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Research Article

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Effects of Ionophore A23187 on Base-Line and Vasopressin-Stimulated Sodium Transport in the Toad Bladder

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ABSTRACT The cation specific ionophore A23187 (Io) is a useful tool for studying the role of intracellular Ca^{++} (Ca^{++})i in physiologic processes. The present studies explore the role of (Ca^{++})i on Na transport in the toad bladder. Scraped bladder cells exposed to 1 μM Io for 60 min took up 100% more ^{45}Ca than control cells. Io, 1 μM, added to the serosal side of bladders incubated in standard Ringers containing 2.5 mM Ca^{++} inhibited short circuit current (SCC) values by a mean of 30% at 60 min and 50% at 90 min. Io did not inhibit SCC significantly in bladders incubated in Ringers containing 0.2 mM Ca^{++}. These data indicate that the effects of Io on SCC depend on the levels of external Ca^{++} and suggest that entry of Ca^{++} into cells mediates the inhibition of base-line SCC. Preincubation of the bladders with either lanthanum chloride or pentobarbital prevented the increased ^{45}Ca uptake produced by ionophore as well as the inhibition of SCC caused by the antibiotic. Vasopressin, antidiuretic hormone (ADH), 10 mU/ml, increased peak SCC by 247% in bladders preincubated for 1 h in Ringers with 2.5 mM Ca^{++} and 1 μM Io and by 318% in control bladders ($P < 0.01$). Bladders exposed to 1 μM Io in Ringers with 0.2 mM Ca^{++} had an increase in SCC after ADH comparable to that observed in controls. Since the effects of ADH on SCC are mediated by cyclic AMP, we tested the effects of Io on cAMP production by scraped toad bladder cells. ADH increased cAMP from 8 to 30 pmol/mg protein in controls but it did not increase cAMP over base-line values in the presence of Io when the Ringers contained 2.5 mM Ca^{++}. Io did not inhibit cAMP production in response to ADH when the Ca^{++} in the Ringers was 0.2 mM. The results indicate that Io inhibits base-line and ADH stimulated SCC by increasing (Ca^{++})i or Ca^{++} bound to the cell membrane. It is suggested that: (*1*) (Ca^{++})i or membrane-bound Ca^{++} plays a key role in base-line and ADH stimulated Na transport in the toad bladder; (*2*) inhibition of ADH stimulated SCC may be due in part to decreased cAMP generation in response to ADH when (Ca^{++})i or membrane-bound Ca^{++} levels are increased.

INTRODUCTION

Previous studies designed to investigate the effects of calcium on sodium and water transport across epithelial membranes were performed by changing the concentration of Ca^{++} in the bathing medium (1–5). Calcium concentrations of 10 mM in the solution bathing the serosal surface of the toad bladder (1, 2) were found to inhibit vasopressin-stimulated osmotic water flow by approximately 65%, but had no effect on the base-line short circuit current or on the vasopressin-stimulated increase in short circuit current (a measure of net sodium transport). Changes in extracellular calcium concentrations may modify cell function, through possible alterations in intracellular calcium concentration, or by direct effects of external calcium on intercellular channels, or on the membrane (6). However, changes in extracellular calcium may not significantly alter cytoplasmic calcium levels due largely to the low permeability of the cell membrane to calcium and to other mechanisms that maintain cytoplasmic calcium concentrations at levels approximately 1,000 times lower than those in the extracellular medium (6). The various mechanisms that function to maintain Ca^{++} concentrations low in the cytoplasm have made it difficult to study the role of intracellular calcium in enzyme and transport regulation in intact

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tissues. Inhibitory effects of calcium on broken cell enzyme preparations (adenyl cyclase, Na-K ATPase) have strongly suggested a regulatory role for intracellular calcium (7, 8). In an effort to study the effects of changes in intracellular calcium on sodium transport across the toad bladder we have tried to modify the levels of cell calcium by utilizing a recently discovered cation-specific ionophore, A23187. This antibiotic has proven to be an important tool in the investigation of the role of intracellular calcium concentrations in cell function in various tissues (9–11). The ionophore A23187 is capable of chelating calcium or magnesium and to affect a marked increase in the uptake of cytoplasmic calcium in numerous tissues which have been studied (9–12). In contrast to the results obtained in the toad bladder when extracellular calcium concentration was increased to 10 mM (see above), we found in the present experiments that addition of the ionophore to the bladder inhibits basal and ADH stimulated sodium transport presumably by increasing intracellular calcium. These effects were markedly blunted when the concentration of calcium in the external bathing medium was decreased from 2–2.5 to 0.2 mM suggesting an important physiologic role for cytoplasmic calcium or membrane-bound calcium in the regulation of basal and antidiuretic hormone (ADH)[1] stimulated sodium transport.

METHODS

All experiments were performed by using the urinary bladder of toads (*Bufo marinus*) of Mexican origin. The animals, obtained during the months of November through May, were kept at room temperature in a tub containing running tap water. The toads were double pithed and in all experiments, before the bladder was removed, the animals were perfused with a Ringer's solution containing either 0.2 or 2.5 mM calcium by injecting the Ringers into the heart and allowing the blood to drain from the liver. The solution used was a standard amphibian Ringers, pH 8.0, of the following composition (in mM/liter): NaCl 112–115, KCl 2.5, $NaHCO_3$ 2.5, glucose 5 mM, and 0.2 or 2.5 mM calcium depending upon the experimental conditions. Magnesium was deleted from the Ringers in all experiments. Experiments involving the measurement of short circuit current (SCC) and potential difference were performed by mounting paired hemibladders in standard Ussing Lucite chambers with an aperture of 7 cm^2. The bladders were continuously bubbled with compressed air and were continuously short circuited. Potential difference measurements were recorded at 5-min intervals. In all experiments the bladders were allowed to stabilize for 2–3 h before the addition of the experimental agents.

Determination of ^{45}Ca uptake. In experiments designed to study the effects of ionophore on calcium uptake the epithelial cells were scraped from quarter bladder segments which had been incubated for 90 min in Ringers containing 0 mM Ca^{++} and 5 mg/ml of collagenase. The isolated cells were suspended in Ringer's solution containing 2 mM calcium and preincubated at 27°C for 10, 30, or 60 min with diluent, dimethyl sulfoxide (DMSO) or 1 μM ionophore before the pulse addition of 0.2–0.5 μCi of $^{45}CaCl_2$. In additional experiments, cells were preincubated for 60 min with 1 μM ionophore alone, or ionophore plus 1 μM pentobarbital, or 50 μM lanthanum chloride. Incubation was continued for an additional 5 min after the addition of the radiolabel and the calcium uptake was stopped by adding 20 ml of cold Ca-free Ringers containing 10 mM ethylene glycol-bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetate (EGTA). The cell pellet was obtained by centrifugation at 270 *g* for 2 min at 4°C with a Sorvall model RC2-B. (Ivan Sorvall, Inc., Norwalk, Conn.) The cells were then washed three times with 30 ml of cold calcium-free Ringers containing 10 mM EGTA. The final cell suspension was placed on Millipore filter disks for separation, the disk containing the cells was then transferred to scintillation vials, and the tissue dissolved overnight at room temperature in 1 ml of 1 N NaOH. Portions were removed for protein determination. The dissolved tissue was then neutralized with 200 μl of 5 N HCl and the mixture was suspended in 10 ml of ScintiVerse. The calcium radioactivity was determined in a liquid scintillation spectrophotometer (model 3390, Packard Instrument Co. Inc., Downers Grove, Ill.). Appropriate quenching corrections were made. Specific activity was obtained by determining ^{45}Ca radioactivity on samples of the incubation medium and by determining the concentration of "cold" calcium by atomic absorption spectrophotometry. Determination of radioactive calcium in the final wash solution revealed that 99% of the radioactivity determined was calcium associated with the cells which could not be removed.

Determination of cyclic AMP levels. In experiments designed to measure the production of cyclic AMP, the bladders were preincubated at 27°C for 1 h in standard Ringers containing either 0.2 mM or 2.0 mM calcium plus 1 μM ionophore or diluent (DMSO). At the end of the 60-min preincubation the bladder sections were then transferred to flasks containing the same Ringers plus 10 mM theophylline. Other flasks contained in addition 10 mU/ml of Pitressin (Parke, Davis & Co., Detroit, Mich.). The incubation was then continued for an additional 30 min at which time the segments were removed, and epithelial cells were scraped free of the supporting tissue into 2 ml of standard Ringers solution containing 10 mM theophylline. The cell suspension was then boiled for 2 min before homogenization in a glass Teflon homogenizing tube. Portions of the cell suspension were then obtained for protein determination by the method of Lowry et al. (13). The remainder was centrifuged at 6,000 *g* for 15 min and the cyclic AMP content in the supernate was determined on duplicate samples by the competitive protein binding assay method of Gilman (14).

Reagents. The A23187 ionophore was a generous gift of Dr. Robert Hamill, Eli Lilly and Company, Indianapolis, Ind. Ionophore was dissolved in DMSO and prepared fresh daily for each experiment. In all instances, DMSO was added to control flasks. Cyclic AMP, pentobarbital, and collagenase were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Pitressin from Parke Davis & Co. Lanthanum chloride was obtained from Fisher Scientific Co. (Pittsburgh, Pa.), and ^{45}Ca from New England Nuclear (Boston, Mass.).

RESULTS

Effects of ionophore on ^{45}Ca uptake by toad bladder epithelial cells. Table I shows the accumulation of

[1] *Abbreviations used in this paper*: ADH, antidiuretic hormone; DMSO, dimethyl sulfoxide, EGTA, ethylene glycol-bis (β-aminoethyl ether) *N,N,N', N'*-tetraacetate, GMP, guanosine 5'-monophosphate; SCC, short circuit current.

TABLE I
Effects of 1 μM Ionophore on ^{45}Ca Uptake by Toad Bladder Epithelial Cells

	A	B	C
	nmol/5 min/mg protein		
Control	4.98±0.49	5.21±0.54	6.13±0.69
Ionophore	4.73±0.55	9.28±1.51	12.89±1.71

The values are the mean±SEM for six experiments. Epithelial cells were incubated for 10, 30, or 60 min (A, B, C) in Ringers with ionophore or diluent, DMSO (control) before the addition of ^{45}Ca. Mean values for ionophore were significantly different from control at 30 and 60 min ($P < 0.01$).

^{45}Ca by toad bladder epithelial cells preincubated with 1 μM ionophore or an identical concentration of DMSO (the diluent) for 10, 30, or 60 min. Radiolabeled Ca was then added and 5 min later Ca^{++} uptake was stopped by the addition of cold EGTA. A significant increase in the accumulation of ^{45}Ca was seen in bladder cells exposed to ionophore for 30 and 60 min. This radioactive calcium was probably associated with the bladder cells per se, since it could not be removed by solutions containing 10 mM EGTA. The exact location of the radioactive calcium accumulation, of course, cannot be determined from these experiments. Since the preincubation, however, was performed in Ringers containing 2 mM calcium, it seems unlikely that the ^{45}Ca accumulation measured was mainly due to nonspecific binding since these sites were presumably already occupied.

Effects of ionophore on SCC. Fig. 1 depicts the effects of adding 1 μM ionophore on SCC and potential difference in a single hemibladder mounted in standard Ringer's solution containing 2.5 mM calcium. The ionophore was added to the serosal side after a prolonged and stable base-line control period. An equal concentration of the diluent (DMSO) added to the control hemibladder was without effect on the SCC or potential difference. Ionophore (1 μM) produced a small increase in SCC, approximately 10–15 min after the addition of the antibiotic. This increase in SCC did not persist and was followed approximately 20 min later by the initiation of a gradual decline in SCC over the next 40–50 min. Ionophore also resulted in a fall in potential difference values which paralleled the decrease in SCC. This was a characteristic response in over 90% of the bladders tested in this manner. Addition of the ionophore to the mucosal media had no effect on SCC. Likewise, experiments in which the bathing media was changed on several occasions in an attempt to reverse the effect of the ionophore were unsuccessful, once the bladder had been exposed to the antibiotic.

Fig. 2 summarizes the effects of serosal addition of 1 μM ionophore on SCC in 12 matched hemibladders exposed to Ringers containing either 0.2 or 2.5 mM Ca^{++}. SCC is expressed as a percent of the mean control SCC recorded during stable readings for 30–45 min before the addition of the antibiotic. Ionophore produced a progressive decline in mean SCC in the 12 bladders mounted in Ringers containing 2.5 mM calcium. The fall in SCC started approximately 30 min after addition of the ionophore and reached levels of approximately 50% of control SCC values 80–90 min after the antibiotic was added. Since the A23187 ionophore is a relatively specific cationic antibiotic, known to affect the transport of both calcium and magnesium (12), we explored the role of changes in calcium concentration on the physiologic effects of the ionophore per se. Since magnesium was excluded from the Ringers bathing the bladder, it seems unlikely that this cation was responsible for the effects observed. When 1 μM ionophore was added to bladders exposed to Ringers containing 0.2 mM Ca^{++}, a concentration of Ca^{++} which had little effect on base-line SCC over the period of observation, there was a slight fall in SCC but not as marked as that seen in the bladders incubated in Ringers containing 2.5 mM Ca^{++}. The decline in SCC produced by ionophore in the low calcium-containing Ringers was not significantly different from the change in SCC observed in control hemibladders exposed to DMSO alone. The fall in SCC produced by ionophore was significantly different ($P < 0.05$–<0.01) at 45 min and subsequent time intervals after addition of the antibiotic in bladders exposed to Ringers containing 2.5 mM Ca^{++} than in those exposed to Ringers containing 0.2 mM Ca^{++}. The results, therefore, suggest that the inhibitory effect of ionophore on SCC and potential difference

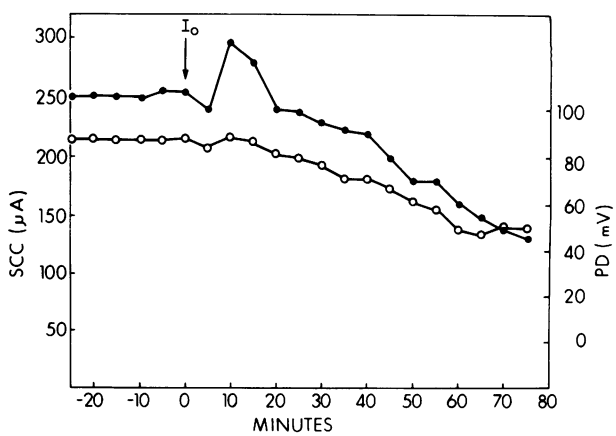

FIGURE 1 Effect of serosal addition of 1 μM ionophore (Io) on SCC (closed circles) and potential difference (open circles) in a single bladder mounted in standard Ringer's solution containing 2.5 mM Ca.

in the toad bladder is related to the concentration of external Ca^{++}.

Effects of pentobarbital and lanthanum chloride on the inhibition of SCC produced by ionophore. To further explore the role of calcium in the inhibition of SCC produced by ionophore, we examined the effects of addition of lanthanum chloride or pentobarbital. Both lanthanum chloride and pentobarbital have been shown to interfere with calcium fluxes in nervous tissue (15, 16). Pentobarbital also seems to affect Ca^{++} fluxes in myocardium (17). The effects of pentobarbital are presumably mediated by inhibiting the movement of Ca^{++} across "specific" channels (16, 18), whereas recent evidence suggests that lanthanum chloride may displace calcium from the ionophore chelating matrix and thereby prevent its uptake (19). Neither of these substances at the concentrations used (1 μM for pentobarbital, 50 μM for lanthanum chloride) had any effect on SCC or potential difference when added alone.

Fig. 3 compares the effects of adding ionophore alone on SCC, at a Ca^{++} concentration of 2.5 mM, with the results obtained when either pentobarbital or lanthanum chloride were present before addition

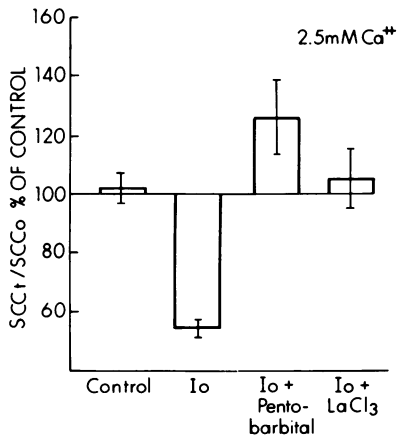

FIGURE 3 Mean values for the ratio of SCC (SCC_t) observed after 60 min of incubation with DMSO, ionophore, ionophore plus pentobarbital, or ionophore plus lanthanum chloride as compared to mean SCC values obtained during 30–45 min before the addition of the different compounds. The addition of DMSO (control) did not affect SCC_t/SCC_o. Addition of ionophore alone produced a decrease in SCC_t/SCC_o values to 54% of control. Both pentobarbital and lanthanum chloride addition together with ionophore prevented a decrease in SCC. Values are the mean and standard error of six experiments.

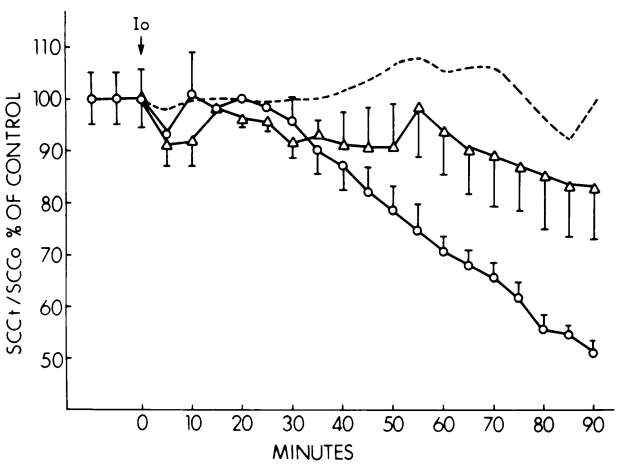

FIGURE 2 Effects of serosal addition of 1 μM ionophore on SCC in 12 matched hemibladders exposed to Ringers containing either 0.2 mM Ca (open triangles) or 2.5 mM Ca (open circles). SCC after ionophore (SCC_t) is expressed as a percent of the mean control SCC recorded during stable readings for 30–45 min before the addition of ionophore (SCC_o). The broken line represents the changes seen in hemibladders in which only DMSO (the diluent) was added. The changes in SCC produced by ionophore in bladders incubated in Ringers containing 0.2 mM calcium (open triangles) were not significantly different from those observed in bladders exposed to DMSO. On the other hand, bladders exposed to ionophore in the presence of 2.5 mM external calcium showed a significant decrease ($P < 0.001$) in SCC (open circles) as compared to DMSO treated bladders or bladders exposed to ionophore and 0.2 mM calcium. Values are mean±SEM.

of the ionophore. The SCC values are expressed as a ratio of the SCC values obtained for 60 min before the addition of ionophore or diluent (DMSO). Addition of ionophore alone inhibited SCC values by approximately 46% after 60 min of incubation. Incubation of the bladders with 1 μM pentobarbital or 50 μM lanthanum chloride for 60 min before the addition of ionophore, however, completely abolished the inhibitory effect of the antibiotic on SCC.

Effect of pentobarbital and lanthanum chloride on Ca^{++} uptake. Table II shows the effects of 1 μM pentobarbital or 50 μM lanthanum chloride on the increased uptake of ^{45}Ca produced by ionophore in toad bladder epithelial cells. Both pentobarbital and lanthanum chloride blocked the increased uptake of ^{45}Ca observed with ionophore.

Effect of ionophore on ADH-stimulated SCC. In vitro experiments with broken cell preparations have suggested that activation of the adenyl cyclase system, responsible for the generation of cyclic AMP, by ADH is profoundly inhibited over a calcium range of 10–100 μM (7). Stimulation of enzyme activity in intact cells, however, may not be affected by external calcium concentrations 1,000 times greater than those necessary to demonstrate inhibition in broken cell preparations. This suggests that the cytoplasmic calcium concentration, not the extracellular calcium concentration, plays a regulatory role in the activity of this enzyme over a fairly narrow range of concentrations. If the effect of the ionophore is to increase cyto-

TABLE II

Effects of 1 μM Pentobarbital or 50 μM Lanthanum Chloride on the Ionophore-Stimulated Uptake of ^{45}Ca by Toad Bladder Epithelial Cells

Control	Ionophore	Ionophore + pentobarbital
5.86±1.56	11.22±2.13*	7.35±0.77‡
Control	Ionophore	Ionophore + lanthanum chloride
5.44±1.11	14.50±3.78*	7.62±1.07‡

The values in nmol/5 min per mg protein are the mean±SEM of four experiments. Epithelial cells were incubated for 60 min in Ringers containing diluent, DMSO (control), or 1 μM ionophore alone, or 1 μM ionophore plus pentobarbital or lanthanum chloride before the addition of ^{45}Ca.

* Values significantly different from control values ($P < 0.01$).

‡ Values not significantly different from control values.

plasmic calcium concentrations, the antibiotic would be expected to inhibit ADH-stimulated adenyl cyclase activity and decrease the production of cyclic AMP. To examine this question we studied the effects of ADH on the SCC of control bladders, exposed to diluent (DMSO), and of bladders preincubated for 1 h with 1 μM ionophore.

Fig. 4 depicts the mean peak response in SCC, expressed as a percent of control, in 12 matched hemibladders after addition of 10 mU/ml of ADH to the serosal media. The increase in SCC in control bladders averaged 318%, a value significantly greater than the mean value of 247% obtained in bladders exposed to 1 μM ionophore for 90 min before the addition of ADH. These results were obtained in bladders incubated in a Ringers containing 2.5 mM Ca^{++}. However, when the calcium concentration of the bathing medium was reduced to 0.2 mM there was no significant difference in the response to ADH between control and ionophore treated bladders. These results again suggest that the inhibitory action of ionophore of the ADH stimulated SCC in the toad bladder is affected by the concentration of external calcium.

Effect of ionophore on cyclic AMP-stimulated SCC. The maximal response in SCC, expressed as a percent of control values, after addition of 50 μM dibutyryl cyclic AMP was measured in bladders previously exposed for 60–90 min to 1 μM ionophore or diluent (DMSO) in Ringers containing 2.5 mM calcium. The results are presented in Fig. 5. There was no difference in the increment in SCC produced by cyclic AMP in bladders exposed to ionophore vs. control bladders. These data again suggest that the inhibition of ADH-stimulated SCC produced by ionophore is mediated, at least in part, by decreased generation of cyclic AMP in response to ADH.

The effect of ionophore on the generation of cyclic AMP in scraped epithelial cells. The cells scraped from quarter bladder segments pretreated with ionophore for 1 h and then exposed to 10 mU/ml of vasopressin for 30 min in Ringers containing 2.5 mM Ca^{++} and 10 mM theophylline, had significantly ($P < 0.01$) lower levels of cyclic AMP than cells obtained from control bladder segments. However, when the incubation medium contained 0.2 mM Ca there was no statistical difference in the levels of cyclic AMP in response to ADH in control vs. ionophore-treated bladders. The results of these experiments are summarized in Fig. 6.

DISCUSSION

The divalent cation specific ionophore A23187 is capable of effecting the transport of calcium and magnesium across numerous tissues including artificial lipid bilayer membranes (9–12). We have utilized this antibiotic in an effort to explore the role of changes in intracellular calcium on the physiologic parameters governing base-line and ADH-stimulated SCC in the toad bladder. Since the Ringers used contained no magnesium, we have assumed that the effects observed are the result of ionophore mediated changes in calcium influx rather than magnesium entry into cells. However, it is somewhat more difficult to exclude a role of decreased intracellular magnesium concentrations in mediating the effects observed with ionophore. A priori, it would not be expected that mag-

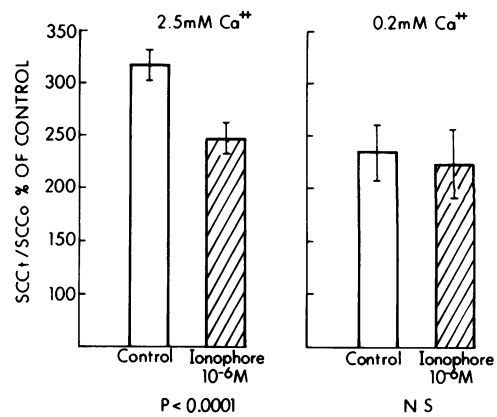

FIGURE 4 Peak increase in SCC expressed as a percent of base-line SCC (SCC_o) in 12 matched hemibladders after addition of 10 mU/ml of ADH to the serosal medium. Bladders were preincubated with ionophore or DMSO (control) before the addition of ADH in Ringers containing 2.5 or 0.2 mM calcium. Ionophore, 1 μM, blocked significantly the increase in SCC produced by ADH when external Ca was 2.5 mM. In bladders exposed to 0.2 mM external calcium the rise in SCC produced by ADH was less than that observed in bladders exposed to 2.5 mM calcium. However, at Ca concentrations of 0.2 mM ionophore did not inhibit the increase in SCC produced by ADH. Values are mean±SEM.

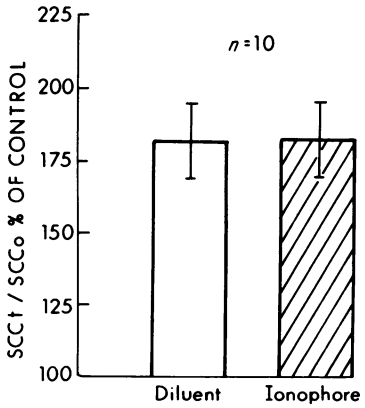

FIGURE 5 Peak increase in SCC (SCC_t) expressed as a percent of base-line values (SCC_0) after addition of 50 μM dibutyryl cyclic AMP in 10 matched hemibladders exposed to 1 μM ionophore or diluent (DMSO) in Ringers containing 2.5 mM calcium. There was no significant difference in the increment of SCC produced by cyclic AMP between control and experimental bladders. Values are mean±SEM.

nesium efflux (loss) from cells would be greater in Ringers containing 2.5 mM calcium than in Ringers containing 0.2 mM calcium. Furthermore, preliminary experiments in which isolated bladder epithelial cells were incubated in small volumes of magnesium-free Ringers with or without 1 μM ionophore for 1 h failed to reveal an increase in the concentration of magnesium in the Ringers.

The results of ^{45}Ca uptake experiments clearly demonstrate that the ionophore is capable of stimulating calcium accumulation by toad bladder epithelial cells. By preincubating the cells in normal calcium containing Ringers and only exposing the cells to the ^{45}Ca for 5 min, we believe to have essentially eliminated a major contribution of nonspecific binding of the radioactive label as well as an effect of ionophore on the "backleak" of calcium. The fact that the 5-min uptake of calcium was greater after 30 and 60 min of incubation with ionophore as compared to 10 min of incubation suggests that the observed effect represents an increase in the accumulation of calcium rather than a simple exchange of radioactive calcium for cold calcium contained within the cell. From our data, however, we cannot exclude the possibility that the accumulation of calcium occurs in the membrane itself and not in the cytoplasm or other intracellular structures such as mitochondria. The inhibitory effects of ionophore on SCC, observed after the serosal addition of this substance, followed a time-course similar to the observed increase in radioactive calcium uptake. That is, the inhibition of SCC occurred after approximately 30 min of exposure to the ionophore. Furthermore, the inhibitory effect of ionophore on SCC was almost completely abolished when the external Ca^{++} concentration was 0.2 mM Ca, strongly suggesting a role for external calcium concentrations in the mediation of the ionophore effects. When we examined the effects of pentobarbital or lanthanum chloride on the inhibitory action of ionophore on SCC, we observed no effect of the antibiotic on SCC. Both pentobarbital and lanthanum chloride blocked the increased uptake of ^{45}Ca produced by ionophore in isolated toad bladder epithelial cells. These findings, therefore, support the concept that the effects of ionophore on SCC are mediated by the entry of calcium into cells. Our data, furthermore, suggest an effect of ionophore-mediated calcium flux on the generation of cyclic AMP in response to ADH. Ionophore, at calcium concentrations of 2.5 mM, partially inhibited the stimulation of SCC produced by ADH. However, when the calcium concentration of the Ringers was 0.2 mM, ionophore did not affect the stimulation of SCC produced by ADH. Likewise, the generation of cyclic AMP was similarly affected. At Ca^{++} concentrations of 2.5 mM the generation of cyclic AMP in response to ADH was significantly decreased by ionophore. On the other hand, the antibiotic did not affect cyclic AMP production in response to ADH when the external Ca^{++} concentration was 0.2 mM. The fact

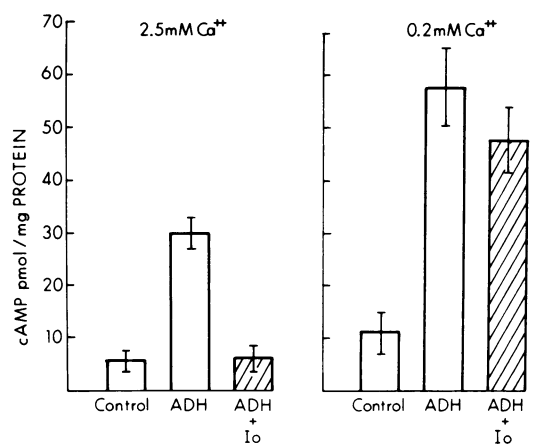

FIGURE 6 Cyclic AMP levels in epithelial cells of bladders before (control) and after exposure to 10 mU/ml of vasopressin for 30 min in Ringers containing 10 mM theophylline and 2.5 or 0.2 mM calcium. A significant decrease in cyclic AMP production in response to ADH was observed in bladders pretreated with ionophore when the external calcium concentration was 2.5 mM. However, exposure to ionophore did not significantly decrease the production of cyclic AMP in response to ADH in bladders incubated in 0.2 mM Ca. The differences in cyclic AMP levels after ADH between bladders exposed to 2.5 mM Ca and 0.2 mM Ca in the absence of ionophore probably represent seasonal variations in toads. Additional experiments conducted in a single batch of toads showed a similar increase in cyclic AMP after ADH at external calcium concentrations of 0.2 or 2.5 mM. This suggests that the differences in cyclic AMP levels seen in the experiments reported here are not due to changes in external calcium. Values are mean±SEM.

that the increase in SCC in response to dibutyryl cyclic AMP was identical in both ionophore-treated and control bladders suggests strongly a calcium-dependent inhibitory role of ionophore on the generation of cyclic AMP. The present findings are somewhat different from those reported by previous investigators who examined the effects of increasing the external calcium concentration on base-line or ADH-stimulated SCC (1, 2). The difference may be related to possible effects of high concentrations of external calcium on intercellular channels and (or) cell membrane function or to the fact that only ionophore effectively mediates an increase in the uptake of calcium from the external bathing medium into the cell interior. The ionophore presumably mediates this increased Ca uptake by either chelating calcium ions and transporting the ion across the lipid bilayer structure into the cell interior or by modifying calcium channels per se and allowing calcium to diffuse into the cell interior along the concentration gradient which normally exists between the external bathing medium and the cell cytoplasmic calcium. While we cannot precisely demonstrate the exact location of the calcium accumulation in these isolated toad bladder epithelial cells, it seems likely from our data that the effect of ionophore is mediated by increased calcium accumulation by the cell from the external media rather than by the release of calcium from mitochondrial stores which may also result in an increase in cytoplasmic calcium concentration. The mechanisms by which the ionophore-mediated calcium uptake has an inhibitory effect on baseline SCC and ADH-stimulated SCC is only speculative at this time. On the basis of in vitro enzymatic studies with the Na-K ATPase and the hormone sensitive adenyl cyclase, it appears that calcium may have a direct inhibitory effect on these enzymes either through an allosteric effect on a membrane-enzyme relationship or through the displacement of other cofactors necessary for enzymatic activation such as magnesium (7, 8). Possibilities might include an effect of changes in intracellular calcium on the permeability to sodium and potassium across the resting membrane, with a resultant decrease in activity of the sodium pump. Large increases in cytoplasmic calcium may have effects on mitochondrial membrane function and could affect the metabolic machinery of the cell itself. This latter possibility seems less likely, however, in view of the fact that the time-course for the inhibition by ionophore is much faster than that necessary for depletion of cell ATP stores. In addition, the increase in SCC, an energy-dependent process, in response to dibutyryl cyclic AMP was unaltered in the presence of ionophore, thereby suggesting that the effects of ionophore are not mediated through an interference with energy metabolism at the level of mitochondria.

It is also possible that changes in intracellular calcium may alter the metabolism of cyclic guanosine 5′-monophosphate (GMP). Calcium has been shown to stimulate the activity of guanylyl cyclase (20), the enzyme responsible for the generation of cyclic GMP. In other tissues, including the parotid gland (21) and liver (22) of rats and dog thyroid slices (23), ionophore A23187 has been shown to increase the intracellular concentration of cyclic GMP. This effect has been shown to be dependent on the external concentrations of calcium (21, 23). It is possible, therefore, that some of the effects observed in the present studies in the toad bladder relate to changes in intracellular cyclic GMP levels mediated by alterations in intracellular calcium or membrane-bound calcium concentrations.

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