Roles of Ca^{2+} and Na^+ on the Modulation of Antidiuretic Hormone Action on Urea Permeability in Toad Urinary Bladder

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Abstract

The present studies probe the role of Ca^{2+} and Na^+ in the stimulation-permeability coupling sequences by which antidiuretic hormone (ADH) induces a cyclic AMP (cAMP)-mediated increase in urea permeability in toad urinary bladder. The following results were obtained: (*a*) Removal of mucosal Na^+ or Ca^{2+} or deletion of serosal Ca^{2+} did not modify ADH action. (*b*) Reduction of the serosal Na^+ concentration to <50 mM inhibited the effects of both ADH and cAMP. The minimal concentration of serosal Na^+ needed for the hormone to elicit its maximal effect was reduced to ~10 mM if serosal Ca^{2+} was concomitantly deleted. (*c*) The Na^+ ionophore monensin produced an inhibition of ADH and cAMP actions that was dependent on the presence of Na^+ and Ca^{2+} in the serosa. (*d*) The Ca^{2+} ionophore A23187 produced a serosal Ca^{2+}-dependent inhibition of ADH effect and did not modify cAMP action. (*e*) Carbachol, which increases Ca^{2+} uptake to the same extent that A23187 does, had no effect on ADH action. (*f*) Quinidine, which releases Ca^{2+} from intracellular stores, produced a large inhibition of the action of ADH but not that of cAMP; the inhibition was greatly reduced if serosal Ca^{2+} was deleted. (*g*) Dinitrophenol and iodoacetate, which also release Ca^{2+} from intracellular pools, had no effect on ADH action. (*h*) The Ca^{2+} channel blocker diltiazem had no effect on ADH action and did not modify the inhibitions produced by deletion of serosal Na^+ or monensin. (*i*) The cyclooxygenase inhibitor indomethacin partially removed the inhibition produced by deletion of serosal Na^+ and almost completely impeded the inhibitions produced by either monensin or A23187. It is concluded: (*a*) Extracellular Ca^{2+}, Na^+ transport rates, and serosal Na^+, in concentrations between 10 and 110 mM, have no participation in modulating the increase in urea permeability produced by ADH. (*b*) Increases in cytosolic Ca^{2+} activity, which are capable of inhibiting the effect of ADH on urea permeability at pre- and/or post-cAMP steps, seem to be highly compartmentalized. (*c*) Endogenous prostaglandins might play a role in the inhibitions produced by absence of serosal Na^+, monensin, or A23187.

Introduction

Antidiuretic hormone (ADH)[1] increases the permeabilities to water and urea in toad urinary bladder through cyclic AMP (cAMP)-mediated mechanisms (1–4). Extensive studies have been done on the cationic requirements of the effect of ADH on water permeability (5–18). Different maneuvers dissociate the effect of ADH on the apical permeation pathways for water and urea (1–4). Therefore, it cannot be inferred from the results obtained in the studies of the relationship between cations and ADH action on water permeability that similar results would be obtained on the effect of ADH on urea permeability, inasmuch as their mechanisms of activation, though both cAMP-mediated, seem to involve discrete cellular pathways and may have different ionic requirements.

The present studies were undertaken to investigate possible roles of Ca^{2+} and Na^+ in the regulation of the stimulation-permeability coupling sequences by which ADH stimulates urea permeability in the toad urinary bladder.

Methods

Toad urinary bladders were isolated from double-pithed female toads (*Bufo marinus*) originating in the Dominican Republic. The bilobed bladder was divided into two hemibladders and each attached as a sac (mucosa inward) to the end of plastic cannulas and rinsed at least twice by exchanging the mucosal and serosal baths for fresh Ringer's solution. In all cases, the volume of the mucosal bath was 5 ml and the volume of the serosal bath was 70 ml.

The composition of the standard Ringer's solution was as follows (in millimoles/liter): NaCl, 110; KCl, 5; $CaCl_2$, 1; glucose, 5; Tris-HCl, 5; pH 8.0–8.2; osmolality, 233 mosmol/kg H_2O.

"Ca^{2+}-free" Ringer's solution was made by omitting $CaCl_2$ from the otherwise unaltered solution; in other cases Ca^{2+}-free Ringer's was made by omitting $CaCl_2$, adding 1 mM EGTA, and titrating the pH of the solution back to pH 8.0–8.2 with 5 N NaOH.

"Na^+-free" Ringer's solution was made by isoosmolal replacement of NaCl with 145 mM Tris-HCl (7). Ringer's solutions other than Na^+-free but with NaCl concentrations < 110 mM were also made by isoosmolal replacement of the corresponding NaCl concentration with Tris-HCl. The use of Tris to substitute for Na^+ instead of choline is due to the following: (*a*) it has been shown that choline increases the production of CO_2 by the isolated toad urinary bladder, whereas Tris does not; and (*b*) it has been suggested that choline may be used as a metabolic substrate and/or may activate bladder metabolism (cf. 7).

When hemibladders were incubated in the absence of a given cation in the serosal bath, the protocol followed was to (*a*) blot hemibladder's serosa with filter paper (Whatman No. 1, Whatman Paper Div., Clifton, NJ); (*b*) wash serosa while stirring for 1 min in the Ringer's from which the cation was omitted; (*c*) blot; (*d*) wash serosa in fresh modified Ringer's for another minute while stirring; (*e*) blot; (*f*) place in fresh modified Ringer's, where they remained for 60 min, stirring at 300–400 rpm. After this period the hemibladders were blotted and placed for 30 min in the modified Ringer's, with or without the permeability stimulant, after which urea permeability was determined. Control hemibladders were treated the same way, but with the standard Ringer's solution throughout.

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1. *Abbreviations used in this paper:* ADH, antidiuretic hormone; DNP, dinitrophenol; IAA, iodoacetate; IBMX, isobutylmethyl xanthine; P_u, urea permeability.

When hemibladders were incubated in the absence of a given cation in the mucosal compartment, the bath was changed a total of five times over a period of 60 min, before determination of urea permeability coefficients.

As the source of ADH, arginine vasopressin (Pitressin, Parke Davis; Morris Plains, NJ) was used, always at a supramaximal dose of 20 mU/ml. To study the effects of the experimental conditions on the post-cAMP steps, a mixture of 5 mM cAMP (Sigma Chemical Co., St. Louis, MO) and 0.4 mM isobutylmethyl xanthine (IBMX; Sigma Chemical Co.) was used. The level of urea permeability (P_u) obtained with this treatment was similar to that achieved with ADH at 20 mU/ml (P_u with ADH: 369 ± 7 nm/s, n = 173; P_u with cAMP plus IBMX: 349 ± 13 nm/s, n = 69).[2]

Monensin and A23187 (Calbiochem-Behring Corp., La Jolla, CA) were prepared as stock solutions in a 1:1 dimethyl sulfoxide-ethanol solvent and added to the serosal Ringer's solution at final concentrations of 1.4×10^{-5} M and 5×10^{-6} M, respectively. Solvent concentration in the experimental bath was 0.1% and was maintained in the control bath as well. Monensin was added 60 min, and A23187 30 min, before stimulation of P_u. Quinidine and carbachol (Sigma Chemical Co.) were used at concentrations of 10^{-3} M and 10^{-4} M, respectively, and added 30 min before either stimulant of P_u. Dinitrophenol and iodoacetate (Sigma Chemical Co.) were used at concentrations of 10^{-5} M and 10^{-4} M, respectively, and added 60 min before ADH.

The Ca^{2+} channel blocker diltiazem (a gift from Dr. C. Van Breemen, Department of Pharmacology, University of Miami, FL) and the cyclooxygenase inhibitor indomethacin (Sigma Chemical Co.) were added to the serosa at concentrations of 2×10^{-4} M and 10^{-5} M, respectively.

When the effects of the drugs were studied in Na^+-free or Ca^{2+}-free Ringer's solution, Na^+ and/or Ca^{2+} were deleted from the serosal bath before addition of any compound, through the same procedure previously described.

To determine coefficients of urea permeability, [^{14}C]urea (New England Nuclear, Boston, MA) was added to the mucosal bath (500 nCi/ml) at appropriate times. The serosal compartment was stirred at 400 rpm to reduce the contribution of unstirred layers to the measured permeability coefficients. Stirring rates were monitored with Hall-effect transistors (10). After 30 min, 1-ml samples were taken from the serosal bath, the hemibladders cut free of the cannulas and placed in tared beakers to determine mucosal volume (the weight of the tissue was not taken into account), and 100-μl samples were taken from the mucosal fluid. The radioactive samples were mixed in 10 ml of Aquasol (New England Nuclear) and scintillation counting was performed in a 1215 Rackbeta (LKB Instruments, Rockville, MD). The disintegrations per minute were derived from the counts per minute, and standard corrections for efficiency and quenching were used.

Permeability coefficients for urea (P_u) were computed from the Fick equation:

$$P_u = J_u^{M-S}[\Delta C_u 4\pi(3V/4\pi)^{2/3}]^{-1}, \quad (1)$$

where J_u^{M-S} is the net increase of [^{14}C]urea, per unit time, in the serosal bath; ΔC_u is the mean concentration gradient of [^{14}C]urea on mucosa and serosa during the period of measurement; and V is the mucosal volume, determined by weight.

Inulin permeability coefficients (P_{in}) were determined in the same way, adding [^{14}C]inulin to the mucosal bath (1 μCi/ml).

Data reduction was performed with the Prophet computer system (Biological Handling Program, Division of Research Resources, National Institutes of Health). Results are expressed as mean±standard error of the mean. Standard programs for curve fitting and t tests for paired data were used. Differences with a probability level $P < 0.05$ were considered statistically significant.

Results

Studies on the role of calcium

EFFECTS OF OMITTING EXTRACELLULAR Ca^{2+}. Table I displays the effects of Ca^{2+}-free baths on basal and stimulated P_u.

Removal of Ca^{2+} from the mucosal bath with addition of 1 mM EGTA, but maintaining 1 mM Ca^{2+} in the serosa, affects neither basal nor ADH-induced P_u, nor, as expected, P_{in}.

In bladders containing 1 mM Ca^{2+} in mucosa, the addition of 1 mM EGTA to a Ca^{2+}-free serosal bath increases basal P_u 28-fold. That this effect is due to the appearance of paracellular permeability pathways is reflected by a 52-fold increase in P_{in}. A similar result is obtained when, in the absence of EGTA, Ca^{2+} is removed simultaneously from mucosal and serosal compartments (11). The incubation of hemibladders in a serosal Ca^{2+}-free bath without EGTA, containing 1 mM Ca^{2+} in mucosa, does not affect basal P_u, P_{in}, or the enhancement of P_u by ADH or cAMP + IBMX.

EFFECTS OF AGENTS THAT INCREASE INTRACELLULAR Ca^{2+} ACTIVITY. To test the effects of an increase in cytosolic Ca^{2+} activity on the stimulation of P_u, we made use of agents that direct or indirect evidence has shown to produce this effect. The results are displayed in Table II.

A23187 and carbachol. Both agents increase Ca^{2+} uptake by the epithelial cells of toad bladder. When Carbachol and A23187 are used at concentrations of 10^{-4} M and 10^{-6} M to 10^{-5} M, respectively, they induce a Ca^{2+} uptake of equal magnitude (13).

Incubation with 10^{-5} M A23187 produced a 50% inhibition of the response to ADH, but did not modify that to cAMP + IBMX. In the absence of serosal Ca^{2+} the inhibition of ADH action on P_u was reduced to 12%.

Contrary to the effect of the calcium ionophore, 10^{-4} M Carbachol did not modify the action of the hormone on P_u.

Quinidine, dinitrophenol (DNP), and iodoacetate (IAA). These agents increase cytosolic Ca^{2+} activity in epithelial cells of the toad bladder, and other cells, by acting on intracellular organelles, decreasing Ca^{2+} uptake, and inducing Ca^{2+} release from these compartments, and also by depressing the extrusion of Ca^{2+} across plasma membranes (14, 17, 19–23).

Incubation of the experimental bladders with 10^{-3} M Quinidine produced an 88% inhibition of the response to ADH. This inhibition was reduced to 24% if quinidine was added to a Ca^{2+}-free serosal medium. This is a real attenuation of the inhibitory effect of quinidine, as can be seen in Table II, because comparison of the responses in matched hemibladders incubated both with quinidine, but controls with 1 mM serosal Ca^{2+} and the experimentals without serosal Ca^{2+} show that the response in the latter is fivefold greater than that obtained in the controls. Quinidine had no effect on the increase in P_u produced by cAMP + IBMX.

2. The reason for using a mixture of cAMP and IBMX is that in the presence of low concentrations of the xanthine the effect of exogenous cAMP on P_u is prolonged and more stable. Nonetheless, several investigators have suggested that methylxanthines may alter cellular Ca^{2+} homeostasis. This may be of particular concern when using IBMX to evaluate the effects of Ca^{2+} on the post-cAMP steps. However, we do not believe that the results reported here have been handicapped by the use of IBMX, in that monitoring intracellular Ca^{2+} activity in isolated epithelial cells of toad bladder with the fluorescent probes Quin-2 and chlorotetracycline showed that IBMX at 4×10^{-4} did not modify Ca^{2+} activity.

Table I. Effects of Removal of Ca^{2+} (with or without Addition of 1 mM EGTA) from the Bathing Media on P_u and P_{in}

Conditions										
Mucosa	Serosa	EGTA	ADH	cAMP + IBMX	P_u	n	P	P_{in}	n	P
					nm/s			*nm/s*		
Ringer's Ca^{2+}-free	Ringer's Ringer's	+	−	−	6±3 19±10	6	NS	0.17±0.03 0.19±0.04	6	NS
Ringer's Ca^{2+}-free	Ringer's Ringer's	+	+	−	352±24 351±22	6	NS			
Ringer's Ringer's	Ringer's Ca^{2+}-free	+	−	−	30±5 847±39	6	<0.001	0.27±0.09 14.31±1.67	6	<0.001
Ringer's Ringer's	Ringer's Ca^{2+}-free	−	−	−	19±6 23±8	6	NS	0.98±0.03 1.87±0.05	7	NS
Ringer's Ringer's	Ringer's Ca^{2+}-free	−	+	−	400±21 394±20	6	NS			
Ringer's Ringer's	Ringer's Ca^{2+}-free	−	−	+	346±22 339±37	12	NS			

Table II. Effects of A23187 (10^{-5} M), Carbachol (10^{-4} M), Quinidine (10^{-3} M), DNP (10^{-5} M) and IAA (10^{-4} M) on Stimulated P_u

Conditions							
Mucosa	Serosa	ADH	cAMP + IBMX	P_u	Change	n	P
				nm/s	%		
Ringer's Ringer's	V A23187	+	−	396±17 211±32	−48±6	9	<0.001
Ringer's Ringer's	Ca^{2+}-free + V Ca^{2+}-free + A23187	+	−	363±28 317±26	−12±3	6	<0.05
Ringer's Ringer's	V A23187	−	+	315±40 329±45	+6±8	9	NS
Ringer's Ringer's	Ringer's Cch	+	−	364±20 349±18	−5±4	9	NS
Ringer's Ringer's	Ringer's Q	+	−	326±39 45±32	−88±8	6	<0.001
Ringer's Ringer's	Ca^{2+}-free Ca^{2+}-free + Q	+	−	462±19 346±18	−24±2	4	<0.05
Ringer's Ringer's	Q Ca^{2+}-free + Q	+	−	79±20 377±82	+379±80	6	<0.001
Ringer's Ringer's	Ringer's Q	−	+	408±18 392±8	−3±6	6	NS
Ringer's Ringer's	Ringer's DNP	+	−	275±28 294±20	−10±7	6	NS
Ringer's Ringer's	Ringer's IAA	+	−	361±21 306±28	−14±10	6	NS

V, vehicle (0.1% dimethyl sulfoxide-ethanol; Cch, carbachol; Q, quinidine; DNP, dinitrophenol; IAA, iodoacetate.

Incubation with either DNP or IAA for 60 min before the hormonal challenge had no effect on the increase in P_u.

Studies on the role of sodium

EFFECTS OF REDUCING THE EXTRACELLULAR Na^+ CONCENTRATION. The results of the experiments in which Tris isoosmotically replaced Na^+ are shown in Table III.

The absence of mucosal Na^+ does not interfere with basal P_u or the effect of ADH on P_u. The absence of serosal Na^+ did not modify basal P_u, and blunted by 60% the increase in P_u triggered by either ADH or cAMP + IBMX.

We tested to see if the complete absence of Na^+ from both mucosal and serosal compartments might alter the response to ADH in a different way than deletion of Na^+ from serosa alone. In these experiments control hemibladders were incubated without serosal Na^+ while the matching experimental hemibladders were incubated with Na^+ deleted from both mucosal and serosal compartments. There was no difference in the action of ADH on P_u between these two conditions, in that the inhibitions of the response to the hormone are comparable.

We attempted to determine the minimal concentration of serosal Na^+ required by ADH to trigger an increase in P_u equal to that obtained when the serosal medium contains 110 mM Na^+. These experiments were carried out in nonpaired hemibladders, and the results are shown in Fig. 1. Decreasing the Na^+ concentration in the serosal bath decreases the effect of ADH on P_u. The mean of the ADH-induced P_u's at the different serosal Na^+ concentrations tested were plotted in a semilogarithmic graph (Fig. 1, *inset*). No difference in P_u can be observed with serosal Na^+ concentrations between 50 and 110 mM. Thus, ~50 mM can be considered to be the minimal serosal Na^+ concentration needed by ADH to trigger a full increase in P_u in toad urinary bladder.

It has been demonstrated that removal of extracellular Na^+ induces an increase in Ca^{2+} uptake by the epithelial cells (24, 25). Therefore, the inhibition of the ADH- and cAMP+IBMX-induced P_u produced by removal of serosal Na^+ could be the result of an increased Ca^{2+} uptake by the epithelial cells, and may not necessarily reflect a requirement for serosal Na^+ to trigger an increase in P_u. To test this possibility the experimental hemibladders were incubated in Ca^{2+}-free Tris-Ringer's while the control hemibladders were placed in standard Ringer's. The results are shown in Table IV. In the absence of serosal Ca^{2+} and Na^+, basal P_u remained unmodified whereas the ADH-induced increase in P_u shows 35% inhibition. This inhibition is less than the 60% inhibition observed when Ca^{2+} was present in the serosal medium (Table III). This could be a genuine attenuation of the inhibition or could be the outcome of variability among different toad batches in the response to ADH, and also of its inhibition by the absence of Na^+. To determine this, control hemibladders were incubated in a Na^+-free serosal bath while the experimental hemibladders were incubated in a serosal Ringer free of Na^+ and Ca^{2+}. In these circumstances the response to ADH was twofold greater in the experimental than in control hemibladders (Table IV).

The concomitant absence of serosal Ca^{2+} and Na^+ from serosa lessens, but does not remove, the inhibition of the effect of ADH on P_u produced by replacing serosal Na^+ with Tris. We determined the minimal serosal Na^+ concentration that in the absence of serosal Ca^{2+} will allow ADH to elicit a response comparable with that obtained with 110 mM Na^+. In nonpaired hemibladders incubated in Ca^{2+}-free solutions, 11 mM serosal Na^+ sufficed to produce a maximal response. Therefore, the nominal absence of serosal Ca^{2+} lowers from ~50 to ~10 mM the serosal Na^+ concentration required for ADH to trigger a full response (see scattergram and inset in Fig. 1).

The same experiments shown in Fig. 1 (nonpaired hemibladders) were repeated for paired hemibladders. Results similar to those seen in Fig. 1 were obtained. Two effects can be defined in the blunting of the ADH-induced increase in P_u produced by reduction of serosal Na^+ (Fig. 2): a major extracellular Ca^{2+}-dependent inhibition and a lesser extracellular Ca^{2+}-independent inhibition.

Whereas the replacement of serosal Na^+ by Tris inhibited the increase in P_u produced by cAMP + IBMX by 60% (Table

Table III. Effects of Replacing NaCl with Tris-HCl in the Bathing Media on P_u

Conditions							
Mucosa	Serosa	ADH	cAMP + IBMX	P_u	Change	n	P
				nm/s	%		
Ringer's	Ringer's	–	–	14±6	−11±7	6	NS
Na^+-free	Ringer's			12±7			
Ringer's	Ringer's	+	–	321±36	+10±8	6	NS
Na^+-free	Ringer's			349±39			
Ringer's	Ringer's	–	–	21±4	+8±6	6	NS
Ringer's	Na^+-free			23±6			
Ringer's	Ringer's	+	–	347±29	−58±4	8	<0.001
Ringer's	Na^+-free			142±15			
Ringer's	Ringer's	–	+	373±34	−62±5	6	<0.001
Ringer's	Na^+-free			136±14			
Ringer's	Na^+-free	+	–	158±20	−5±4	6	NS
Na^+-free	Na^+-free			149±27			

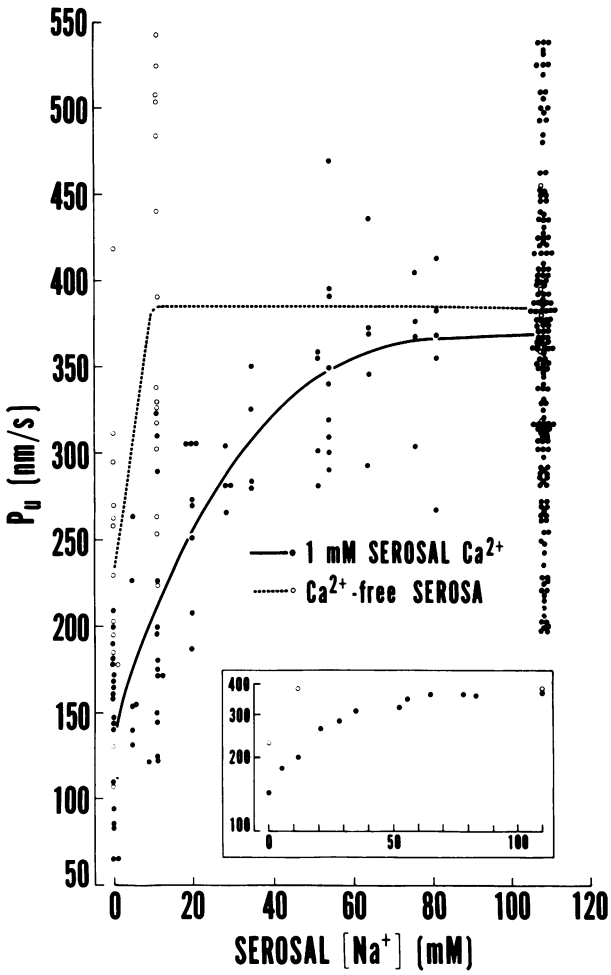

Figure 1. Concentration–response curve of urea permeability coefficients (P_u, ordinate) induced by ADH (20 mU/ml) in the presence of varying concentrations of serosal Na^+ (abscissa), with and without serosal Ca^{2+}. The experiments with serosal Ca^{2+} were carried out in 82 nonpaired hemibladders obtained from 41 toads and exposed to different concentrations of serosal Na^+ (ranging from 0 to 82.5 mM) and compared with the P_u induced by ADH in 110 mM serosal Na^+ in a sample of 173 hemibladders collected from control hemibladders used in different experiments. In no case the two hemibladders obtained from a given toad were exposed to the same low serosal Na^+ concentration, but were randomly incubated in serosal baths with different Na^+ concentrations, which were obtained by mixing full-strength Ringer's solution with Tris-Ringer's in varied proportions. The experiments performed in the absence of serosal Ca^{2+} were done with 34 hemibladders (17 toads). These hemibladders were placed at random in Tris-Ringer's with either 0 mM Na^+ (13 hemibladders) or 11 mM Na^+ (15 hemibladders) and in full-strength (110 mM Na^+) Ringer's (6 hemibladders). All hemibladders were filled with 5 ml of full-strength Ringer's and the serosal bath was repeatedly changed by switching the preparations to different beakers containing 70 ml of Ringer's stirred at 300–400 rpm. In changing from beaker to beaker, the serosal side of the hemibladders was blotted with Whatman No. 1 filter papers. Total incubation time was 60 min for each hemibladder before transfer of the preparation to an ADH-containing experimental Ringer's for 30 min. Then the hemibladders were transferred to fresh experimental Ringer's with ADH, and [^{14}C]urea was added to the mucosa (500 nCi/ml) for a 30 min-period and P_u determined under stirring (400 rpm). Before adding [^{14}C]urea, the serosal Ringer's was sampled and Na^+ concentration determined by flame photometry. The quadratic curve for the serosal Ca^{2+}-containing hemibladders was obtained by curve fitting. Interpolation through the means was done using a third-degree polynomial equation. The curve for the Ca^{2+}-free hemibladders was obtained by linking the means. (*Inset*) Semilogarithmic plot of the mean of the urea permeability coefficients. Ordinate, log P_u; abscissa, serosal Na^+ concentration.

III), the concomitant withdrawal of serosal Ca^{2+} removed this inhibition (Table IV).

EFFECTS OF THE Na^+ IONOPHORE MONENSIN. The foregoing results show that changing the Na^+ gradient across the basolateral membrane produces an inhibition of ADH action on P_u. This effect was further investigated by altering the transmembrane Na^+ gradient through the use of the monovalent cation carboxylic ionophore monensin, which has a greater equilibrium selectivity for Na^+ than for K^+ (26, 27).

In the presence of 110 mM Na^+ in the incubation media the addition of 1.4×10^{-5} M monensin to the experimental hemibladders during 60 min had no effect on basal P_u (control, 35 ± 7 nm/s; experimental, 39 ± 8 nm/s, $n = 6$). The increase in P_u elicited by ADH, on the other hand, was blunted by 55% (Fig. 3 *A*). Monensin concentrations $<10^{-5}$ M had little or no effect on the magnitude of the increase in P_u triggered by the supramaximal dose of ADH employed.

Studies were done on the dependency on serosal Na^+ of the inhibitory effect of monensin on the ADH-induced P_u (Fig. 3 *A*). Because the reduction of serosal Na^+ concentration produces an inhibition of the ADH-induced increase in P_u (Figs. 1 and 2), in these experiments both control and experimental hemibladders were incubated in serosal baths with reduced Na^+ concentrations. Monensin had no effect on the increase in P_u triggered by the hormone at concentrations of serosal Na^+ between 0 and 20 mM. The maximal inhibitory effect of monensin was achieved at concentrations of serosal Na^+ of ~50 mM, which is approximately the minimal Na^+ concentration needed by ADH to elicit a maximal increase in P_u (cf. Figs. 1 and 2).

A dependency on extracellular Ca^{2+} of the inhibitory effect of monensin was assessed. In the absence of serosal Ca^{2+}, monensin blunted the response to ADH by 26%, instead of the 55% inhibition obtained in the presence of serosal Ca^{2+} (Fig. 3 *A*). To exclude the possibility that this attenuation is the outcome of a variable monensin-induced inhibition, experiments were conducted in which a set of hemibladders was incubated with monensin in the presence of 1 mM serosal Ca^{2+} while the matching hemibladders were incubated with the ionophore in a Ca^{2+}-free serosal bath. In these experiments the P_u response to ADH was 103 ± 27% higher in the hemibladders incubated in the Ca^{2+}-free serosal bath than in those whose serosal bath contained Ca^{2+} (Fig. 3 *B*).

Monensin also blunted the response to cAMP + IBMX by 50% (Fig. 3 *C*); i.e., almost to the same extent that the response to ADH was inhibited. Differing from the inhibition of the hormonal effect, in which the ionophore still caused a 26% inhibition in the absence of serosal Ca^{2+}, the deletion of serosal Ca^{2+} removed the inhibitory action of monensin on the cAMP + IBMX-induced enhancement of P_u (Fig. 3 *C*).

Effects of diltiazem and indomethacin

DILTIAZEM. Deletion of serosal Na^+ or incubation with monensin blunted the stimulation of P_u. Part of these inhibitions

Table IV. Effect of Withdrawing Serosal Ca^{2+} on the Inhibition of Stimulated P_u Produced by Replacing Serosal Na^+ with Tris

Conditions							
Mucosa	Serosa	ADH	cAMP + IBMX	P_u	Change	n	P
				nm/s	%		
Ringer's	Ringer's	−	−	22±6	−11±6	6	NS
Ringer's	(Na^+/Ca^{2+})-free			19±8			
Ringer's	Ringer's	+	−	424±39	−35±4	6	<0.001
Ringer's	(Na^+/Ca^{2+})-free			279±32			
Ringer's	Na^+-free	+	−	132±12	+103±21	6	<0.001
Ringer's	(Na^+/Ca^{2+})-free			271±24			
Ringer's	Ringer's	−	+	333±18	+6±16	6	NS
Ringer's	(Na^+/Ca^{2+})-free			336±27			

can be cancelled by deleting extracellular Ca^{2+}, suggesting that an increased Ca^{2+} uptake by the epithelial cells is at least partially responsible for the resulting inhibition.

We tested the effects of 2×10^{-4} M diltiazem, a Ca^{2+}-channel blocker (16, 28), on these inhibitions. The results of these experiments are shown in Table V. A 30-min incubation (six bladders) or 60-min incubation (six bladders) with dialtiazem did not alter the effect of ADH on P_u. Next we incubated the experimental hemibladders with diltiazem for 30 min and then both control and experimental hemibladders were incubated with either Na^+-free serosal Ringer's or with monensin for 60 min, diltiazem being present in the experimental ones

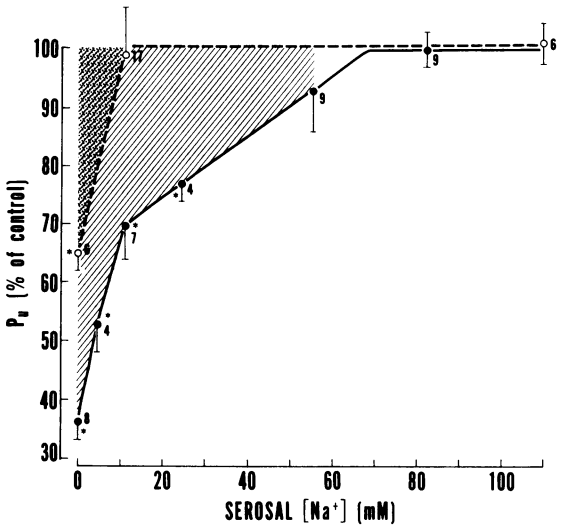

Figure 2. Dependency on serosal Ca^{2+} of the inhibition produced by reduction of the serosal Na^+ concentration (abscissa) of the urea, permeability coefficients (P_u, ordinate) induced by ADH (20 mU/ml). All control hemibladders were filled with full-strength Ringer's solution and incubated with standard Ringer's throughout. Matched experimental hemibladders were incubated with different serosal Na^+ concentrations, with or without serosal Ca^{2+}. Experimental details are identical to those described in the legend to Fig. 1. Each experimental point is the mean±standard error of mean of the number of bladders shown next to each point. Asterisks indicate significant differences ($P < 0.05$–0.001) between matched experimental and control hemibladders. Results are expressed as percent response of the experimental hemibladders as compared with the response of the control hemibladders (100%). —●—, ADH-induced P_u in the presence of serosal Ca^{2+}. - - ○ - -, ADH-induced P_u in the absence of serosal Ca^{2+}. ▨, Ca^{2+}-dependent inhibition of ADH-induced P_u produced by reduction of serosal Na^+ concentration. ▨, Ca^{2+}-independent inhibition of ADH-induced P_u produced by reduction of serosal Na^+ concentration.

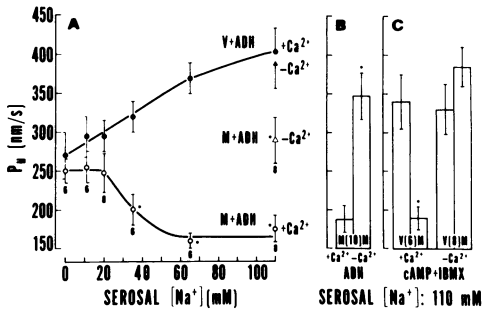

Figure 3. Effects of monensin on the increase in the urea permeability coefficients (P_u, ordinate) in toad bladder elicited by ADH or cAMP + IBMX at different concentration of serosal Na^+, in the presence or absence of serosal Ca^{2+}. (*A*) Effects of monensin at different concentrations of serosal Na^+ (abscissa). Na^+ isosmotically replaced by Tris. Control hemibladders (●) were incubated for 60 min with serosal Ringer's solution of various Na^+ concentrations and 0.1% of dimethyl sulfoxide-ethanol 1:1 (V). The matched experimental hemibladders (○) were incubated the same way but with 1.4×10^{-4} M monensin. Then ADH (20 mU/ml) was added for 30 min and urea permeability determined. In another set of experiments the effect of monensin was tested at a serosal Na^+ concentration of 110 mM, but without serosal Ca^{2+} (▲, △). Each experimental point is the mean±standard error of mean of the n indicated by the number next to the experimental hemibladders. Asterisks (*) in experimental hemibladders indicate a difference as compared with control hemibladders of a level of significance $P < 0.05$–0.001. (*B*) Control and experimental hemibladders incubated with monensin (M) in 110 mM serosal Na^+, in the presence or absence of serosal Ca^{2+}, as indicated, and challenged with ADH (20 mU/ml). Number of experiments in parentheses. $^*P < 0.001$. (*C*) Control hemibladders incubated with 0.1% dimethyl sulfoxide-ethanol 1:1 (V) and experimental hemibladders with monensin (M). Serosal Na^+ concentration: 110 mM, in the presence or absence of serosal Ca^{2+}, as indicated, and challenged with 5×10^{-3} M cAMP and 4×10^{-4} M (cAMP + IBMX). Number of experiments in parentheses. $^*P < 0.001$.

Table V. Effects of Diltiazem on ADH-induced P_u and Its Inhibition by Na^+-free Serosal Baths and Monensin‡*

Conditions					
Mucosa	Serosa	P_u	Change	n	P
		nm/s	%		
Ringer's	Ringer's	367±26	−5±3	12	NS
Ringer's	Ringer's + D	351±22			
Ringer's	Na^+-free	196±17	+3±4	8	NS
Ringer's	Na^+-free + D	199±14			
Ringer's	Ringer's + M	162±23	−7±8	12	NS
Ringer's	Ringer's + M + D	142±20			

D, diltiazem; M, monensin. * 2×10^{-4} M. ‡ 1.4×10^{-5} M.

throughout the procedure. In neither case did Diltiazem attenuate the inhibitions.

INDOMETHACIN. Another effect that the increased cytosolic Ca^{2+} activity may have is to increase the production of endogenous prostaglandins (29), which in turn may be at least partially responsible for the resulting inhibition caused by the experimental maneuvers.

To test this possibility we employed the cyclooxygenase inhibitor indomethacin, incubating the bladders with it during 120 min (with a change to a fresh bath after 60 min), before the introduction of experimental procedures, and with indomethacin being present throughout the rest of the experiment. The results obtained are displayed in Table VI.

Indomethacin did not alter the effect of ADH on P_u (Table VI, part 1). In previous experiments, the absence of serosal Na^+ produced a 60% inhibition of the ADH-induced increase in P_u (Table III) whereas in the presence of indomethacin the inhibition was 40% (Table VI, part 2). To verify that this attenuation of the inhibition was not due to variability, we compared the effect of indomethacin added to experimental hemibladders incubated in the absence of serosal Na^+ with the response of control-matching hemibladders incubated without serosal Na^+ but without Indomethacin. As seen in Table VI, part 2, indomethacin partially cancels the inhibition of the ADH-induced P_u produced by deletion of serosal Na^+ because the response of the experimental hemibladders was significantly greater than that of control hemibladders.

The same protocols shown in Table VI, part 2 were applied to the inhibitions induced by monensin (Table VI, part 3) and A23187 (Table VI, part 4). In the case of the inhibition of the ADH-triggered increase in P_u produced by the ionophores, treatment with indomethacin was more effective than in the case of the incubation with Na^+-free Ringer's solution; particularly in the blunting of the response produced by A23187, which the cyclooxygenase inhibitor almost completely removed.

Table VI. Effects of Indomethacin (10^{-5} M) on ADH-induced P_u and Its Inhibition by Na^+-free Serosal Baths, Monensin (1.4×10^{-5} M) and A23187 (5×10^{-6} M)

	Conditions					
	Mucosa	Serosa	P_u	Change	n	P
			nm/s	%		
1.	Ringer's	Ringer's	369±34	+5±8	8	NS
	Ringer's	Ringer's + I	378±24			
2.	Ringer's	Ringer's + I	450±42	−39±5	8	<0.001
	Ringer's	Na^+-free + I	271±22			
	Ringer's	Na^+-free	234±17	+13±6	9	<0.005
	Ringer's	Na^+-free + I	262±5			
3.	Ringer's	Ringer's + I	400±25			
	Ringer's	Ringer's + I + M	240±27	−34±8	8	<0.001
	Ringer's	Ringer's + M	152±24			
	Ringer's	Ringer's + I + M	209±21	+46±5	10	<0.001
4.	Ringer's	Ringer's + I	386±22			
	Ringer's	Ringer's + I + A23187	311±25	−19±7	8	<0.05
	Ringer's	Ringer's + A23187	193±28			
	Ringer's	Ringer's + I + A23187	297±21	+54±9	8	<0.001

I, indomethacin; M, monensin.

Discussion

Effects of deleting extracellular Ca^{2+}

The absence of Ca^{2+} from the mucosa in the presence of 1 mM serosal Ca^{2+} does not disrupt the epithelium, even if 1 mM EGTA is added to the mucosal Ca^{2+}-free bath. Deletion of serosal Ca^{2+}, maintaining 1 mM Ca^{2+} in mucosa, also preserves epithelial structure. The exclusion of Ca^{2+} from both mucosal and serosal compartments produces structural alterations, with the appearance of large paracellular pathways (11). If 1 mM Ca^{2+} is present in the mucosal bath and 1 mM EGTA is added to a Ca^{2+}-free serosa, the same effect as deleting Ca^{2+} from both compartments results.

When mucosal Ca^{2+} is deleted, the epithelium is capable of responding to ADH with a normal increase in the permeabilities to water, Na^+ (11), and urea. In the nominal absence of serosal Ca^{2+} ADH also produces a normal increase in water and Na^+ permeabilities (11), and ADH or cAMP + IBMX induce a full increase in urea permeation.

From these results we conclude that: (*a*) Extracellular Ca^{2+} is not required for the regulation of the effects of ADH on any of the permeation pathways. This does not exclude possible regulatory roles of Ca^{2+} bound to the basolateral membrane (which may play a role in modulating hormone-receptor interaction, adenylate cyclase activity, activation of phospholipases, synthesis of prostaglandins, etc.) and the apical membrane (which may modulate changes in permeability of water and solute pathways). In other words, at or below physiologic concentrations, Ca^{2+} in the bulk phase does not play any significant role in the activation by ADH of the permeability to water, Na^+, or urea.

(*b*) The main role of extracellular Ca^{2+} is the preservation of tissue structure. Calcium in the mucosal side may be completely absent without consequences for epithelial integrity, as long as Ca^{2+} is present in serosa. Epithelial disrupture ensues if Ca^{2+} in the serosa is absent and EGTA is added,

independently of the presence of 1 mM Ca^{2+} in mucosa. Therefore, integrity of tissue structure is maintained by Ca^{2+} in the serosal compartment, while Ca^{2+} in the mucosa is not required to preserve a normal epithelial structure. The role of serosal Ca^{2+} in preserving epithelial geometry is particularly critical during the ADH-induced increase in bulk water flow (11).

Effects of altering the transmembrane electrochemical Na^+ gradient

EFFECTS OF REDUCING THE EXTRACELLULAR Na^+ CONCENTRATION

At the mucosa. The absence of apical Na^+ does not preclude the action of ADH on P_u. Therefore, we conclude that Na^+ transport rates do not bear any relationship to the effects of the hormone on the mechanisms that trigger the increase in urea permeation.

At the serosa. Altering the transmembrane Na^+ gradients at the basolateral membrane by decreasing the Na^+ concentration below ~50 mM results in the inhibition of the increase in P_u produced by either ADH or cAMP + IBMX. Deletion of serosal Ca^{2+} in bladders incubated in the absence of serosal Na^+ partially restores the response to ADH, decreases the serosal Na^+ requirement for ADH to elicit a full response from ~50 to ~10 mM, and removes the inhibition of the effects of cAMP + IBMX. Thus, the effect of reducing the serosal Na^+ concentration on ADH action on urea permeability can be defined by:

(*a*) A serosal Na^+-dependent (serosal Ca^{2+}-independent) component. This component operates at the pre-cAMP steps, at concentrations of serosal $Na^+ < 10$ mM. The actual concentration of serosal Na^+ that produces the Ca^{2+}-independent inhibition of the pre-cAMP steps that lead to an increase in P_u still has to be tested at Na^+ concentrations between 0 and 11 mM, which we have not done; but its existence is certain inasmuch as, in the absence of serosal Na^+, a drastic reduction of the serosal Ca^{2+} concentration cannot fully restore the response. Which changes in the system are responsible for this part of the inhibition are unknown and still have to be investigated.

(*b*) A serosal Ca^{2+}-dependent (serosal Na^+-independent) component. This component operates at both pre- and post-cAMP steps at concentrations of serosal Na^+ between ~10 and ~50 mM. This effect may be due to a competition between Na^+ and Ca^{2+} for binding sites at the basolateral membrane. The Na^+ bound to the membrane would normally impede Ca^{2+} entry to the cell along the transmembrane electrochemical gradient; deletion of serosal Na^+ would facilitate Ca^{2+} entry into the cell. Another mechanism that could operate under this circumstance, and that does not exclude the one proposed above, would be the decrease in activity of a Na^+/Ca^{2+} exchange mechanism, which under normal transmembrane electrochemical Na^+ gradients would extrude Ca^{2+} from the cell (17, 24, and 25).

From this point of view, concentrations of serosal Na^+ >10 and <50 mM do not allow the hormonal effect on P_u to develop fully because within this concentration range serosal Na^+ would not be high enough to maintain a low intracellular Ca^{2+} activity. This means that in this concentration range extracellular Na^+, per se, is not required for hormonal action. Below ~10 mM serosal Na^+, this effect also takes place, but it overshadows other mechanism(s) that also inhibit ADH action but apparently are independent of extracellular Ca^{2+}. Therefore, at concentrations between 0 and ~10 mM, serosal Na^+ is, per se, a partial requirement for ADH action. Concentrations of serosal $Na^+ > 50$ mM are sufficient to maintain the response of the system to ADH, probably because this concentration is enough to impede an increase in Ca^{2+} uptake by the epithelial cells, maintaining a low intracellular Ca^{2+} activity.

We conclude that the inhibition of the effects of ADH on P_u produced by reducing the concentration of serosal Na^+ below 50 mM is caused by Ca^{2+}-dependent and Ca^{2+}-independent inhibitory mechanisms that operate at pre- and post-cAMP steps. The inhibition of the post-cAMP steps is totally serosal Ca^{2+}-dependent; the inhibition of the pre-cAMP steps is in part dependent on the presence of serosal Ca^{2+} and in part independent of its presence, the latter inhibitory mechanism being activated by concentrations of serosal Na^+ lower than 10 mM.

EFFECTS OF MONENSIN. Results similar to those obtained by reducing the serosal Na^+ concentration were found by adding the Na^+ ionophore monensin to the serosa: (*a*) inhibition of the effects of ADH and cAMP + IBMX on P_u; (*b*) a Ca^{2+}-free serosal Ringer's solution attenuated the inhibitory effect of monensin on ADH action and removed the inhibition of the response elicited by cAMP + IBMX.

We have not determined whether monensin actually produces an increase in Na^+ uptake by the epithelial cells of the toad bladder, but indirect evidence favors the idea that this is the case. The inhibition of ADH action on P_u produced by monensin is dependent on the presence of Na^+ in the serosa: the inhibition seems to be maximal at Na^+ concentration $\geq$50 mM, and no inhibitory effect could be detected at Na^+ concentration $\leq$20 mM. These results strongly point to a specific, Na^+-dependent, effect of monensin on the stimulated P_u. Thus, the inhibitory effect of monensin seems to originate in an ionophore-induced increase in Na^+ uptake by the cells.

In turn, the inhibition is completely (post-cAMP steps) or partially (pre-cAMP steps) dependent on the presence of Ca^{2+} in the serosal bulk phase. Therefore, the question is whether it is the increase in cytosolic Na^+ activity and/or an increase in Ca^{2+} influx across the basolateral membrane that ultimately are the inhibitory factors. Indirect and direct evidence leads us to conclude that monensin does indeed increase the cytosolic Ca^{2+} activity:

(*a*) In different systems, monensin elicits the activation of mechanisms, the triggering of which is attributed to increases in cytosolic Ca^{2+} activity (27).

(*b*) We found that in toad urinary bladders incubated with 110 mM serosal Na^+ the monensin-induced inhibition of ADH action on P_u is reduced by half if the ionophore is added to a serosal bath nominally free of Ca^{2+}. The same maneuver precluded the monensin-induced inhibition of the response to cAMP + IBMX.

(*c*) We tested the effects of monensin on intracellular Ca^{2+} activity in isolated epithelial cells of toad bladder. The cells were incubated with Quin-2, an indicator of cytosolic Ca^{2+} activity (30), and chlorotetracycline, a monitor of Ca^{2+} activity in organelles (31). The results showed that monensin produces a fast, large, and sustained increase in cytosolic Ca^{2+} activity. This is followed by a slow progressive increase in Ca^{2+} activity in organelles, which tends to reduce Ca^{2+} activity in the

cytoplasm, but the buffering mechanisms are not sufficient to lower the high cytosolic Ca^{2+} activity induced by monensin (Hardy, M. A., W. Jy, and D. H. Haynes, unpublished results).

In view of the results obtained, we propose the following hypothesis: monensin increases the intracellular Na^+ activity in the epithelial cells, which, in turn, increases the influx of Ca^{2+} into the cells. This raises the cytosolic Ca^{2+} activity, producing the inhibition of the pre- and post-cAMP steps.

However, the inhibition of the pre-cAMP steps produced by monensin is not totally precluded by deletion of serosal Ca^{2+}. Thus, other mechanisms, besides an increased Ca^{2+} uptake, have to be considered to explain the inhibition that remains after extracellular serosal Ca^{2+} has been omitted.

In erythrocytes monensin induces an electrostatically neutral rapid entry of Na^+ into the cells followed by a loss of intracellular K^+ and H^+. Then H^+ diffuses back into the cell in exchange for K^+. The initial diffusional cationic exchanges take place within the first 1–2 min and reflects the ionic selectivity of the ionophore; the final state of ionic equilibrium is independent of ionophore selectivity, and the transmembrane thermodynamic equilibrium would be given by (27):

$$([Na^+]_o/[Na^+]_i) = ([K^+]_o/[K^+]_i). \quad (2)$$

The result is that the intracellular Na^+ concentration has increased while the K^+ concentration decreased, the final concentrations governed by the ionic equilibrium as reflected in Eq. 2.

In principle, the transmembrane shifts of H^+ would not affect the response to ADH at the pre- or post-cAMP steps inasmuch as we have studied the effects of monensin after 60 min of incubation, i.e., at equilibrium. Still, this does not mean that the cell pH is the same as it was before the drug was added, and it has been shown that alterations of pH modify urea transport (3; cf. 4).

It is unlikely that an increase in cytosolic Na^+ activity, per se, could be the cause of the inhibition in that the activity of an ADH-sensitive adenylate cyclase of rabbit medulla is potentiated by increasing the Na^+ concentration from 0 to 110 mM NaCl (12).

On the other hand, a decrease in K^+ activity inhibits the ADH-sensitive adenylate cyclase of rabbit medulla (12). Therefore, under monensin a loss of cell K^+ could be in part responsible for the inhibition of the pre-cAMP steps.

Another alternative to explain the extracellular Ca^{2+}-insensitive inhibition of the pre-cAMP steps by monensin is that an increased cytosolic Na^+ activity may produce the release of intracellular Ca^{2+} from cytosolic storage and binding sites (cf. 21, 22, 27, and 32). In the absence of serosal Ca^{2+}, monensin would still induce an increase in cell Na^+ activity, which in turn may produce the release of Ca^{2+} from intracellular organelles and also compete with Ca^{2+}-binding sites in the cytoplasm. This could increase the Ca^{2+} activity of a compartment related to the pre-cAMP steps, inhibiting them. From this perspective, although an increased intracellular Na^+ activity would not inhibit, per se, the activation of the pre-cAMP step, it would indirectly contribute to blunt the effect of ADH on it by causing a decreased uptake, and producing release, of Ca^{2+} from intracellular stores.

In conclusion, changes in the transmembrane electrochemical Na^+ gradients at the basolateral membrane, produced by either reducing the Na^+ concentration or adding the Na^+ ionophore monensin, elicit an inhibition of the post-cAMP steps, which is totally dependent on the presence of extracellular Ca^{2+}, and an inhibition of the pre-cAMP steps, which is in part dependent on the presence of extracellular Ca^{2+} and in part dependent on other changes within the system. In the case of the inhibition of the pre-cAMP steps produced by monensin, the extracellular Ca^{2+}-independent inhibition may be caused by changes in intracellular pH, and/or a loss of cell K^+, and/or a release of Ca^{2+} from intracellular stores.

Effects of increasing the intracellular Ca^{2+} activity

The extracellular Ca^{2+}-dependent inhibitions of the pre- and post-cAMP steps obtained by altering transmembrane Na^+ gradients suggest that changes in intracellular Ca^{2+} activity are an important determinant in the regulation of hormonal action on P_u.

This contention was partially confirmed through the use of agents that increase the activity of Ca^{2+} in the cytosol. The agents employed can be divided into two groups: those that increase Ca^{2+} uptake (A23187 and carbachol) and those that increase cell Ca^{2+} activity by their action on intracellular Ca^{2+} stores (quinidine and metabolic inhibitors).

A23187 and carbachol. Both increase Ca^{2+} uptake by the epithelial cells of the toad bladder to the same extent and inhibit the ADH-induced increase in water flow, presumably by acting in the same chain of events, in that their effects are not synergistic (13).

In our experiments A23187 inhibited the pre-cAMP steps only. This effect is in agreement with the reports showing that A23187 decreases both basal and ADH-stimulated cAMP synthesis (6, 9). The inhibition was almost, but not completely, removed by the nominal absence of serosal Ca^{2+}. The A23187-induced inhibition of the pre-cAMP steps that was obtained after removal of serosal Ca^{2+} may be due to Ca^{2+} release from intracellular stores, produced by the ionophore entering the cell. Also, we cannot exclude effects of A23187, which are independent of its action as a Ca^{2+} ionophore.

Carbachol, despite that it induces an increase in Ca^{2+} uptake of similar magnitude to that obtained with A23187 (13), had no effect on the action of ADH on P_u.

Quinidine and metabolic inhibitors. These agents increase cytosolic Ca^{2+} activity by releasing Ca^{2+} from, and decreasing its uptake by, intracellular stores (14, 17, 19, 20–23). Metabolic inhibitors may additionally increase cellular Ca^{2+} activity by depressing Ca^{2+} extrusion across the plasma membrane (21).

Quinidine produced an inhibition of the pre-cAMP steps only, inhibition that was in great part removed by deletion of extracellular Ca^{2+}. The metabolic inhibitors DNP and IAA had no effect on the action of ADH on P_u.

The different effects obtained with these agents are contradictory in terms of what might be expected from their mode of action.

Judged exclusively from the effects on Ca^{2+} uptake, A23187 and carbachol should affect the stimulation of P_u in a similar way, and they did not. One possible explanation is that the Ca^{2+} ionophore and carbachol may increase equally the Ca^{2+} content of the cells, but the cytosolic Ca^{2+} activity increases in different compartments: with A23187 in one that produces the inhibition of the pre-cAMP steps, whereas through carbachol in another that affects neither pre- nor post-cAMP steps. For example, although both A23187 and carbachol increase the uptake of Ca^{2+} by the cells to the same extent, A23187 may

additionally hamper the Ca^{2+}-buffering capacity of the cell but carbachol will not. The result may be that in the cells the Ca^{2+} concentration has increased equally with both treatments, but the Ca^{2+} activity in the cytosol increases more with A23187 than with Carbachol. This speculation should be tested through different procedures.

Other contradictory results were obtained with quinidine, DNP, and IAA. All these agents release Ca^{2+} from intracellular storage sites in toad bladder and different kinds of cells. In principle, all these compounds should have produced equivalent modifications of ADH action on P_u, and they did not.

The effect of quinidine presents a particularly puzzling situation. Quinidine does not increase Ca^{2+} uptake by the cells, but increases cytosolic Ca^{2+} activity by inducing its release from intracellular pools (14). We have confirmed these assertions in isolated epithelial cells of toad urinary bladder using Quin-2 and chlorotetracycline as fluorescent probes of intracellular Ca^{2+} activity (see above): quinidine decreases Ca^{2+} activity in intracellular organelles and elicits a large and sustained increase in cytosolic Ca^{2+} activity (Hardy, M. A., W. Jy, and D. H. Haynes, unpublished results). Accordingly, the inhibitory effect of quinidine should not be modified by the nominal absence of extracellular Ca^{2+}; but Ca^{2+} deletion reduced the quinidine-induced inhibition of the pre-cAMP steps from 88% to 24%. There is no available explanation for this finding, but a hypothesis can be presented. The extracellular Ca^{2+}-independent 24% inhibition would be produced by the increase in intracellular Ca^{2+} activity induced by quinidine; the remainder of the inhibition, which is dependent on extracellular Ca^{2+} and accounts for almost 65% of the effect, might be caused by a direct action of quinidine on the basolateral membrane. The latter contention is supported by our data obtained in erythrocytes (33) and data obtained in frog skin (34). Accordingly, extracellular Ca^{2+} may act as a "cofactor" in the action of quinidine on the membrane and this would be the reason why its presence is required for a full inhibitory effect.

In conclusion, the use of agents that increase Ca^{2+} activity within the cells presents seemingly contradictory results. At the same time they may show that in order for intracellular Ca^{2+} to alter the stimulation of P_u an increase in cytosolic Ca^{2+} activity, per se, may not suffice, and perhaps what is more important is the Ca^{2+} source that produces the increase and in which compartment it increases. Therefore, the regulation by intracellular Ca^{2+} of ADH action on P_u may be highly compartmentalized.

Effect of diltiazem on the Ca^{2+}-mediated inhibition of P_u

In that the inhibition of the stimulation of P_u produced by deletion of serosal Na^+ or by monensin is precluded or diminished by removal of serosal Ca^{2+}, we decided to test the effect of the Ca^{2+}-channel blocker diltiazem on these maneuvers.

At the concentration used, diltiazem blunts the effects of ADH on water permeability by interfering with the activation of adenyl cyclase (16). In our experiments, this effect did not modify the action of ADH on P_u. The inhibitions caused by Na^+ absence or by addition of monensin were not affected by diltiazem. Apparently diltiazem does not interact with the Ca^{2+} channels through which Ca^{2+} enters the cell when the transmembrane electrochemical Na^+ gradient is changed.

Effect of indomethacin on the Ca^{2+}-mediated inhibition of P_u

Prostaglandins inhibit the ADH-induced increase in water permeability (29, 35) by acting at pre- and post-cAMP steps (35); their synthesis can be activated by Ca^{2+} (29). Through the use of indomethacin, we indirectly tested the possibility that the extracellular Ca^{2+}-dependent inhibitions may be mediated by endogenous prostaglandins.

The indomethacin concentration (10^{-5} M) and time of incubation (120 min) employed completely abolishes prostaglandin E_2 production (29). Prostaglandin E_2 inhibits ADH action on both water permeation and P_u, though it is much more effective on the former than on the latter (2). In our experiments, indomethacin did not modify the action of ADH on P_u, while it enhanced the ADH-induced increase in water permeability by 45% (result not shown). These differences are similar to those obtained with naproxen (2).

In the presence of indomethacin, the inhibitions produced by serosal Na^+-free Ringer's solution or monensin were partially removed, but the inhibition produced by A23187 was almost completely nullified.

These results suggest that endogenous prostaglandins are inhibitory and seem to be important determinants of the Ca^{2+}-mediated inhibitions of the ADH-induced P_u. It is possible that indomethacin cannot completely prevent the blunting of the response caused by Na^+ deletion or monensin because, as discussed previously, other mechanisms that are independent of extracellular Ca^{2+} seem to play a role in the inhibition and only the Ca^{2+}-dependent part may be the indomethacin-sensitive fraction of the effect. The inhibition produced by A23187 probably is mostly caused by an increased Ca^{2+} uptake and that may be why the effect is almost totally prevented by indomethacin.

The results obtained with indomethacin must be interpreted with caution, and should not be directly and solely related to effects on synthesis of endogenous prostaglandins. Indomethacin may have additional effects unrelated to prostaglandin biosynthesis, and we have not experimentally determined prostaglandin production under the different experimental procedures employed. To our knowledge an increased prostaglandin biosynthesis has been demonstrated with A23187 (29), but not with low serosal Na^+ or with monensin.

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