$; $P < 0.01$). The DiC8 did not influence water flow in response to 10 mM cAMP (cAMP, 17.3 \pm 4.6 μ l/min; cAMP + DiC8, 18.6 \pm 2.4 μ l/min; $n = 6$) but inhibited that to 0.2 μ M 8-bromo cAMP (8-Bromo cAMP, 34.0 \pm 2.1 μ l/min; 8-Bromo cAMP + DiC8, 25.7 \pm 1.8 μ l/min $n = 4$; $P < 0.02$).

Addition of 50 μ M DiC8 to the mucosal bath did not change the low basal water flows over a 60-min period (control, 1.6 \pm 0.3 μ l/min; DiC8, 2.2 \pm 0.5 μ l/min; $n = 5$).

Effects of RHC-80267 on diglyceride lipase activity, water flow, and prostaglandin synthesis. In this series of experiments, we employed the diglyceride lipase inhibitor RHC-80267 to elevate endogenous diglycerides, and hence increase protein kinase

Table IV. Effects of 200 ng/ml Mucosal PMA on Water Flow and Urea Permeability in the Absence of AVP

Time	Water flow		Urea permeability	
	Control	PMA	Control	PMA
	μ l/min	μ l/min	$\times 10^{-7}$ cm/s	$\times 10^{-7}$ cm/s
-15-0 min	1.7 \pm 0.1	1.9 \pm 0.3	27 \pm 7	34 \pm 7
0-15	1.2 \pm 0.3	3.3 \pm 0.5*	35 \pm 8	56 \pm 10 \
15-30	1.3 \pm 0.3	9.7 \pm 1.1*	35 \pm 9	90 \pm 14*
30-45	1.4 \pm 0.2	13.5 \pm 1.0*	49 \pm 12	104 \pm 16‡
45-60	2.0 \pm 0.3	14.0 \pm 1.0*	58 \pm 12	151 \pm 27‡
60-75	2.1 \pm 0.4	13.2 \pm 0.9*	60 \pm 13	191 \pm 49§

$n = 9$ for water flow data and $n = 6$ for urea data.

* $P < 0.01$.

‡ $P < 0.02$.

§ $P < 0.05$.

Table V. Effect of RHC-80267 on Diglyceride Lipase Activity in 100,000-g Supernatant and Pellet Fractions of Toad Bladder Epithelial Cell Homogenates

	% conversion of [14 C]diglyceride/0.1 mg protein/30 min		
	Control	RHC-80267 (10 μ M)	RHC-80267 (100 μ M)
	%	%	%
Supernatant ($n = 5$)	3.40 \pm 1.06	1.63 \pm 0.50*	0.58 \pm 0.11*
Pellet	10.08 \pm 2.41	8.24 \pm 2.73	3.77 \pm 0.88*

* $P < 0.05$.

C activity. We first verified that RHC-80267 is indeed a diglyceride lipase inhibitor in toad bladder, as it is in platelets (12). As shown in Table V, RHC-80267 inhibited conversion of diglyceride to monoglyceride and fatty acid in a dose-dependent manner, both in the 100,000-g supernatant and the pellet fractions of epithelial cell homogenates.

RHC-80267 (100 μ M) also inhibited diglyceride breakdown in intact epithelial cells that had been prelabeled with [3 H]palmitic acid. Labeled diglycerides increased from 13,770 \pm 2,530 cpm/mg of protein in cells exposed to only AVP (20 mU/ml) to 18,100 \pm 2,750 cpm/mg of protein in those exposed to AVP + RHC-80267; $n = 4$; $P < 0.05$. Labeled triglycerides increased slightly, but not significantly, from 42,000 \pm 3,950 cpm to 47,880 \pm 5,140 cpm, whereas monoglycerides tended to decrease from 5,120 \pm 1,010 cpm/mg of protein to 4,320 \pm 840 cpm/mg of protein. These results are consistent with inhibition of diglyceride lipase by RHC 80267 also in intact cells.

Addition of RHC-80267 to the serosal bath diminished the hydrosmotic response to both low and high doses of AVP, both in the absence and presence of naproxen (Table VI). In addition,

Table VI. Effects of 100 μ M RHC-80267 on Osmotic Water Permeability in the Presence and Absence of Naproxen

	Water flow			
	No naproxen		Naproxen 10 $^{-5}$ M	
	Control	Experimental	Control	Experimental
Basal	2.6 \pm 0.2 $n = 20$	2.6 \pm 0.2	2.5 \pm 0.6 $n = 6$	2.2 \pm 0.8
AVP (0.125-1 mU/ml)	19 \pm 2 $n = 8$	10 \pm 1*	17 \pm 4 $n = 6$	4 \pm 1*
AVP (20 mU/ml)	46 \pm 4 $n = 8$	29 \pm 2*	52 \pm 3 $n = 6$	36 \pm 5‡
cAMP (10 mM)	13 \pm 1 $n = 6$	12 \pm 2		
8-bromo cAMP (0.1-0.2 mM)	28 \pm 4 $n = 6$	7.2‡	16 \pm 3 $n = 6$	5.2*
MIX (4 mM)	Not done		28 \pm 4 $n = 6$	11 \pm 4*

* $P < 0.01$.

‡ $P < 0.02$.

RHC-80267 did not alter PGE₂ content in the serosal bath at the end of the transport experiments (AVP + RHC-80267, 637±120 pg/hemibladder; AVP, 532±113 pg/hemibladder; NS, n = 8). Thus the inhibition of flow by RHC-80267 appears to be completely independent of prostaglandin synthesis. RHC-80267 had no effect on water flow induced by cyclic AMP, but did inhibit the responses to both 8-bromo cAMP and MIX in naproxen-treated bladders (Tables I and III). This pattern is consistent with PGE₂-independent inhibition of AVP-induced water flow via interference with cAMP generation, but may indicate an additional site of inhibition for 8-bromo cAMP.

In contrast to the effects of mucosal PMA, mucosal RHC-80267 did not alter either water flow or urea permeability in the absence of AVP. Both AVP-stimulated and 8-bromo-cAMP stimulated water flows were inhibited by mucosal RHC-80267 (data not shown).

Simultaneous determinations of water flow, cAMP content, and cAMP-dependent protein kinase activity in tissues receiving PMA and RHC-80267. The pattern of transport inhibition suggested that both PMA and RHC-80267 diminished AVP-stimulated water flow predominantly by decreasing cAMP production. We therefore examined water flow, cAMP content, and cAMP-dependent protein kinase activity in a single group of tissues. The in situ protein kinase activity ratio (-cAMP/+cAMP) has been shown to reflect the effective intracellular cyclic AMP concentration (4, 13, 14, 16). In order to avoid the confounding effects of intracellular prostaglandins, all experiments were carried out in the presence of naproxen. As shown in Table VII, PMA decreased the hydroosmotic response to AVP. Consistent with the transport studies, cAMP content was significantly lower in tissues receiving AVP and PMA than in those receiving AVP alone. This was associated with a significantly lower in situ activation of cAMP-dependent protein kinase, as reflected by the lower kinase ratio. For comparison, the protein kinase ratio in the absence of AVP ranges from 0.2 to 0.3, and cAMP concentrations from 20 to 30 pmol/mg protein (4, 13, 14, 16). Thus both our transport and our biochemical studies

Table VII. Effect of PMA on Simultaneously Determined AVP-stimulated Water Flow, cAMP-dependent Protein Kinase, and cAMP Content in Naproxen-pretreated Bladders

Water flow	cAMP content	Kinase ratio
$\mu\text{l}/\text{min}$	$\text{pmol}/\text{mg protein}$	$-\text{cAMP}/+\text{cAMP}$
Control tissues		
55±6	261±74	0.62±0.06
PMA-treated tissues		
44±3*	76±17‡	0.53±0.06‡

Water flows were determined for 30 min after addition of AVP (20 mU/ml) in the absence (control) and presence of 200 ng/ml PMA. All tissues were preincubated with 10 μM naproxen. At the end of the flow experiments, epithelial cells were scraped and prepared for cAMP and kinase determinations. Protein kinase activities (+cAMP) were 2,122±326 and 2,268±303 pmol P_i/mg of protein per min in control and PMA-treated tissues, respectively. Values are means±standard error of the mean for nine paired experiments.

* $P < 0.025$ compared to respective controls.

‡ $P < 0.05$.

suggest that PMA interferes with cyclic AMP generation by a mechanism yet to be defined in toad bladder.

In order to ensure that any PMA carried over from the transport experiments into the assay did not alter the kinase activity itself, we examined the effect of direct addition of PMA to the assay. PMA altered neither the -cAMP or +cAMP kinase activities over a range of 0.2–200 ng/ml ($n = 4$). (PMA was unlikely to activate any calcium-dependent kinase C in the assay, because the assay is carried out in the presence of the calcium chelator EDTA.)

Similar experiments using AVP and RHC-80267 (Table VIII) resulted in data comparable to those with PMA. Again water flow, cAMP content and kinase activity ratio were all decreased in parallel in the RHC-80267-treated tissues compared to those receiving AVP alone. Direct addition of RHC-80267 (0.1–100 μM) to the cAMP-dependent protein kinase assay had no effect ($n = 3$).

Calcium-phospholipid-dependent protein kinase C. Protein kinase activity in the 100,000-g supernate increased when PS/DAG was added to the assay mixture (Table IX). Activities with PS + PMA were comparable to those obtained with PS + DAG. Addition of 2 mM EGTA to chelate calcium markedly inhibited kinase activity to below basal levels, suggesting that the "basal" preparation contained a significant amount of some phospholipid capable of stimulating protein kinase C.

Increasing concentrations of PMA alone increased kinase activity to a modest degree, even in the absence of added phosphatidylserine. Again this is probably because of residual phospholipid in the 100,000-g supernate. The inactive analogue PDD did not stimulate kinase, consistent with reports by others (8).

Protein kinase C activity of the 100,000 g supernate eluted from the DE52 column as a major peak at 30–60 mM NaCl (not shown). No kinase activity could be detected in the NP40 (a nonionic detergent) extract of the 100,000 g pellet either before or after DE52 column fractionation. Consequently, in toad bladder epithelial cells, protein kinase C appears to exist predominantly in the cytosol.

Table VIII. Effect of RHC-80267 on Simultaneously Determined AVP-stimulated Water Flow, cAMP-dependent Protein Kinase, and cAMP Content in Naproxen-pretreated Bladders

Water flow	cAMP content	Kinase ratio
$\mu\text{l}/\text{min}$	$\text{pmol}/\text{mg protein}$	$-\text{cAMP}/+\text{cAMP}$
Control tissues		
70±6	248±48	0.74±0.06
RHC-80237-treated tissues		
51±5*	88±14‡	0.37±0.04*

Water flows were determined for 30 min after addition of AVP (20 mU/ml) in the absence (control) and presence of 100 μM RHC-80237. All tissues were preincubated with 10 μM naproxen. At the end of the flow experiments, epithelial cells were scraped and prepared for cAMP and kinase determinations. Protein kinase activities (+cAMP) were 1,177±71 and 1,200±86 pmol P_i/mg of protein per min for the control and RHC-treated tissues, respectively. Values are means±SEM for six paired experiments.

* $P < 0.01$ compared to respective controls.

‡ $P < 0.05$.

Table IX. Protein Kinase Activity in the 100,000-g Supernatant Fraction of Toad Bladder Epithelial Cell Homogenates

	Protein kinase activity (<i>pmol P</i> /mg protein per 5 min)
Group I (<i>n</i> = 6)	
Basal	960±220
+ PS and DAG	2,860±485 (<i>P</i> < 0.02)
Group II (<i>n</i> = 2)	
Basal	720
+ PS and DAG	2,220
PS and DAG + EGTA (2 mM)	570
+ PMA	
(0.6 ng/ml)	680
(6 ng/ml)	830
(30 ng/ml)	1,080
(60 ng/ml)	1,140
+ PDD (60 ng/ml)	710
+ PS and PMA (30 ng/ml)	2,345

Discussion

Although maneuvers that should alter the cytoplasmic calcium concentration or change the activity of calcium-dependent enzymes (1, 4) have been known for some time to influence the hydroosmotic effect of AVP on water flow, our results represent the first demonstration that calcium-phospholipid-dependent kinase may play a role in this modulation. Taken together with our recent observation (11) that AVP stimulates ³²P-labeled phosphatidylinositol turnover in toad bladder, these findings suggest that AVP can influence epithelial function via effects on protein kinase C, a mechanism that is independent of cAMP, and similar to its effects in hepatocytes (5, 6). The eventual effect of protein kinase C activation appears to be inhibition of AVP-stimulated water permeability, apparently by decreasing cAMP generation though an additional post-cAMP site can not be excluded. This conclusion is based on results obtained with serosal bath addition of PMA, DiC8, and the diglyceride lipase inhibitor RHC-80267. PMA and DiC8, which substitute for diglyceride as activators of kinase C (8, 10), block the hydroosmotic effect of AVP, but not that of cAMP, suggesting that the phorbol ester and the DiC8 act at an "early" site in the cAMP cascade, possibly the activation of adenylate cyclase by AVP. Inhibition required that the phorbol ester be present either before, or simultaneous with, AVP, supporting an action upon some initiating event in the stimulatory cascade. Both DiC8 and RHC 80267 also decreased water flow in response to 8-bromo cAMP, so that an additional "distal" site of inhibition in the cAMP cascade has to be considered.

PMA did increase PGE₂ levels, an effect that has been described in Madin-Darby canine kidney cells (19), and would tend to block the effects of AVP (13, 20). Nonetheless, inhibition of AVP-stimulated transport persisted even when the tissues were incubated with sufficient naproxen to block prostaglandin synthesis completely. Furthermore, inhibition by PMA of AVP-induced water flow persisted in tissues incubated with mepacrine, which inhibits arachidonate release from toad bladders (20) suggesting that PMA inhibits cAMP generation by a mechanism independent of any arachidonic acid metabolites.

PDD, a phorbol ester that does not stimulate protein kinase C (8), did not inhibit water flow even at a concentration 50 times that of the minimum effective PMA concentration tested. These data, together with our demonstration that the toad bladder in fact contains calcium-phospholipid-dependent kinase with the characteristics of protein kinase C, support the hypothesis that the effects of PMA and DiC8 are mediated by kinase C in toad bladder.

We found that PMA inhibited methylisobutylxanthine-stimulated water flow only in naproxen-treated tissues (Table II), but not in those studied without naproxen (Table I). Because PGE₂ also inhibits water flow stimulated by MIX, these findings suggest to us that protein kinase C does not inhibit basal cAMP production when prostaglandin synthesis is intact, but does inhibit the enhanced adenylate cyclase activity observed when inhibition of the enzyme by PGE₂ is eliminated by naproxen incubation (13).

The RHC-80267 experiments approached this problem from another direction. RHC-80267 has been reported to inhibit diglyceride lipase, which, in platelets, leads to accumulation of diglyceride (12). Our results reported here demonstrate that RHC-80267 also inhibits diglyceride lipase activity both in 100,000-g supernatant and pellet fractions of toad bladder epithelial homogenates and in intact epithelial cells. This action would be expected to increase diglyceride levels, and in turn stimulate kinase C activity in the presence of endogenous phospholipid and calcium. As in the PMA experiments, RHC-80267 blocked stimulation of water flow by AVP, but not by cyclic AMP. Furthermore, the inhibition of flow by RHC-80267 was independent of prostaglandin synthesis. RHC-80267 did, however, decrease stimulation by exogenous 8-bromo cAMP. The discrepancy between the results with cAMP and 8-Bromo cAMP can not be explained at present, except to note that results with cAMP and 8-Bromo cAMP have been noted to differ in other respects (22). Therefore, additional "post-cAMP" interference of RHC-80267 with mechanism of action of AVP can not be excluded at present (20).

Our determinations of cAMP content and the cAMP protein kinase ratio provide direct support for the hypothesis that protein kinase C may modulate adenylate cyclase. The lack of inhibition of forskolin-stimulated flow in naproxen-treated tissues suggests that the site of modulation lies either at the receptor or at the regulatory subunit of the cyclase. We would emphasize, however, that our results do not distinguish between a direct effect of protein kinase C on adenylate cyclase (for example, by phosphorylation of cyclase subunits), and an indirect effect involving intermediate steps.

In contrast to the results obtained with serosal addition of PMA, addition of PMA to the mucosal bathing medium enhanced basal water flow, but still inhibited the AVP response. It is believed that fusion of aggregate-containing cell structures with the luminal membrane is responsible for the increased water permeability observed after stimulation with AVP and cyclic nucleotides (23), and PMA is known to facilitate fusion in other systems (24). Indeed, we were able to observe typical aggregates in the luminal membrane of bladder granular cells exposed to mucosal PMA, whereas no such aggregates were observed in the absence of mucosal PMA. These considerations would suggest that both vasopressin-stimulation and exposure to mucosal PMA achieve increased water permeability via the same final fusion pathway. Inasmuch as neither DiC8 nor RHC-802167 in the mucosal bath increased water flow, it is possible that the effect

of mucosal PMA is via a protein kinase C-independent action of PMA.

Based on the results obtained with serosal PMA, DiC8, and RHC-80267, we propose the following model for action of AVP in toad bladder (Fig. 1); in addition to stimulating adenylate cyclase, AVP may also cause breakdown of phosphoinositides to diglycerides and 1,4,5-inositol trisphosphate, similar to its effects in liver (6). The diglycerides in turn stimulate protein kinase C. It should be noted that this neither supports nor precludes any alteration in cytoplasmic calcium concentration, which appears to be sufficient for kinase C activation (9). Indeed diglyceride and PMA enhance the affinity of protein kinase C for calcium (8), so that full activation of the enzyme could occur even at the low concentrations of free calcium present within the cell.

Kinase C activation would in turn inhibit cAMP formation, paralleling a proposal for Leydig cells (25). This inhibition might occur by, for example, phosphorylation of the hormone receptor, which could decrease its affinity for AVP and may represent a mechanism for downregulation of the hormone response. Clearly this hypothesis will require further examination. Additional post-cAMP sites of interaction by protein kinase C can not be excluded at this point.

Formation of the 1,4,5-inositol trisphosphate, on the other hand, can release calcium from intracellular stores (6), thereby providing an additional link between cAMP and calcium in the action of AVP (1).

In summary, our results show that toad bladder epithelial cells contain protein kinase C activity, and that agents which increase kinase C activity inhibit the hydroosmotic effect of AVP with a pattern supporting predominantly inhibition of cyclic AMP generation.

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