Regulation of the Formation and Water Permeability of Endosomes from Toad Bladder Granular Cells

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ABSTRACT Osmotic water permeability (P_f) in toad bladder is regulated by the vasopressin (VP)-dependent movement of vesicles containing water channels between the cytoplasm and apical membrane of granular cells. Apical endosomes formed in the presence of serosal VP have the highest P_f of any biological or artificial membrane (Shi and Verkman. 1989. *J. Gen. Physiol.* 94:1101–1115). We examine here: (*a*) the influence of protein kinase A and C effectors on transepithelial P_f (P_f^{te}) in intact bladders and on the number and P_f of labeled endosomes, and (*b*) whether endosome P_f can be modified physically or biochemically. In paired hemibladder studies, P_f^{te} induced by maximal serosal VP (50 mU/ml, 0.03 cm/s) was not different than that induced by 8-Br-cAMP (1 mM), forskolin (50 μM), VP + 8-Br-cAMP, or VP + forskolin. P_f was measured in endosomes labeled in intact bladders with carboxyfluorescein by a stopped-flow, fluorescence-quenching assay using an isolated microsomal suspension; the number and P_f (0.08–0.11 cm/s, 18°C) of labeled endosomes was not different in bladders treated with VP, forskolin, and 8-Br-cAMP. Protein kinase C activation by 1 μM mucosal phorbol myristate acetate (PMA) induced submaximal bladder P_f^{te} (0.015 cm/s) and endosome P_f (0.022 cm/s) in the absence of VP, but had little effect on maximal P_f^{te} and endosome P_f induced by VP. However, PMA increased by threefold the number of apical endosomes with high P_f formed in response to serosal VP. P_f of endosomes containing the VP-sensitive water channel decreased fourfold by increasing membrane fluidity with hexanol or chloroform (0–75 mM); P_f of phosphatidylcholine liposomes (0.002 cm/s) increased 2.5-fold under the same conditions. Endosome P_f was mildly pH dependent, strongly inhibited by $HgCl_2$, but not significantly altered by GTPγS, Ca, ATP + protein kinase A, and phosphatase action. We conclude that: (*a*) water channels cycled in endocytic vesicles are functional and not subject to physiological regulation, (*b*) VP and forskolin do not have cAMP-independent cellular actions, (*c*) activation of protein kinase C stimulates trafficking of water channels, but does not increase the number of apical membrane water channels induced by maximal VP, and (*d*) water channel function is sensitive to membrane fluidity. By using VP and PMA together, large quantities of endosomes containing the VP-sensitive water channel are labeled with fluid-phase endocytic markers.

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INTRODUCTION

There is strong evidence that transepithelial water permeability in vasopressin-responsive epithelia, including the mammalian kidney collecting tubule and amphibian urinary bladder, is regulated by the targeted movement of vesicles containing water channels between the cell cytoplasm and apical membrane (recent reviews: Handler, 1988; Harris and Handler, 1988; Brown, 1989; Verkman, 1989). Vasopressin addition to the serosal surface of responsive cells is thought to cause insertion of specialized water-permeable membranes into the apical surface by exocytosis; vasopressin removal is thought to cause retrieval of water-permeable membranes by endocytosis. This "membrane cycling hypothesis" is supported by a substantial body of morphological evidence showing correlation between transepithelial water permeability and the location of specialized particle aggregates, visible on freeze-fracture electron microscopy, which are presumed to represent patches of water-permeable membrane (Chevalier et al., 1974; Bourguet et al., 1976; Kachadorian et al., 1977; Muller et al., 1980; Brown et al., 1983).

We have reported functional studies of the formation and transport characteristics of intracellular vesicles from vasopressin-responsive cells. In the kidney collecting tubule of the Brattleboro rat, exogenous vasopressin induced the formation of endosomes with high osmotic water permeability (P_f = 0.04 cm/s) and low activation energy (E_a = 3 kcal/mol), suggesting the presence of functional water channels (Verkman et al., 1988). These water-transporting endosomes were probably in clathrin-coated vesicles (Verkman et al., 1989) and were also induced by DDAVP and oxytocin, and correlated closely with the antidiuretic state of the rat (Lencer et al., 1991). Morphological and functional studies showed that the endosomes formed in response to vasopressin action did not fuse with an acidic prelysosomal compartment, suggesting a unique and specialized mechanism for intracellular processing of endosomes containing the vasopressin-sensitive water channel (Lencer et al., 1990).

Because of the relatively high levels of constitutive endocytosis in the rat and the difficulty of establishing defined physiological conditions for endocytosis, a toad bladder model was developed (Shi and Verkman, 1989). Apical membrane endosomes were labeled with fluid-phase fluorescent markers added to the mucosal solution. It was found that vasopressin induced the formation of endosomes with extremely high P_f (0.1 cm/s) which was inhibited by $HgCl_2$, and low E_a (3 kcal/mol). P_f in labeled vesicles was very low (<0.002 cm/s) in the absence of vasopressin or when endocytosis was turned off by low temperature, azide, or mucosal membrane fixation by glutaraldehyde. The number and P_f of endosomes were influenced by the vasopressin concentration and transepithelial osmotic gradient at the time of endosome labeling in the intact bladder (Shi et al., 1990). Endosomes containing the vasopressin-sensitive water channel had low passive proton permeability and did not contain urea transporters.

We examine here the regulation of endosome formation and water permeability by signaling pathways in the intact bladder, and the hypothesis that water channel function in endosomes is under direct control by physical or biochemical factors. The impetus for this investigation is a series of reports suggesting that toad bladder water permeability is controlled at more than one site by effectors of the protein

kinase A and C pathways, and that water channel function may itself be regulated (see Discussion). We find that effectors acting through the cAMP-dependent pathway have identical effects on P_f in intact toad bladder and in isolated endosomes. However, PMA, acting through protein kinase C, gives a submaximal water permeability response in intact toad bladder and isolated endosomes, but strongly potentiates vasopressin-induced formation of water-permeable endosomes. We find that endosome P_f is not regulated directly by pH, Ca, G proteins, or channel phosphorylation state; however, nonphysiological increases in membrane fluidity are strongly inhibitory.

METHODS

Water Permeability in Intact Bladders

Dominican toads (*Bufo marinus;* National Reagents, Bridgeport, CT) were maintained on wet peak moss and fed worms every 2 d. Excised urinary hemibladders were mounted serosa side out on polyethylene tubing using silk suture. Bladders were initially rinsed with toad Ringer solution (buffer A) containing 110 mM NaCl, 2.5 mM $NaHCO_3$, 3 mM KCl, 2 mM KH_2PO_4, 0.5 mM $MgSO_4$, 1 mM $CaCl_2$, 5 mM glucose, pH 7.8, at 23°C.

Transepithelial osmotic water permeability was measured in intact hemibladder sacs by the gravimetric method of Bentley (1958). Hemibladders were filled with 10 cm^3 of buffer A and bathed in buffer A containing specified ligands for 30–60 min. Solutions were aerated vigorously throughout the experiment. To initiate mucosal-to-serosal osmotic water movement, the mucosal solution was replaced by a fivefold dilution of buffer A with distilled water. The bladder weight was measured every 1.5 min for 6 min before, and >20 min after dilution of the mucosal solution. Initial osmotic volume flow (J_v) was calculated from the slope, evaluated at the time of mucosal dilution, of a quadratic function (bladder wt = $at^2 + bt + c$, where a, b, and c are fitted parameters; $J_v = b$) fitted to the time course of bladder weight from 0 to 12 min after mucosal dilution. Some experiments were performed using paired hemibladders in which water transport in a hemibladder subjected to a test condition was compared with that of a second hemibladder that was maximally stimulated by 50 mU/ml serosal vasopressin. In some experiments ligands were added to the mucosal or serosal buffers at specified times after dilution of the mucosal solution. Transepithelial osmotic water permeability (P_f^{te}) was calculated from the relation $P_f^{te} = J_v/(SV_w\Delta\Pi)$, where J_v is calculated in units of milliliters per minute, S is the surface area of an assumed spherical bladder (22.4 cm^2), V_w is the partial molar volume of water (18 cm^3/mol), and $\Delta\Pi$ is the transepithelial osmotic gradient (mol/cm^3).

Isolation of Fluorescently Labeled Endosomes

Hemibladders were washed and incubated for 30–60 min in buffer A containing specified ligands as described above. Endosomes were labeled by replacement of the mucosal solution with an isosmolar solution of buffer A containing 15 mM 6-carboxyfluorescein (6CF) titrated to pH 7.8 with NaOH. In some experiments endocytosis was inhibited by cooling bladders to 2°C at the time of incubation with 6CF (Harris et al., 1986; Shi and Verkman, 1989). After specified incubation conditions, bladders were washed extensively with buffer B (50 mM mannitol, 5 mM K phosphate, pH 8.5) at 2°C and cells were scraped with a glass slide. All subsequent procedures were performed at 2°C.

Cells were homogenized with a Dounce homogenizer (15 strokes) and a Potter-Elvehjem homogenizer (4 strokes). Debris and heavy membranes were removed by centrifugation (2,000

g, 10 min). A microsomal pellet containing labeled endosomes was prepared by centrifugation of the supernatant at 100,000 *g* for 30 min. The pellet was washed once with >50 volumes of buffer B. The final pellet was homogenized by passage through 23- and 27-gauge steel needles and used immediately. Each hemibladder yielded ~0.5 mg of membrane protein.

Measurement of Osmotic Water Permeability in Endosomes

Osmotic water permeability was measured by a stopped-flow fluorescence-quenching technique described previously (Chen et al., 1988; Ye et al., 1989). The microsomal pellet was suspended to a final concentration of ~0.5 mg protein/ml and subjected to a 60-mM inwardly directed sucrose gradient in a Hi-Tech SF-51 stopped-flow apparatus (Wiltshire, England). Data were acquired at a rate of 5 points/ms with an instrument dead time of 1.7 ms. Fluorescence was excited at 465 ± 5 nm and detected through an OG515 cut-on filter (Schott Glass Technologies, Inc., Duryea, PA). Sample temperature was maintained at 18 or 30°C by a circulating water bath and monitored by an indwelling thermistor.

Each kinetic experiment (512 data points) was repeated 10–15 times for signal averaging before curve fitting. Data were fitted to a double exponential function when there was rapid water transport with a significant change in fluorescence in under 50 ms; a single exponential fit was adequate when rapid water transport was absent. P_f (centimeters per second) was calculated from time constants and endosome surface-to-volume ratio (6×10^5 cm^{-1}) as described previously (Shi and Verkman, 1989).

Chemicals

6CF was purchased from Molecular Probes Inc. (Junction City, OR). Vasopressin (pitressin) was obtained from Parke-Davis (Morris Plains, NJ). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Solutions of forskolin, 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), and 3-isobutyl-1-methylxanthine (IBMX) were prepared immediately before use. 4-Phorbol-12-myristate 13-acetate (PMA) and 4-phorbol 12,13-dideconate (PDD) were added from 1-mM stock solutions in ethanol. Hexanol and chloroform were added from 2-M stock solutions in ethanol.

RESULTS

Regulation of Water Permeability by the cAMP-dependent Pathway

Vasopressin, forskolin, the cell-permeable cAMP analogue 8-Br-cAMP, and the phosphodiesterase inhibitor IBMX are effectors of different steps in the cAMP-dependent pathway for activation of water permeability. Fig. 1 *A* shows the time course of hemibladder weight in response to a 1:5 dilution of mucosal solution giving a 175 mOsm serosal-to-mucosal osmotic gradient. Experiments were performed using paired hemibladders in which one hemibladder was exposed to 50 mU/ml serosal vasopressin. Water permeability was normalized to that for the vasopressin-treated bladder to account for variations in the responsiveness of bladders from different toads. In 32 vasopressin-treated hemibladders from different toads, water flow was 0.11 ± 0.02 ml/min (SD) with a range of 0.05–0.18 ml/min, giving a water permeability coefficient (P_f^{te}) of 0.028 ± 0.004 cm/s. Bladders with water flow <0.07 ml/min were found in <10% of experiments and were excluded from the analysis. Those bladders were generally associated with ill-appearing toads with poor skin turgor.

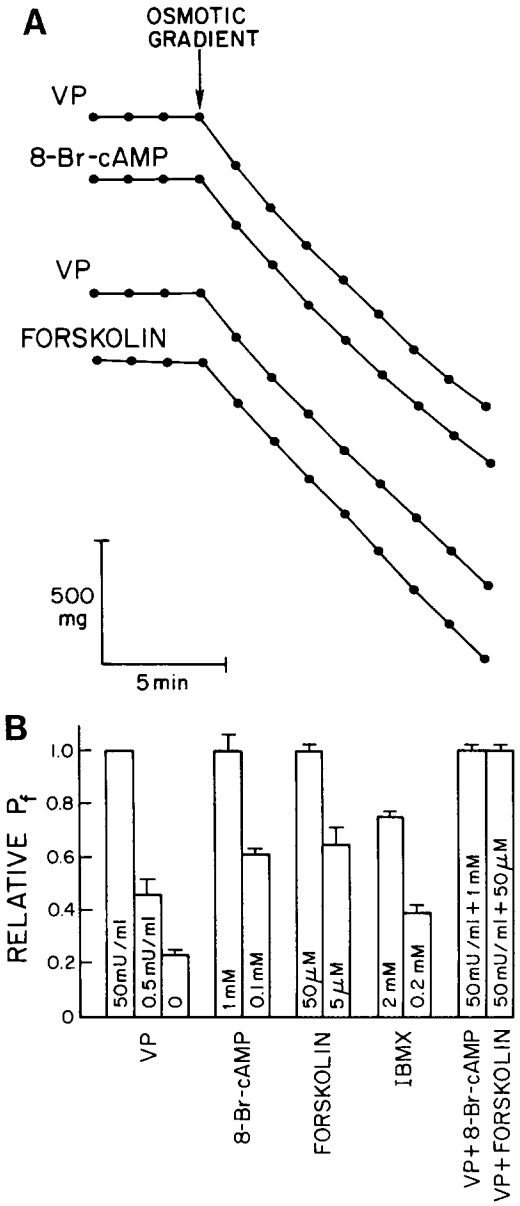

FIGURE 1. Activation of water transport in intact toad urinary bladders by effectors of the cAMP-dependent protein kinase pathway. *A*, Paired hemibladders were incubated for 30 min at 23°C with vasopressin (50 mU/ml), 8-Br-cAMP (1 mM), or forskolin (50 μM). Where indicated, the mucosal solution was made hypoosmotic by replacement with a fivefold dilution of buffer A with distilled water. Initial bladder weight was 10 g; curves were displaced in the *y* direction for clarity. *B*, Data are summarized (mean ± SE) for measurements performed on three to six hemibladder pairs. Relative P_f was normalized to unity for the vasopressin-treated bladder. All bladders were incubated with specified effectors for 30 min before mucosal dilution.

Paired data shown in Fig 1 *A* indicate that bladder P_f^{te} stimulated by saturating concentrations of vasopressin, 8-Br-cAMP, and forskolin did not differ significantly. The averaged results for a series of paired experiments are given in Fig. 1 *B*. P_f^{te} stimulated by vasopressin + forskolin or vasopressin + 8-Br-cAMP, was not different from P_f^{te} stimulated by vasopressin, 8-Br-cAMP, or forskolin alone. These data support the conclusion that effects of maximal vasopressin, forskolin, and 8-Br-cAMP are neither different nor additive. The data show a half-maximal response in

P_f for [vasopressin] ~0.5 mU/ml, [8-Br-cAMP] ~0.1 mM, and [forskolin] ~5 μM. The P_f induced by IBMX alone at high concentration (2 mM) was ~70% of the maximum response.

Fig. 2 shows the osmotic water permeability of endosomes derived from bladders treated with vasopressin, 8-Br-cAMP, and forskolin. As reported previously, vasopressin induced the formation of a population of endosomes with very high water permeability ($P_f \sim 0.1$ cm/s) and with a time course of fluorescence quenching of <10 ms (Shi and Verkman, 1989). This rapid quenching was not observed in the

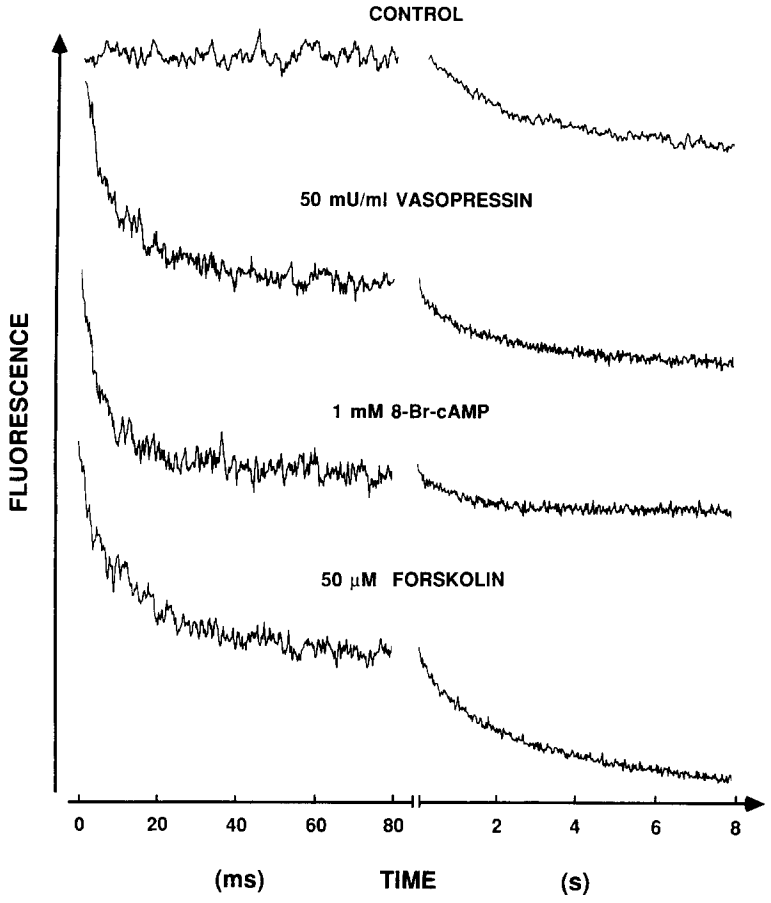

FIGURE 2. Water permeability in endosomes from bladders treated with effectors of the cAMP-dependent protein kinase pathway. Endosomes were labeled for 15 min with 6CF in the presence of vasopressin (50 mU/ml), 8-Br-cAMP (1 mM), or forskolin (50 μM) in the serosal solution as described in Methods. The time course of 6CF fluorescence is shown from stopped-flow experiments in which microsomes containing the 6CF-labeled endosomes were subjected to a 60-mM, inwardly directed sucrose gradient. Fluorescence decreased because of outward osmotic water movement and decreased endosome volume, resulting in increased 6CF concentration and fluorescence self-quenching. Data are shown over two time scales, 0–80 ms and 0.1–8 s.

absence of vasopressin, or when endocytosis was inhibited (four preparations). A second, slower component of water permeability over 1–2 s was present when endocytosis was turned off, and probably represents water transport in closed membrane fragments that have nonspecifically entrapped mucosal 6CF.

In paired studies in which one hemibladder was treated with 50 mU/ml vasopressin, P_f in endosomes from bladders treated with vasopressin, 8-Br-cAMP, and forskolin were 0.10 ± 0.01, 0.12 ± 0.01, and 0.08 ± 0.02 cm/s (SE, four preparations), respectively; the relative signal amplitudes, proportional to the number of labeled endosomes, were 1.00 (vasopressin), 1.08 ± 0.1 (8-Br-cAMP), and 1.15 ± 0.2 (forskolin). Therefore, the number and P_f of endosomes induced by maximal vasopressin, 8-Br-cAMP, and forskolin did not differ significantly. Taken together with results in the intact bladder, these data suggest that the action of vasopressin and forskolin on water transport are mediated exclusively through the cAMP-dependent pathway.

Regulation of Water Permeability by the Protein Kinase C Pathway

Fig. 3 *A* shows the effects of phorbol esters on transepithelial water permeability in intact bladders. Addition of 1 μM PMA to the mucosal solution resulted in a rapid but small stimulation of water permeability compared with control bladders (top two curves). This increase was not observed when PMA was replaced by the inactive phorbol ester analogue PDD, or when PMA was present in the serosal instead of mucosal solution (not shown). Preincubation of bladders for 60 min with mucosal PMA gave a submaximal P_f (bottom three curves); there was no effect of a 60-min incubation with PDD. Fig. 3 *B* gives a summary of results from a series of bladders. The effects of maximal mucosal PMA and serosal vasopressin were not additive; bladder P_f was not greater than that induced by vasopressin alone.

Fig. 3 *C* shows the water permeability of endosomes isolated from bladders treated with PMA. PDD had no effect on water transport in endosomes. The effect of combined mucosal PMA and serosal vasopressin on the fluorescence kinetic signal was striking. Although P_f in endosomes from bladders treated with PMA + vasopressin was similar to P_f in endosomes from vasopressin-treated bladders (Table I), the signal amplitude was increased by 3.0 ± 0.4-fold when PMA was present. These results suggest that PMA caused a marked increase in the endocytosis of water channels that were inserted into the apical membrane by the action of vasopressin. Mucosal PMA alone induced a population of vesicles with P_f lower than that induced by maximal serosal vasopressin (0.022 ± 0.002 cm/s; SE, six separate preparations). Rapid water permeability was absent when PMA was replaced by PDD. In addition, the signal was absent when bladders were cooled to 2°C during the 15-min incubation with mucosal 6CF (three preparations, not shown), indicating that the signal component with P_f = 0.022 cm/s arises from a population of endocytic vesicles.

Experiments were carried out to investigate whether the water permeability characteristics of endosomes formed by the action of PMA + vasopressin were the same as those formed by the action of vasopressin alone. First, as noted above, the rapid fluorescence decrease was absent when bladders were cooled to inhibit endocytosis. Second, the calculated activation energies (E_a) from P_f measurements at

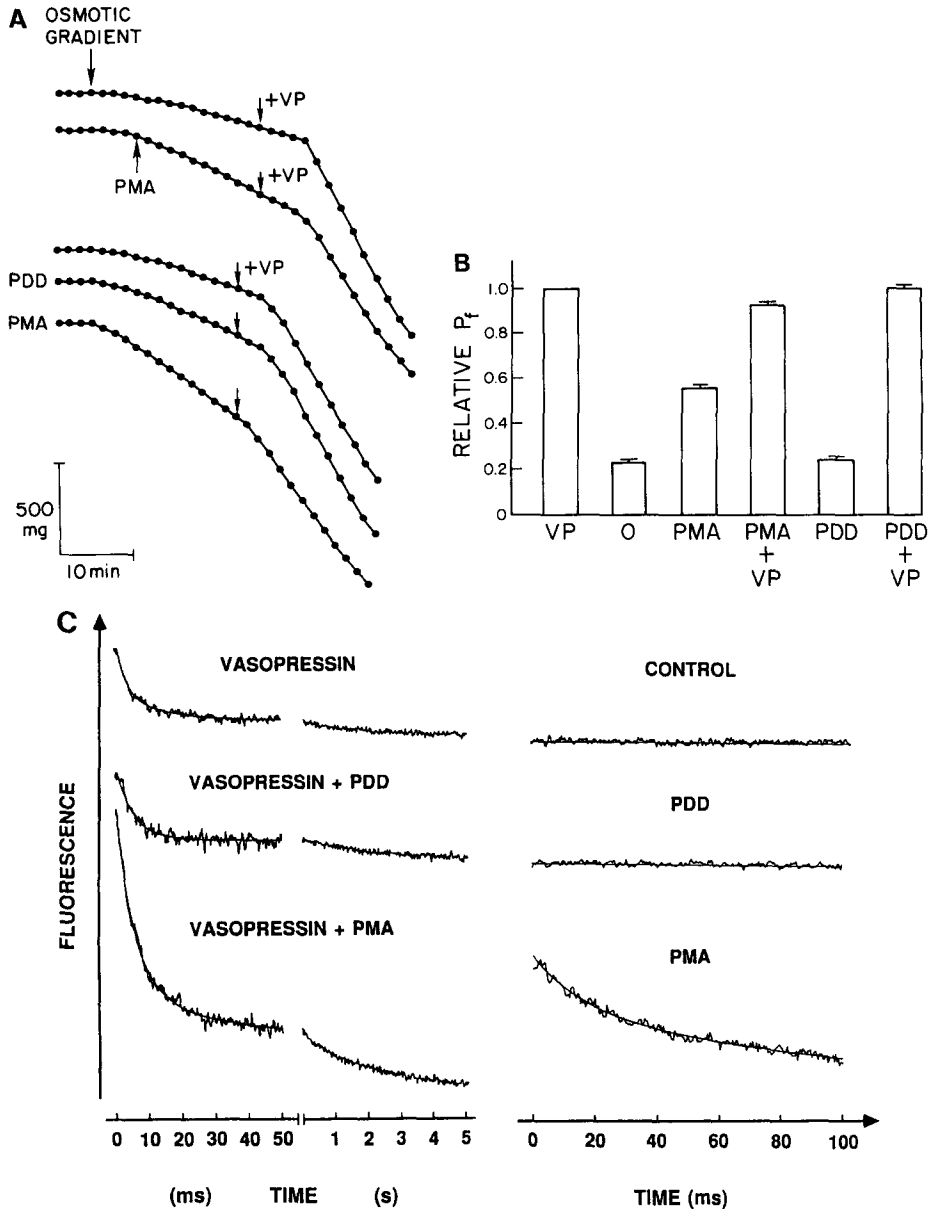

FIGURE 3. Effect of phorbol esters on water permeability in intact toad bladder and isolated endosomes. *A*, In the top two curves, bladders were incubated for 30 min in buffer A in the absence of vasopressin. Where indicated, the mucosal buffer was diluted fivefold and mucosal PMA (1 μM) and serosal vasopressin (50 mU/ml) were added. In the bottom three curves, bladders were incubated for 60 min with no hormone, mucosal PDD (1 μM), or mucosal PMA (1 μM). The mucosal solution was diluted (without changing PDD or PMA concentrations), and serosal vasopressin (50 mU/ml) was added where indicated. *B*, Data are summarized (mean ± SE) for measurements performed on four hemibladder pairs. Relative P_f was normalized to unity for the vasopressin-treated bladder. All bladders were incubated with specified effectors for 60 min before mucosal dilution. *C*, Bladders were incubated for 60 min with serosal vasopressin (50 mU/ml), mucosal PDD (1 μM), or mucosal PMA (1 μM) as indicated. Endosomes were labeled with 6CF for 15 min by replacing the mucosal solution with an isosmotic solution containing 15 mM 6CF, and PMA or PDD if present initially. A microsomal pellet was isolated and endosome water permeability was determined as described in the legend to Fig. 2.

TABLE I

Water Transport Characteristics of Vasopressin-induced Endosomes

	+VP	+VP + PMA
P_f (*cm/s*)	0.11 ± 0.01	0.08 ± 0.01
Relative signal amplitude	1.00	3.0 ± 0.4
E_a (*kcal/mol*)	3.0 ± 1	3.5 ± 1
% Inhibition by 1 mM $HgCl_2$	72 ± 12	68 ± 10

Data are the mean ± SE for paired experiments performed on three to five separate endosome preparations. Incubation conditions for hemibladders were 50 mU/ml serosal vasopressin (+VP) or 50 mU/ml serosal vasopressin + 1 μM mucosal PMA (+VP + PMA) as described in Methods.

18 and 30°C did not differ significantly (Table I). Finally, 1 mM $HgCl_2$ inhibited P_f by the same extent in both classes of endosomes. Taken together, the similar P_f and E_a values and the $HgCl_2$ inhibition suggest that PMA acting through protein kinase C strongly stimulates endocytosis of membrane patches containing the vasopressin-sensitive water channel.

Is the Vasopressin-sensitive Water Channel Regulated Directly?

Experiments were carried out to examine the influence of biochemical maneuvers that are known to regulate a variety of ion channel and membrane transport processes. Endosomes that contained the vasopressin-sensitive water channel were labeled with 6CF in toad bladders incubated with 50 mU/ml serosal vasopressin. Possible regulatory conditions and ligands were tested in the isolated endosome preparation.

Table II shows the influence of solution pH on endosome water permeability. Endosomes were isolated in pH 8.5 buffer to minimize 6CF leakage, and then incubated for 20 min with buffers at specified pH to set intravesicular pH. There was little 6CF leakage at this time even at the lowest pH, and the self-quenching characteristics of 6CF were nearly pH independent (Ye et al., 1989). The data show a small but significant influence of pH on endosome water permeability in the range of pH 6.5–9.0.

TABLE II

Influence of pH on Endosome Water Permeability

pH	*n*	Time constant	P_f
		ms	*cm/s*
6.5	6	7.5 ± 1	0.074 ± 0.01
7.5	4	6.1 ± 0.9	0.091 ± 0.01
8.5	5	5.9 ± 0.7	0.096 ± 0.01
9.0	5	8.3 ± 1.5	0.067 ± 0.01

Endosomes labeled with 6CF were isolated from bladders treated with 50 mU/ml serosal vasopressin. Endosomes were incubated with 50 mM mannitol, 5 mM K phosphate at the specified pH for 20 min before stopped-flow experiments. Endosomes were subjected to a 60-mM, inwardly directed sucrose gradient in the absence of a pH gradient to measure P_f. Data are mean ± SE for experiments performed on *n* separate preparations.

Fig. 4 shows the effect of several possible modulators of endosome water permeability. 1 mM $HgCl_2$ inhibited endosome P_f by 74%. The organic mercurial pCMBS inhibited P_f weakly under the conditions of the experiment. GTPγS, at a concentration that fully activates ion channel–associated G proteins, had no effect on

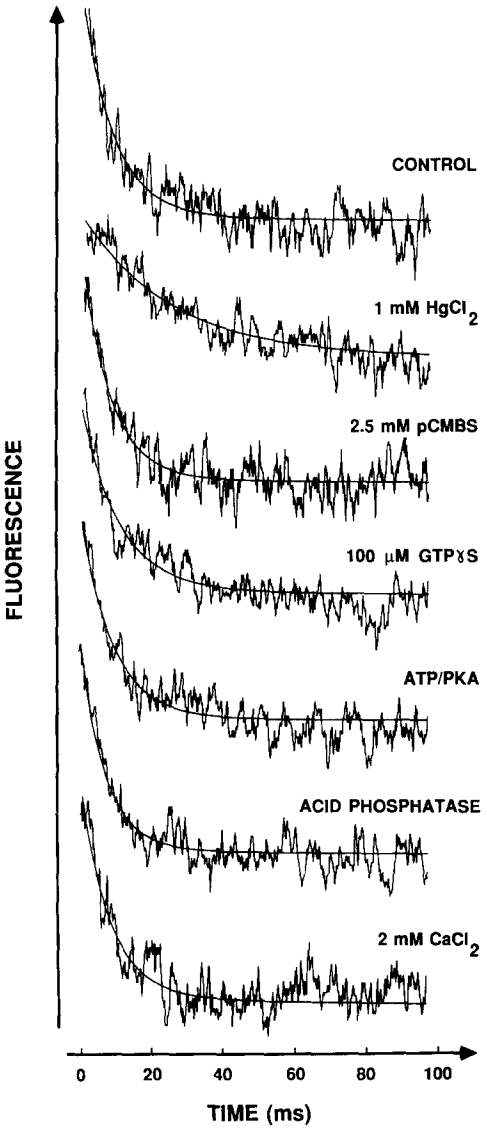

FIGURE 4. Influence of potential regulatory ligands on P_f of endosomes containing water channels. Bladders were incubated with 50 mU/ml serosal vasopressin for 30 min and labeled with mucosal 6CF as described in Methods. Isolated endosomes were incubated for 10 min at 23°C, pH 7.5, with specified ligands just before stopped-flow measurements. Concentrations: 1 mM ATP and 167 U/ml of the protein kinase A catalytic subunit (PKA), 50 U/ml acid phosphatase.

water permeability. Possible alteration of the state of water channel phosphorylation by ATP and the catalytic subunit of protein kinase A, or by acid phosphatase, did not affect P_f significantly. Addition of Ca or EGTA to the buffer had no effect. Table III summarizes results from a series of experiments. Endosome water permeability was

not altered by any of the biochemical maneuvers tested that might correspond to physiologically relevant modes of water channel regulation. In addition, the second data column in Table III indicates that latent water permeability could not be "unmasked" in endosomes labeled in the absence of vasopressin.

Influence of Membrane Fluidity on Water Permeability

The effect of two chemically unrelated "membrane fluidizing" agents, hexanol and chloroform, were examined. Fig. 5 *A* (*left*) shows that endosome water permeability decreased with increasing hexanol concentration in the range 0–75 mM. At 75 mM hexanol in the aqueous phase, it is estimated that the membrane hexanol/phospholipid mole ratio is ~1:200 based on hexanol partitioning data given by Seeman (1972). The effect of 75 mM hexanol was not time dependent; the fluorescence

TABLE III

Influence of Potential Modulators of Water Channel Function on Endosome P_f

Modulator	Concentration	P_f +VP	P_f −VP
	mM	*cm/s*	*cm/s*
None		0.099 ± 0.01	<0.001
$HgCl_2$	0.5	0.063 ± 0.008	<0.001
	1	0.026 ± 0.005	<0.001
pCMBS	1	0.098 ± 0.008	<0.001
	2.5	0.073 ± 0.01	<0.001
GTPγS	0.1	0.097 ± 0.009	<0.001
ATP	1	0.088 ± 0.01	<0.001
ATP + PKA	167 U/ml	0.080 ± 0.01	<0.001
cAMP	0.1	0.095 ± 0.01	<0.001
Acid phosphatase	50 U/ml	0.110 ± 0.02	<0.001
$CaCl_2$	2	0.110 ± 0.03	<0.001
EGTA	2	0.100 ± 0.01	<0.001

Endosomes were labeled with 6CF in bladders treated (+VP) or not treated (−VP) with 50 mU/ml serosal vasopressin. Endosomes were incubated with potential modulators for 10 min at 23°C before stopped-flow experiments. Data are mean ± SE for measurements carried out on three to five separate preparations.

curve shape was not different when measured at 1 or 30 min after hexanol addition to the endosome suspension. The effect of 75 mM hexanol was reversible; 10-fold dilution of 0.1 ml of a 5 mg/ml suspension of microsomes containing 75 mM hexanol with buffer not containing hexanol gave a P_f not different than that measured at 0 hexanol (0.09 ± 0.02 cm/s, diluted microsomes; 0.10 ± 0.01 cm/s, 0 hexanol; SE, two preparations).

The effect of hexanol and chloroform on endosome water permeability was opposite to that observed in PC liposomes (Fig. 5 *A*, *right*). Hexanol and chloroform gave a dose-dependent increase in the water permeability of PC liposomes as measured by a stopped-flow light scattering technique. P_f values for the measurements in endosomes and PC liposomes are summarized in Fig. 5 *B*. Hexanol and chloroform decreased P_f in endosomes, but increased P_f in PC liposomes; note that

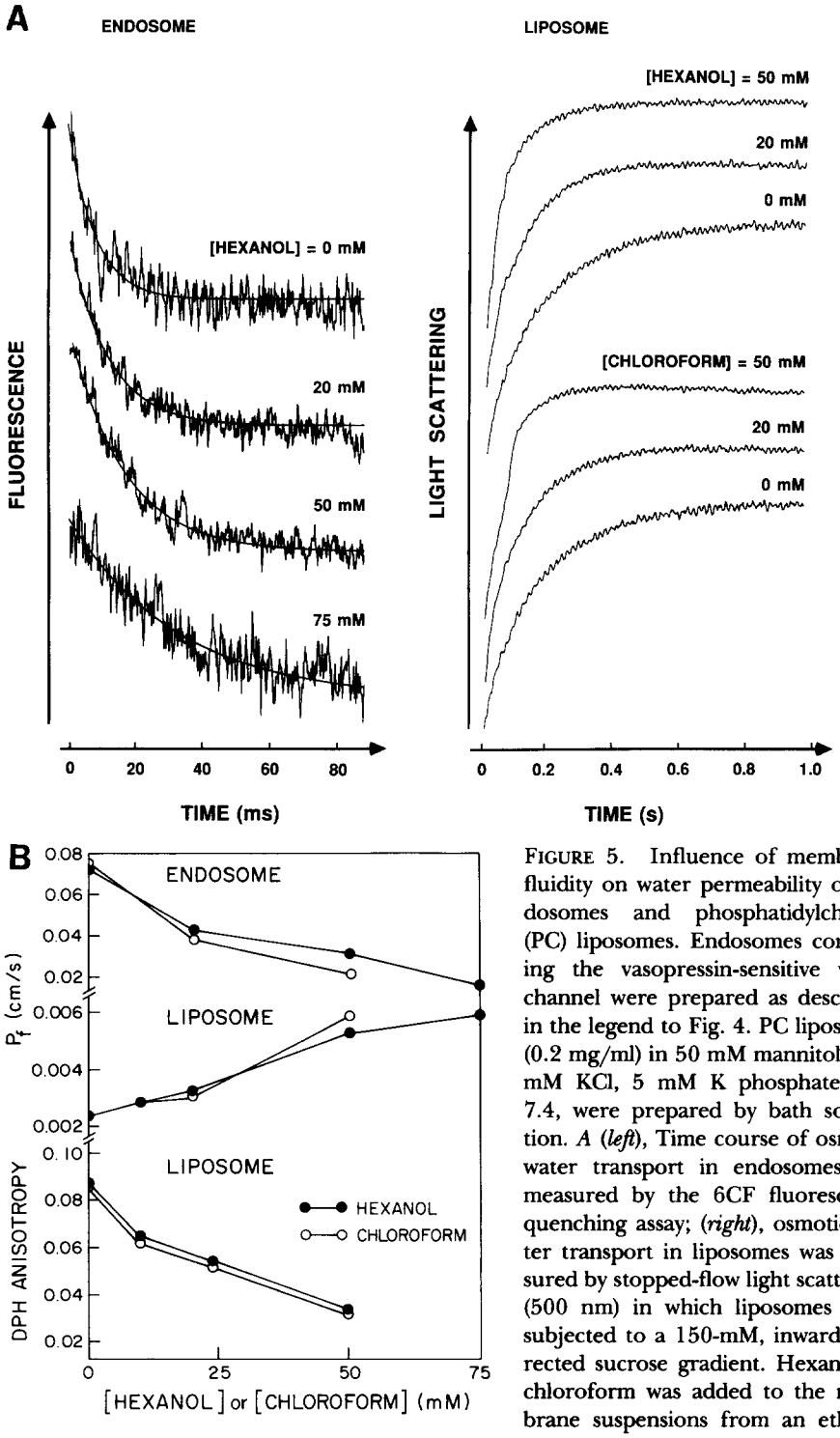

FIGURE 5. Influence of membrane fluidity on water permeability of endosomes and phosphatidylcholine (PC) liposomes. Endosomes containing the vasopressin-sensitive water channel were prepared as described in the legend to Fig. 4. PC liposomes (0.2 mg/ml) in 50 mM mannitol, 100 mM KCl, 5 mM K phosphate, pH 7.4, were prepared by bath sonication. *A* (*left*), Time course of osmotic water transport in endosomes was measured by the 6CF fluorescence quenching assay; (*right*), osmotic water transport in liposomes was measured by stopped-flow light scattering (500 nm) in which liposomes were subjected to a 150-mM, inwardly directed sucrose gradient. Hexanol or chloroform was added to the membrane suspensions from an ethanol stock solution while vortexing vigorously just before experiments. *B*, P_f values (means for three preparations) are given for endosomes (SE < 0.01 cm/s) and liposomes (SE < 0.001 cm/s) containing hexanol or chloroform. Steady-state anisotropy of diphenylhexatriene (DPH) was measured in a suspension of liposomes (0.2 mg/ml) containing 0.5 uM DPH and hexanol or chloroform. Anisotropy (SE < 0.005) was measured in an SLM 48000 fluorimeter using Glan-Thompson polarizers in the T-format as described previously (Illsley et al., 1988).

P_f in endosomes is much higher than that in liposomes which do not contain water channels. In endosomes that were inhibited by 1 mM $HgCl_2$ (P_f = 0.024 cm/s), addition of 50 mM hexanol decreased P_f further by 11 ± 8% (two preparations).

To compare the potencies of hexanol and chloroform as "membrane fluidizing" agents, steady-state DPH anisotropy was measured in liposomes suspended in different concentrations of the fluidizing agents. The data in Fig. 5 *B* show similar potencies of hexanol and chloroform as evidenced by the similar magnitude of the decreases in DPH anisotropy. This result is consistent with the similar membrane/buffer partition coefficients for hexanol and chloroform (13 and 18, respectively) given by Seeman (1972) and strengthens the correlation between membrane fluidity and water permeability. Similar fluidity studies could not be performed in endosome

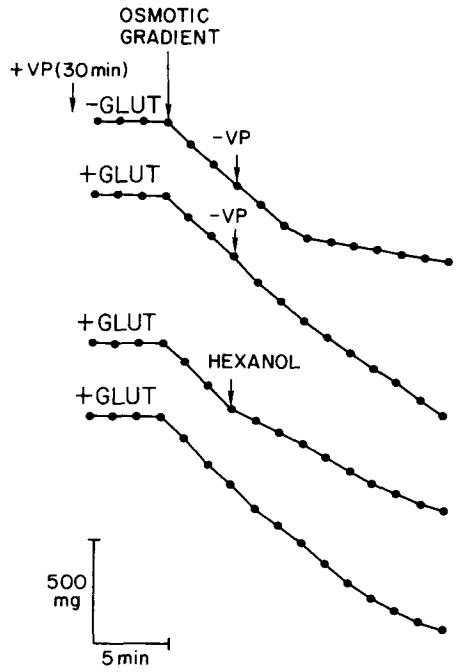

FIGURE 6. Influence of hexanol on transepithelial water permeability in fixed toad bladders. Bladders were incubated with 50 mU/ml serosal vasopressin for 30 min. The mucosal surface was fixed with 1% gluraraldehyde for 10 min where indicated (+ glut). The mucosal solution was then replaced by a 1:5 dilution of buffer A to initiate mucosal-to-serosal osmotic water movement. Top curves, demonstration of efficacy of gluteraldehyde fixation. Vasopressin was removed from the serosal solution where indicated. Bottom curves, effect of 50 mM mucosal hexanol on water permeability. Where indicated the dilute mucosal solution was replaced by the same solution containing 50 mM hexanol.

membranes because selective labeling of endosomal membrane in the microsomal suspension with fluorescent probes of membrane fluidity was not possible. The carrier solvent ethanol, at its maximal concentration (3.3 mol%), had no effect on P_f in endosomes or PC vesicles, or on DPH anisotropy.

It was next examined whether the hexanol-related decrease in P_f of the vasopressin-sensitive water channel in endosomes was also observed for water channels in the apical membrane of intact toad bladders. Bladders were incubated with vasopressin and water channels were fixed in the apical membrane by mucosal glutaraldehyde (Eggena, 1972). The top curves in Fig. 6 demonstrate the efficacy of glutaraldehyde fixation; in three sets of experiments, removal of serosal vasopressin resulted in a rapid decrease in water permeability of the unfixed bladder, but little

effect in the fixed bladder. The bottom curves demonstrate that addition of mucosal hexanol caused a rapid decrease in the water permeability of the fixed bladder. In four sets of experiments, bladder water permeability was inhibited by 41 ± 4% (SE) after hexanol addition. It is likely that this decrease represented a decrease in activity of apical membrane water channels because the cellular mechanisms responsible for regulation of water permeability were bypassed by the fixation procedure.

DISCUSSION

The aims of this study were: (*a*) to investigate the regulation of formation and water permeability of endosomes in intact toad bladder by protein kinase A and C pathways, and (*b*) to determine whether the activity of the vasopressin-sensitive water channel in endosomes is regulated by biochemical or physical factors. These studies were based on the observation that vasopressin induced the formation of endosomes in toad bladder with very high water permeability (Shi and Verkman, 1989). Endosomes were labeled in the intact toad bladder with 6CF, an impermeant fluid-phase marker that undergoes concentration-dependent fluorescence self-quenching. Osmotic water permeability was measured in a microsomal fraction containing the labeled endosomes by a stopped-flow quenching assay which was developed and validated in studies of water transport in membrane vesicles from kidney proximal tubule (Chen et al., 1988). The fluorescence assay was used in these studies as a functional marker of water channels in endocytic vesicles prepared from bladders treated with hormonal effectors.

Vasopressin, forskolin, and cAMP act at different steps in the cAMP-dependent protein kinase pathway. Vasopressin and forskolin cause cAMP production by action at the receptor and catalytic subunits, respectively, of adenylyl cyclase. If there are no cAMP-independent effects of vasopressin and forskolin, it is predicted that the water permeability in intact toad bladder and the regulation of endosome water permeability would be the same for saturating concentrations of vasopressin, forskolin, or 8-Br-cAMP, or any combination of these activators. Kachadorian et al. (1987) found differences in the water permeability, and the relationship between water permeability and particle aggregate density, of bladders treated with vasopressin, forskolin, and cAMP. They suggested the existence of a second, nonapical membrane barrier to transepithelial water movement that is regulated by vasopressin and not by cAMP. Our results do not support this hypothesis. In studies in which an osmotic gradient was applied after bladder incubation with saturating vasopressin, forskolin, or 8-Br-cAMP, or combinations of vasopressin with forskolin or 8-Br-cAMP, bladder P_f did not differ significantly. Similarly, the number and P_f of endosomes prepared from bladders treated with saturating vasopressin, forskolin, or 8-Br-cAMP were not different. We therefore find no evidence for cAMP-independent mechanisms of vasopressin and forskolin action. This conclusion is consistent with results of Levine and Jacoby (1987) showing similar bladder P_f and P_d for vasopressin, 8-Br-cAMP, and forskolin simulation.

In toad bladders, activation of protein kinase C by phorbol esters was associated with massive exocytosis of granules and possibly other membranes, and gross alterations in the morphology of the apical membrane (Masur et al., 1985; Wade et al., 1986). It is possible that the increase in P_f^{te} due to PMA is the result of a

considerable increase in apical membrane surface area (Masur and Massardo, 1987) without exocytosis of water channels. Our results show the contrary. We find that PMA acting through protein kinase C caused a submaximal increase in bladder water permeability in the absence of vasopressin, but did not augment maximal P_f^{te} in the presence of vasopressin. It is unlikely that the increase in P_f^{te} from PMA alone was due to fusion of granules, because P_f in isolated purified granules has been found to be extremely low (0.0005 cm/s; Verkman and Masur, 1988). Endosomes prepared from bladders treated with PMA had a high water permeability, although it was less than that in endosomes prepared from vasopressin-treated bladders. These results indicate that action of PMA through protein kinase C is associated with insertion of water channels into the apical membrane that are subsequently retrieved by endocytosis. The density of water channels in endosomes formed by action of PMA appears to be low, similar to results reported for endosomes formed by the action of submaximal vasopressin (0.05–0.5 mU/ml) in the presence of a serosal-to-mucosal osmotic gradient (Shi et al., 1990).

It was surprising that the amplitude of the fast fluorescence signal was increased threefold when paired hemibladders were treated with vasopressin and PMA compared with vasopressin alone. The water permeability characteristics of endosomes prepared from vasopressin/PMA-treated bladders were not different from those of endosomes prepared from vasopressin-treated bladders. Assuming that the amplitude of the fluorescence signal is proportional to the number of endosomes containing water channels, it is concluded that PMA increases the rate of cycling of membranes containing water channels without increasing bladder P_f beyond that due to vasopressin alone. However, the assumed linearity between signal amplitude and endosome number or total volume must be viewed cautiously. Alterations in intracellular trafficking could result in different kinetics of transfer of the fluid phase marker to cell cytoplasm or intracellular structures that do not fractionate in the microsomal pellet. Methodology to study the trafficking of individual labeled endosomes in intact bladders and the fluorescence of individual isolated endosomes are required to determine endosome number with certainty. Recognizing the potential difficulties in the analysis of amplitude data, concurrent use of vasopressin and phorbol esters maximizes the number of endosomes containing the vasopressin-sensitive water channel for studies of water channel physiology and purification.

The influence of cAMP and protein kinase C agonists on water permeability in rabbit cortical collecting tubule has been examined in a number of studies. Activation of the cAMP pathway by vasopressin, forskolin, or cAMP analogues causes a marked increase in transepithelial osmotic water permeability (Dillingham et al., 1984; Nadler et al., 1986; Kuwahara et al., 1988; Ando et al., 1989). Activation of protein kinase C inhibits the vasopressin response at a post-cAMP site of action (Ando et al., 1987). In addition, there are complex actions of intracellular calcium and prostaglandins that may be of physiological importance (Nadler et al., 1986; Ando et al., 1989). Effects of protein kinase C activation on membrane trafficking have not been examined in kidney collecting tubule.

It is not known whether water channels are always fully functional or whether they are activated and inactivated in response to physiological stimuli. Such a regulatory mechanism would probably not be necessary if membrane shuttling were very

efficient; however, it might provide a secondary mode of fine control of water permeability. Several studies have raised the possibility that water channel function is graded. Kachadorian et al. (1978, 1985, 1986, 1987) found that the membrane density of particle aggregates in toad bladder did not in many circumstances correlate well with transepithelial water permeability. In frog urinary bladder, Parisi and Bourguet (1984) found that an acute rise in P_{CO_2} decreased water permeability without altering the frequency of particle aggregates. Based on these and previous results (Parisi et al., 1981; Chevalier et al., 1983), it was suggested that acid pH (6.5) closed water channels directly. In the kidney collecting tubule, kinetic analysis of the pre–steady state turn-on of P_f after vasopressin addition was consistent with a process of water channel activation following insertion into the apical membrane (Kuwahara and Verkman, 1989). Taken together, these studies raise the possibility that the water permeability of particle aggregates may not be constant.

Based on studies of ion channel and membrane transporter regulation, possible regulatory mechanisms for water channel function include pH, transporter phosphorylation state, channel-associated G proteins, and calcium concentration. Table II showed a small (~20%) decrease in endosome P_f at pH 6.5 and 9.0 compared with 7.5 and 8.5. However, we were unable to modify significantly the P_f of endosomes by the other regulatory mechanisms. In contrast, endosome P_f was sensitive to temperature and $HgCl_2$, and in a previous study, to vasopressin concentration and osmotic gradient size (Shi et al., 1990), showing that the measurement process was sensitive to changes in membrane water permeability. In addition, the biochemical methods used to regulate transporter phosphorylation and channel-associated G proteins are well established from previous work (Valdivia et al., 1988; Bae and Verkman, 1990). These findings, therefore, do not support the hypothesis that the water permeability of individual water channels is regulated under physiological conditions. In light of the studies of Kachadorian and co-workers, our results raise questions about the quantitative interpretation of particle aggregates, at least as assessed by present morphological techniques, in terms of water-transporting units. Of course, it is recognized that the presence of regulatory mechanisms that were not examined here cannot be ruled out.

Little is known about the molecular composition of the water channel. It has been assumed that the water channel is a protein; however, specialized lipids may comprise part or all of the water pathway. In toad bladder, radioiodination of apical membrane proteins that were internalized by endocytosis gave a series of labeled proteins that are candidate components of the water channel (Harris et al., 1987, 1988). In the red blood cell that contains a mercurial-inhibitable water channel, neither protease digestion (Benga et al., 1983) nor radiation inactivation (Dix et al., 1985) altered P_f; however, both maneuvers abolished the inhibitory potency of mercurials. These results suggested that a large protein, which was inactivated by radiation, might regulate a radiation and proteolysis-insensitive water pathway composed of lipids or small proteins. In recent work, mRNA coding for kidney and erythrocyte water channels has been expressed in *Xenopus* oocytes, suggesting that the water channel is a protein (Zhang et al., 1990).

One way to distinguish between lipid- and protein-mediated pathways has been to examine effects of membrane fluidity on transport (Forman et al., 1985; Ives and

Verkman, 1985; Golchini and Kurtz, 1988). We find that two chemically unrelated membrane fluidizing agents hexanol and chloroform, decrease endosome water permeability. At the same concentrations, these agents increase the water permeability and membrane fluidity of artificial PC liposomes. The inhibitory effects of hexanol on endosome P_f were reversible, showing that hexanol did not extract water channels from the membrane. Hexanol had little effect on P_f in endosomes treated with $HgCl_2$, in which water permeability through channels was partially inhibited. The lack of an increase in P_f with hexanol addition may be due to the high P_f in $HgCl_2$-inhibited endosomes compared with P_f in liposomes not containing water channels. Hexanol was also shown to decrease the water permeability of gluteraldehyde-fixed bladders when present in the mucosal solution.

It must be recognized that effects of membrane fluidizing agents on cell function must be interpreted with caution because addition of membrane perturbants may cause multiple specific and nonspecific effects. Assuming that the primary effect of hexanol and chloroform is that of increasing bulk membrane fluidity, our results suggest that increased membrane fluidity is deleterious to the function of the water channel, possibly by altering the conformational state of a protein or specialized lipid component of the water pathway. Using an anisotropy imaging technique to map cell membrane fluidity (Fushimi et al., 1990), it was shown that serosal vasopressin, at a concentration that increased P_f in cortical collecting tubule >15-fold, did not alter apical membrane fluidity (Fushimi and Verkman, 1990). Taken together, these findings suggest that control of membrane fluidity has little importance in the vasopressin hydroosmotic response.

In summary, the data reported here show that endosome formation and water permeability are regulated by effectors of the protein kinase A and C pathways in intact toad bladder granular cells. The water permeability of isolated endosomes containing the vasopressin-sensitive water channel was not regulated by a number of physiologically relevant biochemical maneuvers, suggesting that the membrane trafficking of fully functional water channels accounts for the cellular action of vasopressin on water permeability. The molecular identity of the water channel remains a major unanswered question.

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