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COMPARISON OF THE EFFECTS OF INCREASED INTRACELLULAR CALCIUM AND ANTIDIURETIC HORMONE ON ACTIVE SODIUM TRANSPORT IN FROG SKIN

A STUDY WITH THE CALCIUM IONOPHORE A23187

ROBERT S. BALABAN and LAZARO J. MANDEL

Department of Physiology, Duke University Medical Center, Durham, NC 27710 (U.S.A.)

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Summary

The addition of the Ca^{2+} ionophore A23187 (1 μM) to the inside solution of the frog skin resulted in an approx. 40% transient increase in the active influx of Na^+ and ionic conductance, which decayed to an approx. 13% steady-state stimulation after 1—2 h. A23187 had no effect from the outside solution. A23187's stimulatory action is most likely the result of the ionophore's ability to increase intracellular Ca^{2+}. This contention is supported by the following experimental results: (1) reintroduction of Ca^{2+} into a Ca^{2+}-free inner solution stimulated Na^+ transport only in the presence of A23187: (2) Mg^{2+} would not mimic these effects, and (3) EGTA in the inner solution would inhibit the A23187 response.

The stimulation of active transport and ionic conductances elicited by A23187 were found to be very similar to those caused by antidiuretic hormone. Several lines of evidence suggest that A23187 may by-pass steps in the normal antidiuretic hormone stimulatory process: (1) A23187 and antidiuretic hormone are apparently non-additive; (2) A23187 acts three times faster than antidiuretic hormone; (3) A23187 stimulates antidiuretic hormone-insensitive frog skins, and (4) results from other laboratories indicate that A23187 does not increase cyclic AMP concentrations.

It is speculated that an increase in free intracellular Ca^{2+} may be a step in the normal antidiuretic hormone stimulatory process. This increase in intracellular Ca^{2+} may in turn stimulate active sodium transport by increasing the Na^+ permeability of the outer 'rate-limiting' membrane.

Abbreviations: ADH, antidiuretic hormone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetra-acetic acid.

Introduction

Antidiuretic hormone (ADH) has been shown by numerous investigators to stimulate active Na^+ transport and increase water permeability in anuran skin and urinary bladder preparations [1—3]. ADH is believed to act by binding to specific basal membrane sites which results in an increase in the concentration of the second messenger cyclic adenosine 3',5'-phosphate (cyclic AMP) within the epithelial cells [1,4]. This increase in cyclic AMP is hypothesized to generate, through an as yet undetermined pathway, the stimulation of sodium transport and the increase in water permeability. Evidence for this hypothesis has been obtained by the mimicry of the ADH effect by exogenously applied cyclic AMP and by the addition of theophylline, which inhibits the cellular breakdown of cyclic AMP [5]. In addition, intracellular cyclic AMP concentrations have been demonstrated to increase after the application of ADH to these preparations [1,6].

Recently, it has been proposed that an increase in intracellular Ca^{2+} also occurs during the stimulation of target tissues by cyclic AMP-mediated peptide hormones [7,8]. This contention has been supported by the following observations in various peptide hormone-sensitive systems: (1) extracellular Ca^{2+} is required for peptide hormone action [9,10]; (2) Ca^{2+} efflux increases during hormonal stimulation [10]; (3) total cellular Ca^{2+} content increases [11] during hormonal stimulation, and (4) the effects of the hormones and cyclic AMP have been mimicked by the use of Ca^{2+} ionophores, whose action is to increase the amount of intracellular Ca^{2+} [8,12—14].

Due to the low concentration of intracellular free Ca^{2+} (less than 10^{-7} M) and the small size of most 'tight' epithelial cells, Ca^{2+}-sensitive optical dye and microelectrode studies to measure the free intracellular Ca^{2+} concentration in these cells are technically unfeasible at this time. One method, as mentioned above, for determining whether intracellular Ca^{2+} is involved in a hormone stimulation process in epithelia has been to increase the intracellular Ca^{2+} content with a Ca^{2+} ionophore, and observe whether this perturbation mimicked the entire or a portion of the hormone effect. This rationale is similar to that used by Sutherland et al. [15] for the identification of a cyclic AMP-mediated process, namely, the exogenously applied cyclic AMP must mimic the effect of the hormone.

The Ca^{2+} ionophore A23187 is a carboxylic acid antibiotic that is crystallized from *Streptomyces chartreusensis*. A23187 has the specific property of complexing divalent cations, in a 2 : 1 ratio of A23187 to cation, forming a neutral lipid-soluble complex [16,17]. This ionophore has been used in a number of experimental preparations to increase the Ca^{2+} concentration of the cytosol of the cells (10^{-7}—10^{-6} M) by allowing the Ca^{2+} in the extracellular space (10^{-3} M) to permeate into the cells. In addition, the ionophore has also been shown to release Ca^{2+} from intracellular stores, such as the mitochondria [16]. The movement of Ca^{2+} by A23187 is believed to involve the exchange of Ca^{2+} for either H^+ or Mg^{2+}, or result in the net movement of A23187 with Ca^{2+}. However, in each case no charged species moves across the membrane, thus no direct effect of A23187 is seen on the membrane potential or resistance.

The purpose of this study was to investigate the effect of increased intra-

cellular Ca^{2+} on active Na^+ transport in the isolated frog skin preparation with the use of the Ca^{2+} ionophore A23187 and to compare this effect with that of ADH.

Methods and Materials

The abdominal skins of bullfrogs (*Rana catesbeiana*) were mounted as flat sheets (3.14 cm^2 in area) between lucite chambers equipped with solution reservoirs similar to those described by Schultz and Zalusky [18].

The open circuit potential across the skin was measured with calomel electrodes, and current was passed through the skin via Ag|AgCl electrodes. Both pairs of electrodes were connected to the solution reservoirs with 4% agar bridges having a composition identical to that of the bathing solution in the chambers. An automatic voltage clamp that compensated for the resistance of the solution between the agar bridges was used to pass the appropriate current through the skin to clamp the membrane potential at zero mV.

The short circuit current (I_{sc}) or, that current necessary to maintain the transepithelial potential at 0 mV, was taken to represent the active outside to inside flux of Na^+ as demonstrated by Cereijido et al. [19] and by ^{22}Na flux experiments conducted in some of the experimental conditions of this investigation. ^{22}Na influx experiments were performed by methods previously described [20]. The transepithelial resistance was calculated (using Ohm's law) from the voltage deflections caused by constant current pulses delivered across the open-circuited skin from a constant current generator (Nuclear Chicago, Model 7510). The composition of the Ringer solutions used in all experiments (unless specified differently) was (in mmol/l): NaCl (85), CaCl (1), KCl (5), $NaHCO_3$ (24) and glucose (2), mixed and aerated with 95% O_2/5% CO_2 gas to obtain a solution pH of 7.4.

A23187 was obtained as a gift from Dr. R.L. Hamill of Lilly Research Laboratories, Indianapolis, IN. Fresh stock solutions of 0.4 mM A23187 in methanol were made up weekly. Methanol added by itself at concentrations used in this study was found to have no significant effect on either the short-circuit current or the resistance of the tissue. Pitressin (Parke-Davis) was used as the source of ADH.

Results

The effect of A23187 on the I_{sc} of the bullfrog skin was investigated by the addition of 1 μM A23187 to either the inside (blood side) or outside (pond side) solutions bathing the epithelium. Application of 1 μM A23187 to the outside of six skins resulted in no effect or a slight inhibition (Fig. 1). In contrast, addition of A23187 to the inside solution resulted in a prolonged transient increase in the I_{sc} of 46.9 ± 7.2% (± S.E.) in 18 skins. At the end of 1—2 h a steady-state I_{sc} was reached which was sometimes above and other times below the initial I_{sc}, with a few skins demonstrating an oscillatory behavior before reaching a steady state. On the average, the steady-state I_{sc} after ionophore addition was above the initial I_{sc} by 13.6 ± 7.9% (not significantly different from zero).

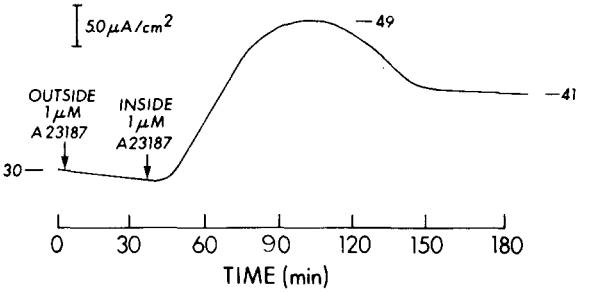


Fig. 1. Effect of 1 μM A23187 in the outside and inside solutions on the I_{sc} of the frog skin. The numbers represent values of I_{sc} (μA/cm²) at the points indicated in this and all other figures.

To investigate whether this increase in I_{sc} was due exclusively to an increase in active sodium influx, ^{22}Na influx experiments were performed before and after the addition of A23187 in six skins. The ^{22}Na influx was the equivalent of 30.2 ± 2.8 μA/cm² while the measured I_{sc} was 27.5 ± 3.0 μA/cm² in the same control period. These two numbers are not statistically different although the former probably contains a small component of passive Na^+ influx through the shunt pathway [20]; the results indicate that the I_{sc} does represent the active Na^+ influx in the control situation. ^{22}Na influx experiments conducted at the peak of the A23187 response, a 20 min period, resulted in a measured Na^+ influx stimulation of 32.1 ± 4.0% ($P < 0.005$ as compared to control) and a stimulation of the average I_{sc} over the same time period of 30.1 ± 3.8% ($P < 0.005$ as compared to control). These two values are not significantly different from each other ($P > 0.1$) indicating that the increase in I_{sc} caused by A23187 is the result of an increase in active Na^+ influx across the epithelium.

The effect of A23187 on the transepithelial resistance was investigated by using open-circuited preparations through which hyperpolarizing constant current pulses were passed transepithelially (see Methods and Materials). Fig. 2 illustrates such an experiment in which A23187 had no effect on the transepithelial resistance from the outside solution ($n = 5$); however, from the inside solution 1 μM A23187 caused a 40.3 ± 6.7% ($P < 0.005$ as compared to controls) transient decrease in the resistance of eight skins. The steady-state transepithelial resistance, after the transient decrease, was found to be 10.2 ± 6.4% (not significantly different from zero) lower than the control resistance. The time course of this resistance change was similar to that observed for the Na^+ transport stimulated in paired short-circuited skins. The percent change in resistance at both the peak and the steady-state values in individual skins was found to be proportional to the change in the I_{sc} occurring in paired short-circuited skins (i.e. a large increase in I_{sc} was always found with a large decrease in resistance and vice versa).

The baseline or tissue-generated transepithelial potential was observed to follow two kinetic patterns with equal frequency: (1) the baseline potential would increase with the decrease in resistance and then slowly return to near control values (Fig. 2), and (2) a biphasic response, in which the baseline potential would rapidly decrease with the decreasing resistance and then reverse, with the

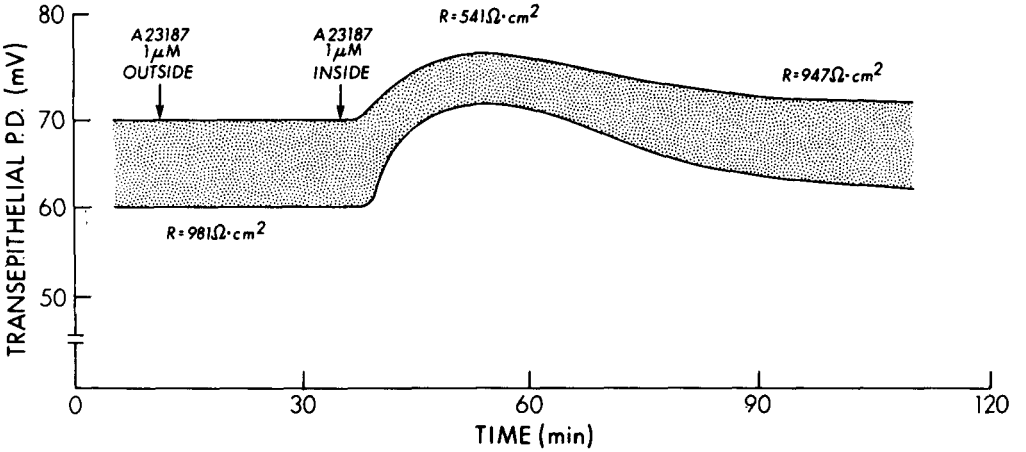


Fig. 2. Effect of 1 μM A23187 in the outside and inside solutions on the resistance and transepithelial potential. The upper line represents the magnitude of the transepithelial voltage in response to a hyperpolarizing 32 μA current pulse of 400 ms duration at a frequency of 0.2 pulse/s. The distance between the upper and lower lines represent the magnitude of the voltage pulse. The resistances shown were calculated using Ohm's law.

potential increasing above control and then returning to near control values (not shown).

The effect of Ca^{2+} in the inside bathing solution on the A23187 stimulation of Na^+ transport was studied by the addition of A23187 in the presence and absence of Ca^{2+} in the inside solution. A Ca^{2+}-depleted inside solution was obtained by first washing the inside chamber with a 1 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*'-tetraacetic acid (EGTA) Ca^{2+}-free Ringer solution of sufficient volume to exchange the entire volume of the chamber ten times. The EGTA/Ringer solution was then replaced with a Ca^{2+}-free Ringer solution not containing EGTA. In these experiments and in similar experiments by other investigators [21], this treatment was found to have no effect on the Na^+-transporting properties of this epithelium. Since the frog skin has a large Ca^{2+} pool in its connective tissue [21], this treatment probably does not remove all the Ca^{2+} from the inner solution. However, it should significantly reduce the concentration of Ca^{2+} and, thus, will be referred to as a Ca^{2+}-depleted condition. Addition of the ionophore after the EGTA treatment caused a 40% (±9.7) peak stimulation of I_{sc} in seven skins (Fig. 3a and b), essentially the same as that obtained in regular Ringer. However, a subsequent addition of 2 mM Ca^{2+} to the inner solution containing the Ca^{2+}-depleted Ringer, caused at additional increase of 21% (±5.2) in the I_{sc} of seven skins. (Fig. 3a and b). This latter response appeared to be specific for Ca^{2+} because addition of Mg^{2+} would not mimic the effect (Fig. 3a); furthermore, subsequent addition of 1 mM EGTA, a specific chelator of Ca^{2+}, decreased the steady-state I_{sc} level obtained after the reintroduction of Ca^{2+} (Fig. 3b). Ca^{2+} reintroduced in the absence of the ionophore did not result in stimulation of I_{sc}, nor did the subsequent addition of EGTA lead to an inhibition.

The stimulation of I_{sc} caused by A23187 was then compared to the ADH stimulation in paired skin experiments. ADH (20 mU/ml) and A23187 (1 μM)

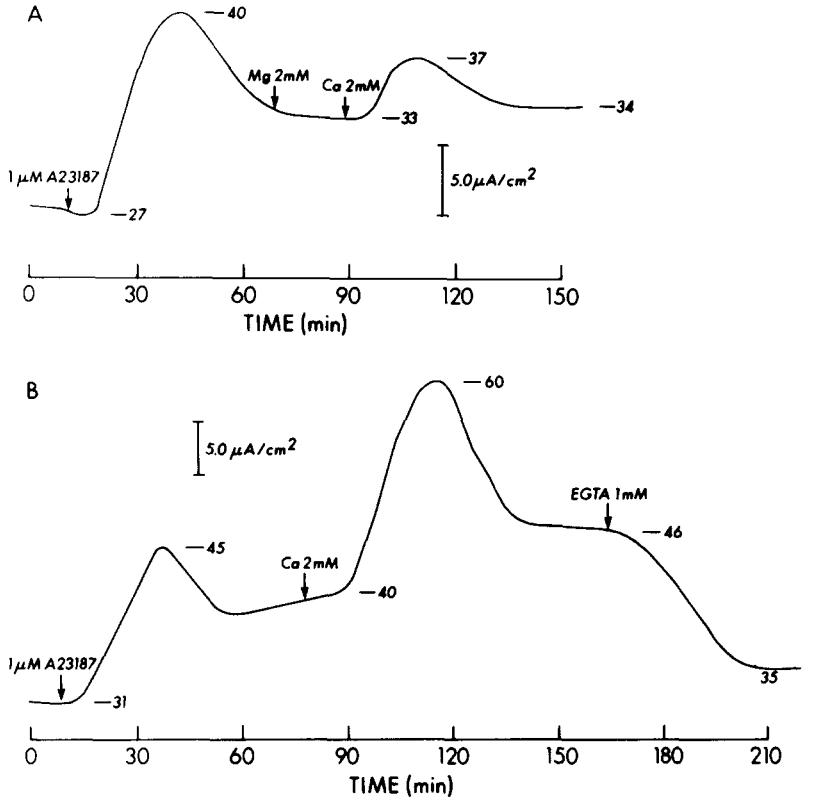


Fig. 3. Responses of I_{sc} to A23187 and other additions in Ca^{2+}-depleted inside solutions. Ca^{2+}-depleted conditions were obtained by initially washing the inside chamber with an EGTA/Ringer solution (see text). (A) After the transient response produced by the ionophore, subsequent addition of Mg^{2+} (2 mM) caused no response, but Ca^{2+} (2 mM) addition elicited another transient increase in I_{sc}. (B) After the transient response to A23187, subsequent addition of Ca (2 mM) produced another transient increase in I_{sc}. After the second transient subsided, addition of EGTA (1 mM) to the inside solution decreased I_{sc} to its original level.

were added at the same time to the inside solution of different paired frog skins to compare the kinetics of onset of the two responses. Fig. 4 illustrates such an experiment. It was found that the time to onset of the stimulation by A23187 was 5.5 min (±0.32) in five skins while the ADH response was found to be consistently slower, with an onset time of 17.5 min (±1.4).

To test whether the effects of these two agents were additive, A23187 was added to skins after they had achieved a new steady state following ADH administration; the opposite experiment was also performed, adding ADH to skins after the transient response to the ionophore. Both agents caused a transient increase in I_{sc} (Fig. 4) when added after the other agent, ADH causing a 17.4% (±4.0) increase and A23187 a 15.2% (±2.1) increase. The magnitudes

Fig. 5. Effect of A23187 on the I_{sc} of ADH-insensitive frog skins. In paired skins, 1 μM A23187 was added to the inside solution either by itself (lower trace) or after the addition of 100 mU/ml of ADH (upper trace).

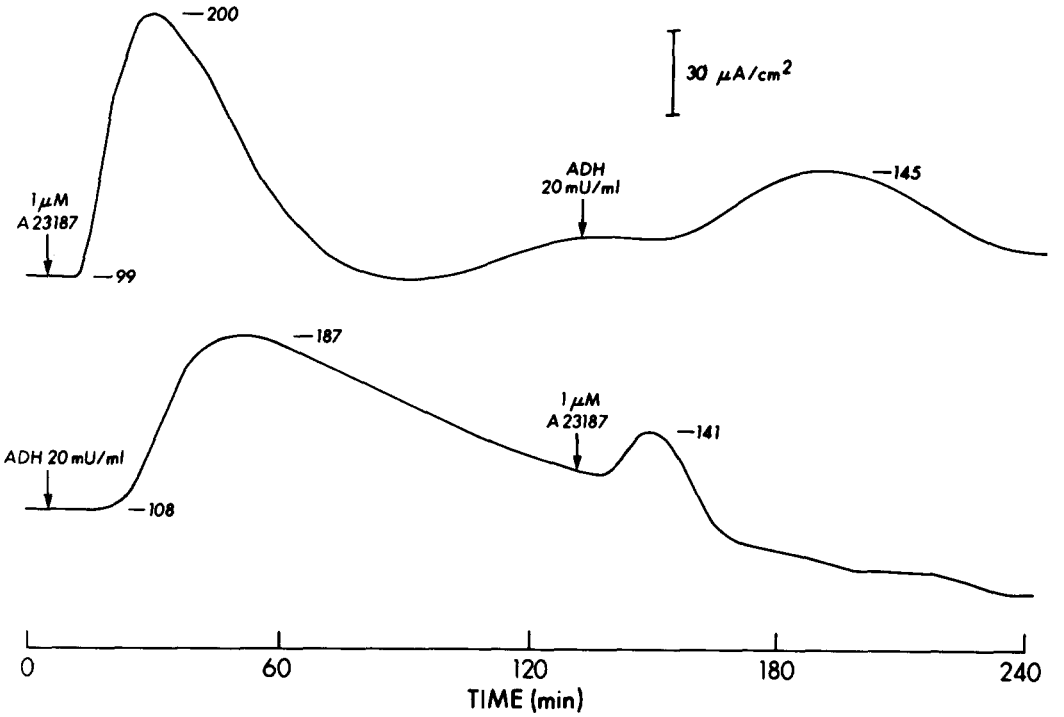
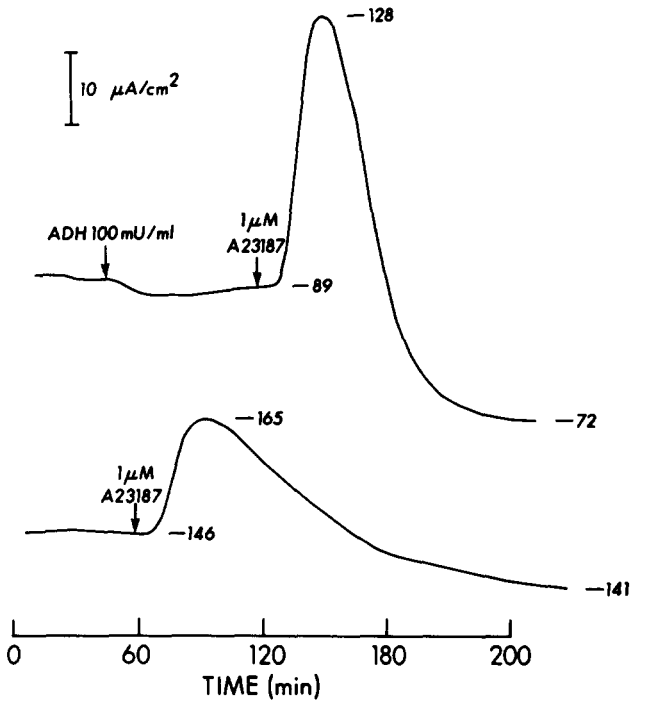


Fig. 4. Comparison of the I_{sc} stimulation produced by A23187 and ADH in frog skin. 1 μM A23187 and ADH 20 mU/ml were added to the inside solution of different paired skins simultaneously. Subsequently, 1 μM A23187 was added to the ADH skin (bottom trace) and 20 mU/ml ADH to the A23187 skin (upper trace) on the inside solution at the times indicated.



of both increases were rather small compared to the initial stimulations obtained by the individual agents; in addition, no stimulation of the steady-state I_{sc} was observed after addition of the second agent and, in fact, a small inhibition in steady-state I_{sc} was usually observed. When a similar addition of a second agent was made at the peak of the response to the first agent, no further increase in the I_{sc} was seen; the only change in I_{sc} observed under those conditions was an apparent prolongation of the transient response.

Several times during this investigation skins were observed to be totally insensitive to ADH in concentrations up to 100 mU/ml. However, these three paired skins did respond to A23187 (Fig. 5) in the presence (by 37.4 ± 3.2% peak increase in I_{sc}) or absence (by 32.6 ± 5.0% peak increase) of ADH.

Discussion

These data demonstrate that A23187, when added to the inside solution of the frog skin, results in a stimulation of the active transepithelial movement of Na^+. This effect of A23187 is most probably the result of an increase in cytoplasmic Ca^{2+} caused by A23187's ability to transport Ca^{2+} down its electrochemical gradients from the extracellular space and intracellular Ca^{2+} pools into the cell cytosol, as has been proposed in various other preparations. Ca^{2+} was implicated as the effector of A23187 action in this preparation by the following experimental results: (1) reintroduction of Ca^{2+} into a Ca^{2+}-depleted inner solution stimulates Na^+ transport only in the presence of A23187; (2) Mg^{2+} will not mimic these efects even though A23187 may complex this ion [16], and (3) EGTA, a specific chelator of Ca^{2+}, will inhibit the A23187 response. The inability to demonstrate a complete dependence of the A23187 effect on extracellular Ca^{2+} in this preparation is likely the result of the large Ca^{2+} pool located in the connective tissue adjacent to the inner membrane of this epithelium [21], and the ability of A23187 to release Ca^{2+} from intracellular pools.

The active transepithelial movement of Na^+ in the frog skin is believed to occur in two steps: (1) a passive entry of Na^+ across the outer membrane of the epithelium, and (2) the active extrusion of Na^+ into the inside solution at the basolateral membrane by $(Na^+ + K^+)$-ATPase [22]. Of these two steps, the passive entry of Na^+ across the outer membrane is believed to be rate limiting for the entire transepithelial movement of Na^+ [23]. Thus, the simplest mechanism by which the A23187-induced increase in intracellular Ca^{2+} could stimulate Na^+ transport would involve intracellular Ca^{2+} increasing the passive entry of Na^+ across the outer membrane of this epithelium. This hypothesis is supported by observations in other tissues which show that intracellular Ca^{2+} does indeed increase cation permeability; these tissues include: red cells [24], spinal motor neurons [25], and Aplysia nerve cells [26]. Furthermore, since 75—90% of the transepithelial resistance of the frog skin is found in the outer membrane [27], one would expect a simultaneous decrease in the transepithelial resistance if the Na^+ permeability of this membrane was increased. Fig. 2 illustrates that after the addition of A23187 a transient decrease in transepithelial resistance does indeed occur which mimics the kinetic pattern, and is proportional to, the Na^+ transport stimulation response. This decrease in resis-

tance observed with the addition of A23187 is unlikely to be due to the actual A23187-mediated movement of Ca^{2+} across the membranes of the epithelium, for the following reasons: (a) this interpretation would not explain the transient nature of the resistance response; (b) results from other laboratories [16], as well as experiments conducted on phosphatidylethanolamine lipid bilayers in our laboratory (Balaban, R.S. and Hall, J.E., unpublished observations), indicated that A23187 moves Ca^{2+} across membranes in an electrically silent manner, and (c) direct evidence for the stimulation of epithelial membrane conductances by increased intracellular Ca^{2+} was obtained by Rose and Lowenstein [28] who found that the intracellular injection of Ca^{2+} near the plasma membrane in blowfly salivary gland epithelial cells resulted in a decrease in the resistance of this membrane. In their study, however, the ionic specificity of the conductance changes or the transepithelial transport properties of this epithelium were not investigated.

The failure of A23187 to affect Na^+ transport or transepithelial resistance from the outside solution (Figs. 1 and 2) is difficult to explain, for one would expect the ionophore to transport Ca^{2+} into the cells from either the outer or inner solutions. However, this behavior is not unique to frog skin, since A23187 has also been shown to be ineffective in stimulating Cl^- secretion from the mucosal or outside solution of the rabbit ileum while serosal additions caused a considerable stimulation of Cl^- secretion [13].

Another mechanism through which A23187 could stimulate Na^+ transport would be for the ionophore to elevate cyclic AMP levels within the skin, which in turn would stimulate sodium transport. Several findings argue against this mechanism: (1) A23187 has been demonstrated not to affect the cyclic AMP levels in various other epithelial preparations where the ionophore mimics the cyclic AMP effect [12—14]; (2) the adenyl cyclase isolated from the toad urinary bladder has been found to be inhibited by Ca^{2+} [30], while the concentration of Ca^{2+} is likely to be increased by A23187, and (3) it has been recently demonstrated that addition of A23187 to the inside solution of isolated *Rana temporaria* frog skin did not result in an increase in cyclic AMP concentration [31]. Although these authors did not measure active transport in this study, the ionophore concentrations used were similar to those used in the present study.

In addition, several other possible 'second' messengers triggered by A23187 or Ca^{2+}, such as, cyclic GMP [32], prostaglandins [33], or microtubule assembly [34] could also cause the observed change in active Na^+ transport. However, little information is presently available to evaluate these possibilities.

Ca^{2+}-dependent A23187 mimicry of cyclic AMP and cyclic AMP-mediated hormone effects on active transport processes have been demonstrated in various epithelial preparations, such as, rabbit colon [12], rabbit ileum [13] and in the frog cornea [14]; in these preparations, the stimulatory effect of the hormones and A23187 was an increase in active Cl^- transport. In addition, preliminary results from Nielsen [35], indicate that A23187 increases Na^+ transport in *R. temporaria* frog skin. In the frog bladder, Hardy [36] has shown that A23187 causes an ADH-like hydrosmotic effect, while Wiesmann et al. [37] have reported that A23187 leads to an inhibition of I_{sc} in toad bladder. Examination of the data from the latter publication as well as experi-

ments conducted in our laboratory indicate that a transient stimulation of I_{sc} does occur in the toad bladder but is usually followed by a steady-state inhibition below the initial I_{sc}. This discrepancy between the toad bladder and frog skin may be the result of their different abilities to regulate intracellular Ca^{2+} and withstand the potentially toxic mitochondrial uncoupling properties of A23187 [16]. An increased active efflux and sequestration of Ca^{2+} along with the uncoupling properties of A23187 may potentially result in an energy-depleted state and subsequent cell damage or disfunction. This situation apparently does not arise in the frog skin because the tissue will usually maintain a stimulated rate of Na^+ transport after A23187 in excess of 90 min.

The effects of A23187 and ADH in this preparation are both quantitatively and qualitatively similar (Fig. 4). The apparent non-additive nature of these two agents may indicate that their actions converge on the same stimulatory process within the skin. Three types of results suggest that A23187 may be by-passing steps in the normal ADH stimulatory process: (1) A23187 acts on the skin three times faster than ADH, even though the Stokes radius of these molecules differ by only 20%; (2) A23187 stimulates ADH-insensitive skins, and (3) A23187 stimulates Na^+ transport without affecting intracellular cyclic AMP concentrations in frog skin (as discussed earlier), while ADH has been demonstrated to increase cyclic AMP [31]. Thus, A23187 may be affecting a step in the ADH stimulatory process at some point after the ADH-induced increase in cellular cyclic AMP. However, this interpretation is not unique; for example, it is possible that A23187 stimulates Na^+ transport through a different pathway than ADH, and the non-additive nature of A23187 and ADH may be the result of either transport of metabolic limitations.

ADH is believed to stimulate Na^+ transport by raising the levels of cyclic AMP within the epithelial cells which, by an as yet undetermined mechanism, results in an increase in Na^+ conductance across the rate-limiting apical membrane [1,38]. It may be speculated that the ADH-stimulated rise in intracellular cyclic AMP causes an increase in intracellular Ca^{2+} [8], which may serve as a feedback inhibitor of cyclic AMP production [8,39]. The increase in Ca^{2+} may in turn cause the increase in Na^+ permeability of the outer membrane and subsequently stimulate active sodium transport. A23187, by directly causing an increase in intracellular Ca^{2+} could induce a rapid stimulation of Na^+ transport, by-passing the ADH binding and cyclic AMP-stimulated Ca^{2+} influx steps. Evidence that increased intracellular Ca^{2+} does occur during ADH stimulation has been obtained in the frog bladder by Pietras et al. [11] who found an increase in total cellular Ca^{2+} in isolated frog bladder cells with the addition of ADH and in toad bladder by Cuthbert and Wong [10] who observed an increase in mucosal Ca^{2+}-Ca^{2+} exchange after the serosal addition of either ADH or cyclic AMP. Also, Snart [40] found evidence for increased release of Ca^{2+} from rat kidney mitochondria isolated after the addition of ADH to kidney slices.

The transient nature of the Na^+ transport responses to both ADH and A23187 may represent similar transients induced on the intracellular Ca^{2+} concentrations. The initial rise in intracellular Ca^{2+}, caused by the agents, may be followed by increased cellular reaccumulation (into the mitochondria), active efflux of Ca^{2+} and/or Ca^{2+} stimulated feedback inhibition of Ca^{2+} influx [11], which would cause a return of Ca^{2+} activity in the cell cytosol to near

control levels. The ability of epithelial cells to regulate intracellular Ca^{2+} was demonstrated by Rose and Lowenstein [28] in salivary glands; where injected Ca^{2+} was found to be isolated at the injection point by a metabolic-dependent process which was assumed to be mitochondrial uptake. The same situation may also exist in frog skin because metabolic inhibitors will initially stimulate active Na^+ transport before causing a sustained inhibition (Balaban, R.S. and Mandel, L.J., unpublished observations). This type of regulatory mechanism not only explains the transient nature and occasional oscillatory responses of Na^+ transport in response to A23187 but also the variability found in the steady-state I_{sc} after A23187 and/or ADH stimulation. Evidence for this type of regulatory mechanism has also been found in studies showing that cellular calcium oscillates during ADH stimulation [41].

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