

# Correlates of Aldosterone-induced Increases in $\text{Ca}_i^{2+}$ and $\text{Isc}$ Suggest That $\text{Ca}_i^{2+}$ Is the Second Messenger for Stimulation of Apical Membrane Conductance

David Petzel, Michael B. Ganz, Eric J. Nestler, Jonathan J. Lewis, James Goldenring, Fehmi Akcicek, and John P. Hayslett  
Departments of Internal Medicine, Psychiatry and Surgery, Yale School of Medicine, New Haven, Connecticut 06510

## Abstract

Studies were performed on monolayers of cultured  $\text{A}_6$  cells, grown on permeable filters, to determine the second messenger system involved in the aldosterone-induced increase in electrogenic sodium transport. Addition of aldosterone ( $1 \mu\text{M}$ ) to the solution bathing the basal surface of cells caused both an increase in  $\text{Isc}$  and threefold transient rise in intracellular calcium  $\text{Ca}_i^{2+}$  after a delay of  $\sim 60$  min. Because both events were inhibited by actinomycin D and cyclohexamide, they appeared to require transcriptional and translational processes. Addition of BAPTA to the bathing media to chelate  $\text{Ca}_i^{2+}$  reduced  $\text{Isc}$  and the delayed  $\text{Ca}_i^{2+}$  transient;  $50 \mu\text{M}$  BAPTA inhibited  $\text{Isc}$  and the rise in  $\text{Ca}_i^{2+}$  by  $> 80\%$ . Further studies suggested that the action of aldosterone to increase  $\text{Isc}$  may be dependent on a calcium/calmodulin-dependent protein kinase, because W-7 and trifluoperazine reduced the aldosterone-induced  $\text{Isc}$  in a dose-dependent manner. Taken together, these observations suggest that calcium is a second messenger for the action of aldosterone on sodium transport, and suggest, for the first time, that agonists which bind to intracellular receptors can utilize, via delayed processes dependent on *de novo* transcription and translation, intracellular second messenger systems to regulate target cell function. (*J. Clin. Invest.* 1992. 89:150–156.) **Key words:**  $\text{A}_6$  cells • calcium/calmodulin-dependent kinase • electrogenic sodium transport • intracellular calcium

## Introduction

Aldosterone stimulates electrogenic sodium transport in some target epithelia, such as urinary toad bladder (1), mammalian cortical collecting tubule (2), and distal large intestine (3). This expression of hormone action is initiated only after a latent period of 60–90 min, and is dependent on mRNA (4) and protein synthesis (5). Experiments in intact tissue and cultured amphibian cells suggest that the aldosterone-induced increase in electrogenic sodium transport can be viewed as involving an early phase lasting 6–12 h, and a delayed or chronic phase, that persists as long as agonist is present. The early phase is characterized by a gradual increase in apical membrane conductance for sodium, due to activation of amiloride-sensitive sodium

channels located in the apical membrane or insertion of pre-formed channels into the cell membrane (6, 7). During the chronic phase, hormone action stimulates the production of  $\alpha$  and  $\beta$  subunits of Na-K-ATPase (8). Physiological studies have demonstrated both a higher density of activated sodium channels, with unchanged current amplitude per channel, in apical membrane (9), and a higher turnover of sodium at the basolateral membrane in epithelium exposed chronically to aldosterone (10).

Although considerable progress has been made in identifying mechanisms for signal transduction of primary messengers that bind to cell surface receptors and cause rapid changes in cell expression, there are few insights into second messenger systems that subserve agonists, like aldosterone, that bind to cytosolic or nuclear receptors and have more delayed and often tonic effects. Exposure of target epithelium to aldosterone is not associated with an increase in cAMP levels (11). It seemed possible, therefore, that the transcription-dependent second messenger system recruited during the early phase of aldosterone-induced sodium transport involves alterations in intracellular calcium ( $\text{Ca}_i^{2+}$ ) and turnover of phosphatidylinositol. The present study was designed to test this hypothesis in cultured  $\text{A}_6$  cells, derived from kidney of *Xenopus laevis*.

## Methods

Experiment were performed on  $\text{A}_6\text{D}_2$  cells, a clone of  $\text{A}_6$  cells, kindly provided by Gregory Grillo, Walter Reed Research Institute, Washington, DC. This clone exhibits a more robust response to aldosterone than the parent cell line and retains hormone responsiveness through at least passage 100. Cells were grown to confluency, during passages 80–100, on millicell-HA cups (Millipore Corp., Bedford, MA), that had an active surface area of  $0.6 \text{ cm}^2$ . Cells were maintained in media which contained DMEM (Gibco, Grand Island, NY), glutamine (2 mM), penicillin ( $100 \text{ U} \cdot \text{ml}^{-1}$ ) and streptomycin ( $100 \mu\text{g} \cdot \text{ml}^{-1}$ ) and  $\text{NaHCO}_3$  (8 mM). Cells were grown at  $27^\circ\text{C}$  in a humidified incubator gassed with  $1\% \text{ CO}_2$ , and were passaged at intervals of  $\sim 7$ – $10$  d. Cells were maintained in media during all experiments performed in this study, except in experiments where estimates of intracellular calcium were performed when amphibian Ringer solution was used. The Ringer's solution contained (in mM): NaCl 110, KCl 3,  $\text{KH}_2\text{PO}_4$  1,  $\text{CaCl}_2$  1,  $\text{MgSO}_4$  0.5, glucose 5, Hepes 10, pH 7.6. The potential difference ( $V_T$ ) across confluent monolayers was measured with Ag/AgCl<sub>2</sub> electrodes (STX-2) and current was passed with a model DVC-1000 voltage current clamp apparatus (WPI Industries, New Haven, CT) to estimate transepithelial resistance ( $R_T$ ), which ranged from 2,000 to 4,000  $\Omega \text{ cm}^2$ . The equivalent short circuit current,  $\text{Isc}$ , reported to equal net sodium transport by  $\text{A}_6$  cells (12), was calculated from the open circuit estimates of  $V_T$  and  $R_T$ .

Preliminary experiments indicated that basal and hormone stimulated  $\text{Isc}$  was unaffected by ETOH and DMSO in a dilution of 1:100 or more. In these experiments, aldosterone, added to media on the basal side of the membrane, was used in the maximal stimulatory concentration of  $1 \mu\text{M}$ , dissolved in ETOH in a dilution of 1:100, and ETOH, in the same dilution, was added to bathing solution of all control tissues.

This work has previously been reported in preliminary form.

Address correspondence to John P. Hayslett, M.D., Dept. of Medicine, Section of Nephrology, Yale School of Medicine, 333 Cedar St., LMP 2073, New Haven, CT 06510.

Received for publication 06 February 1991 and in revised form 22 July 1991.

*J. Clin. Invest.*

© The American Society for Clinical Investigation, Inc.

0021-9738/92/01/0150/07 \$2.00

Volume 89, January 1992, 150–156

The chemical probes used to evaluate the mechanism of action of aldosterone were dissolved in DMSO in a dilution of at least 1:100.

Estimates of the activity of  $\text{Ca}_i^{2+}$  were made with Fura-2/AM (5  $\mu\text{M}$ ) using a model LS-5B spectrofluorometer (Perkin-Elmer Corp., Pomona, CA) as previously reported by this laboratory (13). In these experiments  $\text{Ca}_i^{2+}$  was estimated by measuring the ratio of fluorescence of emitted light at 510 nm after excitation at 340 and 380 nm (14). Intracellular calcium was also determined in some experiments using the fluorescent probe Indo-1/AM (5  $\mu\text{M}$ ). Estimates of  $\text{Ca}_i^{2+}$  were made from the ratio of emitted light at 410 and 485 nm, when cells were excited by light at 340 nm. Cells were grown to confluency on plastic cover slips, and the same solvents for aldosterone and experimental probes were used as in whole membrane experiments.

These experiments were initiated before it was possible to estimate  $\text{Ca}_i^{2+}$  with fluorescent probes in cells grown on a porous membrane, because of the unavailability of a porous membrane that was transparent and had low autofluorescence. It was important, however, to demonstrate that changes in  $\text{Ca}_i^{2+}$  exhibited by cells grown on a nonporous substratum were comparable to aldosterone-induced changes in cells grown on a porous membrane. Additional experiments were therefore, performed when a suitable membrane became available. Cells were grown on Falcon membranes (Becton Dickinson Co., Lincoln Park, NJ) attached to the bottom of culture cups, with an active membrane area of 0.6  $\text{cm}^2$ , in studies designed to demonstrate the effect of aldosterone on electrogenic  $\text{Na}^+$  transport, and cells were grown on the same membrane in studies performed to estimate  $\text{Ca}_i^{2+}$ . Experimental conditions were identical to those performed on Millipore Corp. filter paper and on nonporous plastic cover slips.

The level of 1,4,5- $\text{IP}_3$  was estimated in cells grown to confluency on millicell-HA cups by a sensitive and specific competitive binding radio-receptor assay, as recently reported (15). These cells were handled under the same conditions used in transport studies, and estimates of  $\text{IP}_3$  were obtained immediately before sacrifice for biochemical assay.

Actinomycin D, cyclohexamide, trifluoperazine, spironolactone, and aldosterone were purchased from Sigma Chemical Co. (St. Louis, MO), while the acetoxymethyl ester of 5,5'-dimethyl-BAPTA ( $\text{Me}_2\text{BAPTA}$ ), Fura-2/AM, Indo-1/AM, A23187, ionomycin, W-7, and W-5 were obtained from Molecular Probes Inc. (Eugene, OR).

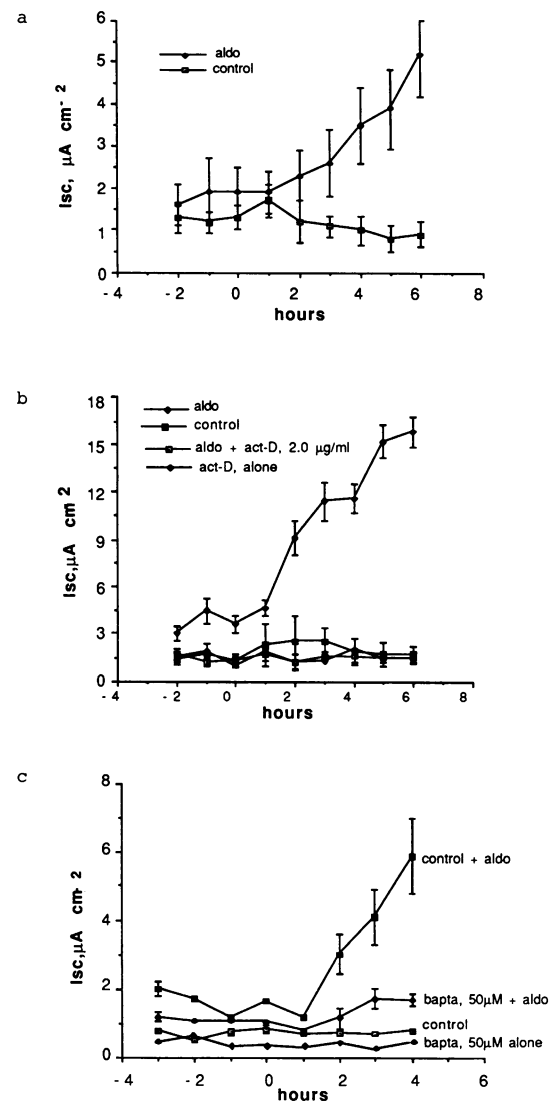
## Results

Exposure of  $\text{A}_6\text{D}_2$  monolayers to aldosterone, 1  $\mu\text{M}$ , stimulated more than a fivefold increase in  $\text{Isc}$  within 6 h. As shown in Fig. 1 *a* the rise in  $\text{Isc}$  was delayed at least 1 h after addition of the hormone at time zero; in control tissue  $\text{Isc}$  fell slightly during the same time interval. A further rise of  $\text{Isc}$  was observed on continued exposure to aldosterone, increasing to a maximal level of 15–20  $\mu\text{A} \cdot \text{cm}^{-2}$  at  $\sim 15$  h (data not shown). The level of  $\text{Ca}_i^{2+}$  measured by Fura-2, in a typical experiment (Fig. 2 *a*), exhibited a small transient rise of  $\sim 30$  nM immediately after addition of aldosterone to bathing solution, and a larger, threefold rise in  $\text{Ca}_i^{2+}$  between 60 and 70 min after addition of the agonist. In 21 experiments, the delayed calcium transient showed an average onset of  $62 \pm 8$  min (mean  $\pm$  SE) after exposure to aldosterone, from a mean basal level of  $92 \pm 6$  nM to  $302 \pm 12$  nM, and returned to a level indistinguishable from baseline after  $7.3 \pm 2.8$  min.

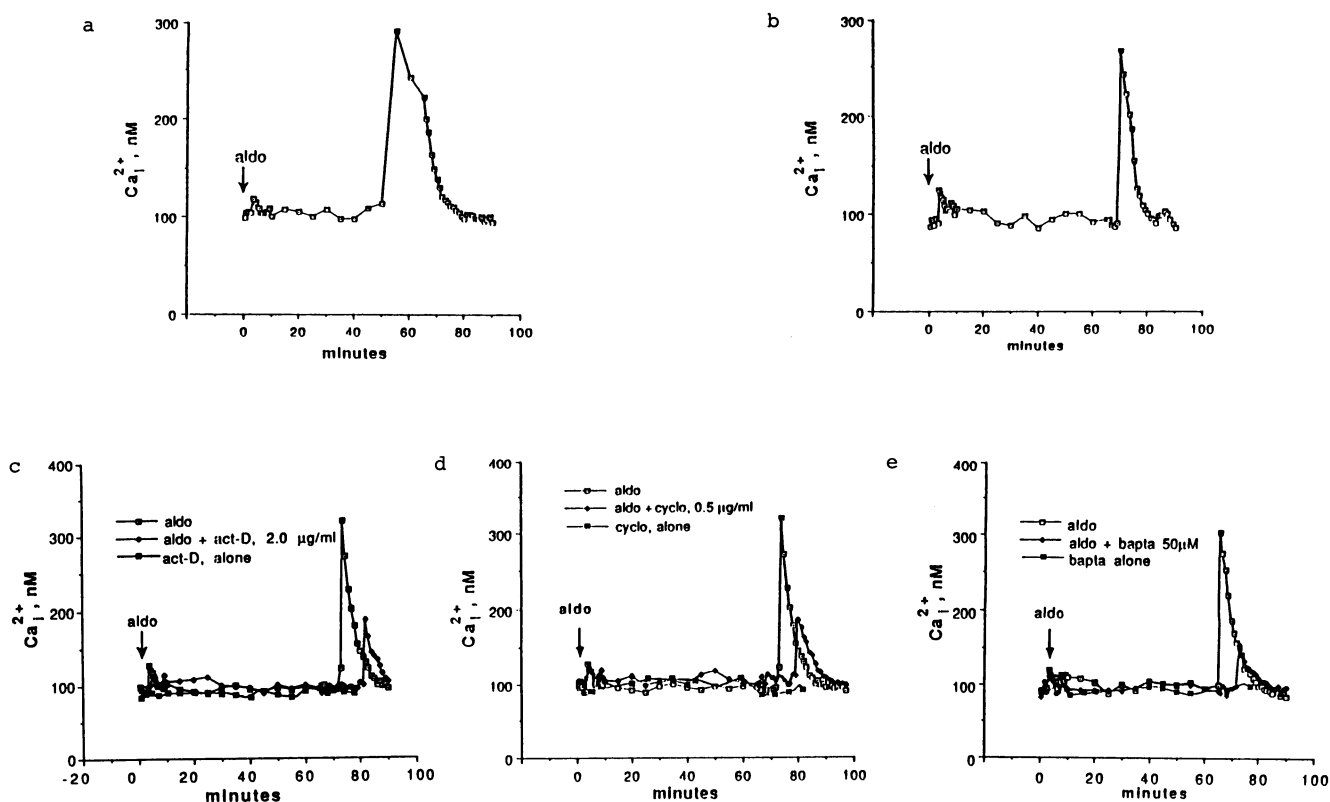
Additional experiments were performed to determine whether the aldosterone-induced increase in  $\text{Ca}_i^{2+}$  found in cells grown on a nonporous surface reflected the effect of aldosterone on intracellular calcium levels in cells grown to confluency on a porous membrane. Initial studies demonstrated that aldosterone stimulated a similar maximal increase in  $\text{Isc}$  by cells grown on the Falcon membrane compared to levels exhibited by cells grown in the Millipore filter. Exposure of

monolayers to aldosterone, 1  $\mu\text{M}$ , resulted in a rise of  $\text{Isc}$  from a basal value of  $1.8 \pm 0.2$  to  $19.6 \pm 2.74$   $\mu\text{A} \cdot \text{cm}^{-2}$  after 15 h. The level of  $\text{Ca}_i^{2+}$  was estimated with Fura 2/AM and, as in experiments performed on nonporous plastic culture slips,  $\text{Ca}_i^{2+}$  increased transiently, after a delay of  $\sim 60$ –70 min, to a maximum level of  $269 \pm 19$  nM ( $n = 6$ ). The results of a typical experiment are shown in Fig. 2 *b*.

To determine whether the aldosterone induced increase in  $\text{Ca}_i^{2+}$  was due, at least in part, to release from intracellular pools, the effect of the hormone on inositol triphosphate ( $\text{IP}_3$ ) was determined. As shown in Fig. 3 *a*, aldosterone exposure was associated with an increase in the level of  $\text{IP}_3$  from a basal



**Figure 1.** Action of aldosterone to increase  $\text{Isc}$ . (a) Effect of aldosterone, 1  $\mu\text{M}$ , on equivalent short circuit ( $\text{Isc}$ ,  $\mu\text{A} \cdot \text{cm}^{-2}$ ) vs. time, compared to monolayers not exposed to aldosterone. Data points are mean  $\pm$  SE obtained from eight experiments in which four cups were employed for control and experimental groups. Aldosterone was added at zero time in this and other experiments in this figure. (b) Effect of actinomycin D, 2  $\mu\text{g} \cdot \text{ml}^{-1}$ , on the aldosterone-induced increase in  $\text{Isc}$ . (c) Effect of BAPTA (acetoxymethyl ester of 5,5'-dimethyl-BAPTA), in a concentration of 50  $\mu\text{M}$ , on  $\text{Isc}$ . BAPTA was added to media 3 h before aldosterone. Values are mean  $\pm$  SE of three cups in each group; similar results were observed in three additional experiments.



**Figure 2.** Effect of aldosterone on  $\text{Ca}_i^{2+}$  in typical individual experiments. (a) Effect of aldosterone,  $1 \mu\text{M}$ , on the activity of intracellular calcium,  $\text{Ca}_i^{2+}$ . Cells were grown on a plastic cover slip. (b) Effect of aldosterone,  $1 \mu\text{M}$ , on  $\text{Ca}_i^{2+}$  in cells grown on a porous Falcon membrane. (c) Effect of actinomycin D,  $2 \mu\text{g} \cdot \text{ml}^{-1}$ , on aldosterone-induced increase in  $\text{Ca}_i^{2+}$ . Monolayers treated with aldosterone alone and actinomycin D alone are shown for comparison. (d) Effect of cyclohexamide,  $0.5 \mu\text{g}/\text{ml}$ , on aldosterone-induced increase in  $\text{Ca}_i^{2+}$ . Monolayers treated with aldosterone alone and cyclohexamide alone are shown for comparison. (e) Effect of BAPTA,  $50 \mu\text{M}$ , on aldosterone-induced increase in  $\text{Ca}_i^{2+}$ . Monolayers treated with aldosterone alone and BAPTA alone are shown for comparison.

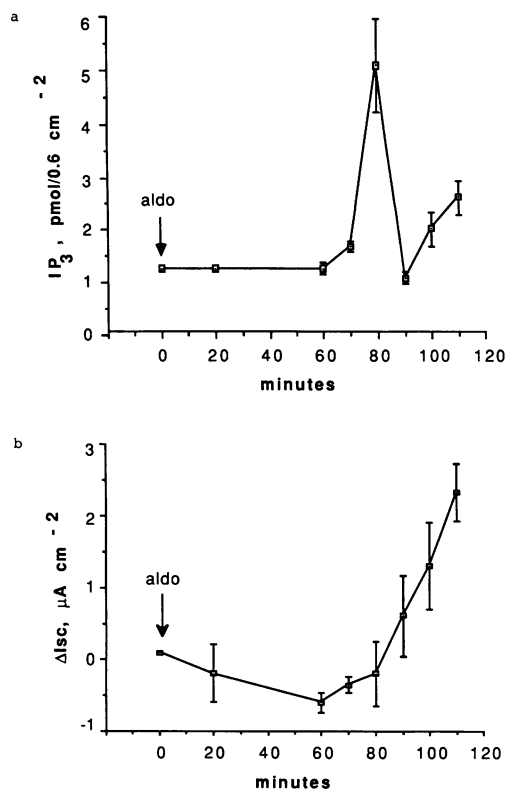
level of  $1.2 \pm 0.1 \text{ pmol}/0.6 \text{ cm}^2$  ( $n = 6$ ) to  $4.6 \pm 0.8$  at  $\sim 70$  min after addition of the agonist. Similar changes were found in three experiments. Concurrent measurements of  $\text{Isc}$  (Fig. 3 b) in the same culture cups used for estimates of  $\text{IP}_3$  showed that the rise in  $\text{Isc}$  began  $\sim 70$  min after addition of aldosterone, indicating a temporal coincidence of the two phenomena. These results, taken together, showed that aldosterone increased  $\text{Ca}_i^{2+}$ , the turnover of phosphatidylinositol, and  $\text{Isc}$ , each after a delay of  $\sim 70$  min.

Because previous experiments in toad bladder (4, 5) indicated that the aldosterone-induced increase in  $\text{Isc}$  was dependent on a transcriptional process and new protein synthesis, further studies were performed to determine whether the delayed aldosterone-induced rise in  $\text{Ca}_i^{2+}$  was dependent on the same factors. Fig. 1 b shows that preincubation of monolayers with actinomycin D ( $2.0 \mu\text{g}/\text{ml}$ ) completely inhibited the action of aldosterone to increase  $\text{Isc}$ . This concentration of actinomycin D inhibited the production of mRNA in amphibian cells by 80% (16). Fig. 2 c illustrates the effect of the same dose of actinomycin D on the delayed calcium transient. Compared to the control response in the same experiment, the rise in  $\text{Ca}_i^{2+}$ , although not substantially delayed in onset, was inhibited in magnitude. In seven experiments the average inhibition of the delayed calcium transient was  $64 \pm 7\%$ . As in the previous report on cultured toad bladder cells (16), the concentration of actinomycin D did not reduce transepithelial resistance (data not shown), used as an index of monolayer viability and differentiation. Similar effects were observed with an inhibitor of

protein synthesis. Preincubation with cyclohexamide ( $0.5 \mu\text{g}/\text{ml}$ ) prevented the hormone-induced increase in  $\text{Isc}$  (data not shown) and, as illustrated by a typical experiment Fig. 2 d, markedly blunted the rise of the delayed calcium transient. In eight experiments, the delayed  $\text{Ca}_i^{2+}$  transient was inhibited by an average of  $70 \pm 5\%$ . Transepithelial resistance was not reduced. Previous studies in  $\text{A}_6$  cells showed that this concentration of cyclohexamide inhibited new protein synthesis by 75% (17). Taken together, these experiments suggest that aldosterone induces an increase in  $\text{Isc}$  and  $\text{Ca}_i^{2+}$  via processes dependent on mRNA and protein synthesis.

To determine whether the aldosterone-induced increase in  $\text{Isc}$  was dependent on the delayed rise in  $\text{Ca}_i^{2+}$ , cells were loaded with BAPTA, reported to chelate  $\text{Ca}_i^{2+}$  (18). The addition of BAPTA to culture media bathing cells inhibited the aldosterone-induced increase in  $\text{Isc}$  in a dose-dependent manner;  $1 \mu\text{M}$  by  $43 \pm 9\%$ ,  $10 \mu\text{M}$  by  $66 \pm 10\%$ ,  $50 \mu\text{M}$  by  $100 \pm 13\%$  (Fig. 1 c), and  $100 \mu\text{M}$  by  $100 \pm 10\%$ , in three separate experiments. Fig. 2 e shows a marked attenuation in the delayed  $\text{Ca}_i^{2+}$  transient. In 11 experiments,  $50 \mu\text{M}$  BAPTA inhibited  $\text{Ca}_i^{2+}$  by an average of  $81 \pm 7\%$ . Basal levels of  $\text{Ca}_i^{2+}$  were not affected by  $50 \mu\text{M}$  BAPTA, similar to previous reports (19), and there was no evidence that cell viability was affected at this concentration. After incubation of monolayers with  $50 \mu\text{M}$  BAPTA for 24 h, the usual aldosterone-induced increase in  $\text{Isc}$  was observed immediately after removal of BAPTA.

Because aldosterone-stimulated electrogenic sodium transport was apparently dependent on a rise in  $\text{Ca}_i^{2+}$ , further studies



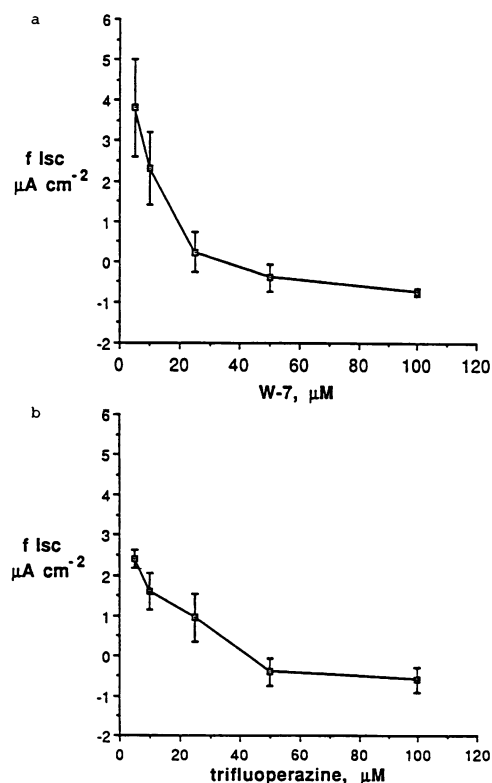
**Figure 3.** The effect of aldosterone on IP<sub>3</sub> levels and I<sub>sc</sub>. (a) Time-related levels of IP<sub>3</sub> after addition of aldosterone, 1 μM, to the basal solution of monolayers grown on millicel-HA cups. (b) The change in I<sub>sc</sub>, compared to the value at time zero, estimated immediately before cells were taken for bioassay of IP<sub>3</sub> at various time intervals. Similar results were obtained in three separate experiments. The fall in IP<sub>3</sub> levels after 20–30 min was probably due to degradation, because lithium was not employed in the assay.

were performed to identify the calcium dependent process involved. As shown in Fig. 4 a, the calmodulin inhibitor W-7 caused a concentration-dependent inhibition of the aldosterone-induced rise in I<sub>sc</sub>. At a concentration near the reported K<sub>i</sub> of 30 μM (20, 21) for calmodulin-dependent enzymes, inhibition was nearly complete. This effect did not appear to be due to a nonspecific toxic effect, in as much as W-5, another compound in the W series with a K<sub>i</sub> of 200 μM for these enzymes, failed to inhibit aldosterone action (data not shown). Fig. 4 b shows that trifluoperazine, another class of calmodulin inhibitor, also blocked the action of aldosterone to increase I<sub>sc</sub> in a dose-dependent manner. Complete inhibition was achieved at a concentration of ~ 20 μM, which approximates the K<sub>i</sub> for calmodulin-dependent enzymes reported in other tissues (20, 22).

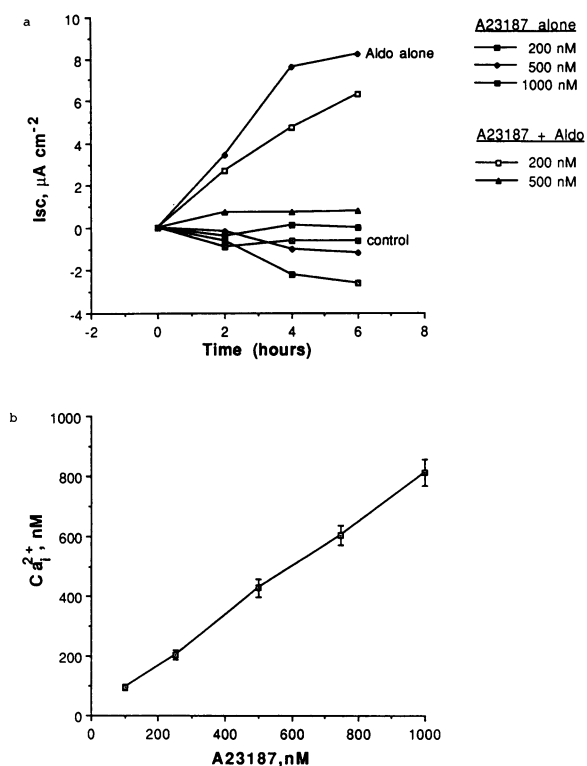
Because previous studies suggest that chronic elevations of Ca<sub>i</sub><sup>2+</sup> reduced active transport of sodium through changes in apical conductance for sodium (23, 24), it was of interest to assess the effect of chronic increases in Ca<sub>i</sub><sup>2+</sup> on basal and aldosterone-induced sodium transport. Because direct measurements of Ca<sub>i</sub><sup>2+</sup> were not performed in earlier studies we correlated increases in Ca<sub>i</sub><sup>2+</sup>, in a range spanning physiological levels to supraphysiological values, with whole membrane estimates of electrogenic sodium transport. In these experiments A23187, or ionomycin, as used in previous reports, was added in increasing concentrations from 100 to 1,000 nM to the bathing solution, which contained 1 mM of CaCl<sub>2</sub> (6.0 mg · dl<sup>-1</sup> free

calcium by a calcium-selective electrode). Estimates of Ca<sub>i</sub><sup>2+</sup> were made with Indo-1/AM, and corrections were made for autofluorescence from measurements made in the absence of cells. The basal level of Ca<sub>i</sub><sup>2+</sup>, measured with Indo-1/AM, was 93 ± 9 nM, similar to the value observed with Fura-2/AM.

The addition of the calcium ionophore to the bathing solution resulted in an immediate rise in Ca<sub>i</sub><sup>2+</sup>, which reached a steady state in ~ 2 min and persisted unchanged for at least 90 min. As shown in Fig. 5 b, the concentration of 250 nM of A23187 caused a rise in Ca<sub>i</sub><sup>2+</sup> to a high physiological level of 205 ± 16 nM, similar to the level observed with aldosterone stimulation, whereas concentrations of 500 nM or more were associated with values of Ca<sub>i</sub><sup>2+</sup> that exceed 400 nM. The concentration 1,000 nM of ionomycin, used in many previously reported experiments (23), caused Ca<sub>i</sub><sup>2+</sup> to rise to a level of 741 ± 36 nM. Levels of Ca<sub>i</sub><sup>2+</sup> exceeding 2,000 nM have been reported after addition of 3 μM of ionomycin (25). Fig. 5 a demonstrates that addition of 200 nM of A23187 to bathing solution had no apparent effect on either basal or aldosterone-induced I<sub>sc</sub>. In contrast, concentrations of 500 nM and 1,000 nM of A23187, associated with increases in Ca<sub>i</sub><sup>2+</sup> to 430 ± 29 and 800 ± 45 nM, respectively, inhibited I<sub>sc</sub>. The concentration of 500 nM of A23187 completely inhibited the effect of aldosterone, but not basal I<sub>sc</sub>, whereas 1,000 nM of the ionophore reduced basal transport of sodium. Because changes in transepithelial resistance may reflect cellular differentiation and viability, it should be noted that at high concentrations of A23187 resistance of monolayers, not exposed to aldosterone, decreased by 47%



**Figure 4.** Effect of calmodulin antagonists on aldosterone-induced rise in I<sub>sc</sub>. Fractional I<sub>sc</sub> was calculated as follows:  $f I_{sc} = (I_{sc\ 3h} - I_{sc\ 0h}) / I_{sc\ 0h}$  Aldo + Antagonist -  $(I_{sc\ 3h} - I_{sc\ 0h}) / I_{sc\ 0h}$  Control. Each data point represents mean ± SE of three experiments at each antagonist concentration, in which three monolayers were assayed in control and experimental groups. (a) Effect of W-7 on aldosterone-induced I<sub>sc</sub>. (b) Effect of trifluoperazine on hormone-stimulated I<sub>sc</sub>.



**Figure 5.** The concentration-dependent effect of A23187 on  $\text{Ca}_i^{2+}$  and  $I_{sc}$ . (a) Effect of A23187, in various concentrations, on basal and aldosterone-induced  $I_{sc}$ . A23187 and aldosterone were added to bathing solution at time zero. The concentration of free calcium in the bathing solution,  $6.0 \text{ mg} \cdot \text{dl}^{-1}$ , was the same in experiments conducted to estimate  $\text{Ca}_i^{2+}$  and in transport studies. In transport experiments, there were three cups in each group. Two additional experiments demonstrated the same results. (b) effect of various concentrations of A23187, added to bathing solution, on  $\text{Ca}_i^{2+}$  estimated with Indo-1/AM.

(500 nM) and 54% (1,000 nM); resistance was unchanged by 200 nM of the ionophore.

Because previous studies have shown that glucocorticoids have a unique action to stimulate sodium transport (26), it was possible that the action of aldosterone to stimulate electrogenic sodium was caused by cross-over binding to glucocorticoid receptors. Studies were therefore, performed to determine whether the effect of aldosterone on  $I_{sc}$  was due to activation of the mineralocorticoid receptor. The action of aldosterone,  $1 \mu\text{M}$ , on  $I_{sc}$  was estimated in the absence and in the presence of spironolactone, a competitive inhibitor of the hormone due to occupancy of the mineralocorticoid receptor, in concentrations of 100 and 200  $\mu\text{M}$ . Spironolactone was added to media bathing the basal surface of monolayers 30 min before the addition of aldosterone. After 6 h exposure to aldosterone,  $I_{sc}$  increased from the basal level of  $0.9 \pm 0.2$  to  $2.5 \pm 0.5 \mu\text{A} \cdot \text{cm}^{-2}$  ( $n = 4$ ). Exposure to aldosterone plus 100- and 200-fold higher concentrations of spironolactone resulted in 94 and 75% inhibition of hormone-stimulated  $I_{sc}$ , compared to the effect induced by aldosterone alone. These results suggest that the action of aldosterone to stimulate electrogenic sodium transport was due to activation of the mineralocorticoid receptor.

## Discussion

Insights into mechanisms that modulate sodium channels in the apical membrane of epithelial cells are confounded by a

marked variation in the types of cation channels which have been studied. Although amiloride-sensitivity was demonstrable in most cases, there was heterogeneity in conductance and ion sensitivity and, in some preparations it was not determined that the channels selected for analysis participated in transepithelial movement of sodium. This heterogeneity may, at least in part, explain conflicting results in experiments designed to determine regulatory factors that activate amiloride-sensitive sodium channels. Exposure of frog skin to phorbol esters, for example, activated amiloride-sensitive sodium transfer across the epithelium (27). Under similar conditions, however, basal sodium absorption was inhibited in rabbit cortical collecting tubule (28) and cultured  $A_6$  cells (29). Phorbol esters also inhibited sodium uptake into LLC-PK cells via amiloride-sensitive sodium channels, although it was not shown that this pathway is involved in transepithelial movement of sodium (30). Recent evidence has also been provided that suggests that a G protein may regulate sodium channel activity in some cell types, because introduction of  $\text{GTP}\gamma\text{S}$  into membrane vesicles prepared from toad bladder epithelium stimulated amiloride-sensitive sodium transport severalfold (31), and direct application of  $\text{GTP}\gamma\text{S}$  and the  $\alpha_{i-3}$  subunit of  $G_i$  to the solution bathing the cytosolic surface of excised patches of apical membrane derived from rat renal inner medullary collecting tubule cells increased the open probability of nonselective cation channels (32). In addition, recent evidence suggests that the apical sodium channel in  $A_6$  cells grown on impermeable surfaces is activated by the  $\alpha_{i-3}$  subunit of  $G_i$  (33), and that the action of  $G_i$  is mediated through the regulation of phospholipase and lipoxigenase activities (34). It is not known, however, whether this signal transduction pathway is sensitive to known stimuli of sodium channel activity, such as aldosterone or arginine vasopressin.

The present experiments were performed to determine the second messenger system for the cellular action of aldosterone to increase amiloride-sensitive sodium transport in the early phase of hormone action in high-resistance monolayers of amphibian cells with highly selective sodium channels (35). Previous studies have shown no evidence of active potassium secretion (36). Although recent studies have provided important insights into the second messengers for cell expression induced by agonists that bind to cell surface receptors, there is little understanding of the internal transmission of signals generated by agonists, such as aldosterone, which interact with cytosolic or nuclear receptors that initiate transcriptional processes. However, because two major signal pathways have been identified for agonists which bind to surface receptors, one involving cyclic adenosine monophosphate (cAMP) and another involving calcium and products of phosphatidylinositol hydrolysis, we sought to determine whether aldosterone, via a transcription directed process, utilized one of these major second messenger pathways. The possibility that calcium/phosphatidylinositol is involved in the action of aldosterone seemed more likely because previous studies have indicated that aldosterone did not increase cAMP in toad bladder cells (11). Previous studies have shown, however, that the second messenger system involved in the cellular action of aldosterone interacts with the system utilized by arginine vasopressin, an agonist that binds to surface receptors, because prior exposure to aldosterone amplifies the action of vasopressin-induced increase in  $I_{sc}$  and cellular levels of cAMP (11, 36). Previous workers have suggested that these effects occur because of inhibition of the phosphodiesterase that degrades cAMP (11).

The present experiments strongly suggest that aldosterone stimulates electrogenic sodium transport by increasing  $\text{Ca}_i^{2+}$  transiently, possibly as a consequence of increasing the turnover of inositol phospholipid to produce  $\text{IP}_3$ . Observations that support this notion include (a) the temporal coincidence of increases in  $\text{Isc}$ ,  $\text{Ca}_i^{2+}$ , and  $\text{IP}_3$ , (b) the dependency of both the delayed rise in  $\text{Ca}_i^{2+}$  and the aldosterone-induced increase in  $\text{Isc}$  on both transcriptional and translational processes, and (c) experiments with the lipid-soluble calcium chelating agent BAPTA that showed a dependence of the rise in  $\text{Isc}$  on the delayed calcium transient. The effect of aldosterone to increase  $\text{Ca}_i^{2+}$  was observed in cells grown to confluency on porous, as well as nonporous surfaces. Moreover, it seemed likely that the action of aldosterone to stimulate  $\text{Isc}$  was dependent on activation of mineralocorticoid receptors, because spironolactone markedly attenuated the response.

This study, however, did not show that the transient rise in  $\text{Ca}_i^{2+}$  preceded activation of apical membrane conductance. In contrast to agonists that bind to cell membrane receptors and cause rapid changes in cell expression, the slow gradual increase in  $\text{Isc}$  precludes a precise determination of the time when the increase in  $\text{Isc}$  begins. It seems likely that this limitation would prevail even if estimates of  $\text{Isc}$ , or apical membrane permeability, and  $\text{Ca}_i^{2+}$  were made concurrently in the same cell.

The present studies suggest that calcium, as a second messenger, causes activation of a calmodulin-dependent enzyme, possibly a protein kinase, to increase apical membrane conductance. There is a possibility, in the use of synthetic molecular probes, that observed effects in whole cell preparations result from a mechanism other than the one intended. Inhibition of aldosterone-induced  $\text{Isc}$ , however, with different classes of calmodulin inhibitors, in a dose-dependent manner and in concentrations near the  $K_i$  of these agents for calmodulin-dependent enzymes reported in other tissues, strongly suggest a single action of W-7 and trifluoperazine, namely inhibition of a calmodulin-dependent enzyme.

Previous studies that implicate intracellular calcium as a second messenger for various agonist-induced alterations in cell expression in nonexcitable tissues have shown that  $\text{Ca}_i^{2+}$  is sustained at levels of 80–100 nM, except for transient increases that immediately precede changes in cell expression, even when sustained increases in the turnover of cellular calcium are associated with long lasting cellular action (38). Recently, it has also been shown that transiently elevated levels of  $\text{Ca}_i^{2+}$  may occur in an oscillating manner in some cell types, but increases lasting more than a few minutes have not been observed (38). In these studies, using fluorescent dyes to measure  $\text{Ca}_i^{2+}$ , the high transient levels of  $\text{Ca}_i^{2+}$  ranged from 200 to ~ 500 nM. Despite this information on the behavior of intracellular calcium there are numerous reports (see reference 23 for review) that suggest that chronic increases in  $\text{Ca}_i^{2+}$  down-regulate apical sodium channels and reduce transepithelial movement of sodium in epithelial cells, in contrast to the rise in electrogenic sodium transport found in this study. Because the design of those studies involved chronic sustained elevations of  $\text{Ca}_i^{2+}$  after addition of calcium ionophores in concentrations of 1  $\mu\text{M}$  or more to the outside bathing solution, it was of interest to determine the effect of ionophores in various doses on  $\text{Ca}_i^{2+}$  and the effect of chronic elevations of  $\text{Ca}_i^{2+}$  on basal and stimulated electrogenic sodium transport in cultured  $A_6$  cells.

The present studies show that  $\text{Ca}_i^{2+}$  was increased to supra-physiological levels by A23187 and ionomycin in concentra-

tions of 500 nM or more, and that under these conditions both basal and hormone-induced increases in electrogenic sodium transport were reduced. The possibility that this alteration in sodium transport is not related to a physiological control mechanism for down-regulation of apical sodium channels is suggested by both the large increases in  $\text{Ca}_i^{2+}$  and fall in the resistance of monolayers of transporting cells.

Previous reports have shown that the calcium-dependent action of some agonists, that which bind to cell membrane receptors, can be simulated when  $\text{Ca}_i^{2+}$  is increased by the addition of calcium ionophores to external bathing solution. Similar observations, however, have not been made in studies designed to evaluate the signal transduction system for agonists that involve the production of transcription-dependent effector proteins. The absence of a rise in  $\text{Isc}$  when  $\text{Ca}_i^{2+}$  was chronically increased to high physiological levels with A23187 cannot therefore be evaluated on the basis of previous comparable experiments. It is possible that an integrated transduction system for the cellular action of aldosterone requires a factor(s) in addition to increased  $\text{Ca}_i^{2+}$  to activate apical sodium channels. Because the initial increase in apical membrane sodium conduction is gradual in the first hour or two after exposure to aldosterone and results in small changes in  $\text{Isc}$ , it is also possible that an ionophore-induced increase in  $\text{Ca}_i^{2+}$  did initiate transient changes in apical conductance that were not detectable. In glomerulosa cells, for example, the A23187 induced increase in  $\text{Ca}_i^{2+}$  produces only a transient rise in aldosterone release, while the calcium-dependent action of angiotensin II is sustained for as long as the agonist is present (39).

A novel finding in this study, in addition to providing information on the second messenger system for the cellular action of aldosterone, is the delayed increase in  $\text{Ca}_i^{2+}$ . Although an increase in  $\text{Ca}_i^{2+}$  secondary to the interaction of agonist and receptor is established for many types of agonists, the initial onset of the rise of  $\text{Ca}_i^{2+}$  typically occurs very rapidly, as do changes in cellular function. In contrast, many hormones derived from thyroid, adrenal, or germinal tissues, which are characterized by slower responses and gradual deactivation, bind to intracellular receptors (40) and require gene transcription to induce changes in cellular function. The present study is the first demonstration, to our knowledge, that links calcium as a second messenger to a cellular expression of this class of hormones. These studies support the view that aldosterone and other hormones that act through intracellular receptors produce many of their physiological actions through the transcriptional regulation of intracellular second messenger pathways.

## Acknowledgments

The authors wish to thank Leena Kalghatgi for excellent technical assistance.

This work was supported by grant DK 18065 from the National Institutes of Health, and by a Veteran's Administration Career Development Award (M. B. Ganz).

## References

1. Crabbe, J. 1961. Stimulation of active sodium transport by the isolated toad bladder with aldosterone in vitro. *J. Clin. Invest.* 40:2103–2110.
2. Schwartz, G. J., and M. B. Burg. 1978. Mineralocorticoid effects on cation transport by cortical collecting tubules in vitro. *Am. J. Physiol.* 235:F576–F585.
3. Edmonds, C. V., and V. C. Marriott. 1970. Sodium transport and short-circuit current in rat colon in vivo and the effect of aldosterone. *J. Physiol. (Lond.)* 210:1021–1039.
4. Porter, G. A., B. Bogoroch, and I. S. Edelman. 1964. On the mechanism of

- action of aldosterone on sodium transport. The role of RNA synthesis. *Proc. Natl. Acad. Sci. USA*. 52:1326-1333.
5. Edelman, I. S., R. Bororoch, and G. A. Porter. 1963. On the mechanism of action of aldosterone on sodium transport. The role of protein synthesis. *Proc. Natl. Acad. Sci. USA*. 50:1169-1176.
  6. Civan, M. M., and R. E. Hoffman. 1971. Effect of aldosterone on electrical resistance of toad bladder. *Am. J. Physiol.* 220:324-330.
  7. Palmer, L. G., and N. Speez. 1986. Stimulation of apical Na permeability and basolateral Na pump of toad urinary bladder by aldosterone. *Am. J. Physiol.* 250:F273-F281.
  8. Geering, K., M. Claire, H. P. Gaeggeler, and B. C. Rossier. 1985. Receptor occupancy vs. induction of Na-K-ATPase and Na<sup>+</sup> transport by aldosterone. *Am. J. Physiol.* 248:C102-C108.
  9. Palmer, L. G., J. H.-Y. Li, B. Lindeman, and I. S. Edelman. 1982. Aldosterone control of the density of sodium channels in the toad urinary bladder. *J. Membr. Biol.* 64:91-102.
  10. Halevy, J., E. L. Boulpaep, N. J. Binder, and J. P. Hayslett. 1987. Aldosterone increases the maximal turnover rate of the sodium pump. *Pflugers Arch. Eur. J. Physiol.* 410:476-480.
  11. Stoff, J. S., J. S. Handler, and J. Orloff. 1972. The effect of aldosterone on the accumulation of adenosine 3':5'-cyclic monophosphate in toad bladder epithelial cells in response to vasopressin and theophylline. *Proc. Natl. Acad. Sci. USA*. 69:805-808.
  12. Perkins, F. M., and J. S. Handler. 1981. Transport properties of toad kidney epithelia in culture. *Am. J. Physiol.* 241:C154-C159.
  13. Ganz, M. B., S. K. Pekar, M. C. Peretto, and R. B. Sterzel. 1988. Arginine vasopressin promotes growth of rat glomerular mesangial cells in culture. *Am. J. Physiol.* 255:F898-F906.
  14. Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440-3450.
  15. Bredt, D. S., R. J. Mourey, and S. H. Snyder. 1989. A simple, sensitive, and specific radioreceptor assay for inositol 1,4,5-triphosphate in biological tissues. *Biochem. Biophys. Res. Commun.* 159:976-982.
  16. Rossier, B. C., H. P. Gaeggeler, and M. Rossier. 1978. Effects of 3' deoxyadenosine and actinomycin D in RNA synthesis in toad bladder: analysis of response to aldosterone. *J. Membr. Biol.* 41:149-166.
  17. Verrey, F., E. Schaerer, P. Zaerkler, M. P. Paccolat, K. Geering, J. P. Kraehenbuhl, and B. C. Rossier. 1987. Regulation by aldosterone of Na<sup>+</sup>, K<sup>+</sup>-ATPase on RNAs, protein synthesis and sodium transport in cultured kidney cells. *J. Cell Biol.* 104:1231-1237.
  18. Tsien, R. Y. 1980. New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis and properties of prototype structures. *Biochemistry*. 19:2396-2404.
  19. Foskett, J. K., P. J. Gunter-Smith, J. E. Melvin, and R. J. Turner. 1989. Physiological localization of an agonist-sensitive pool of Ca<sup>2+</sup> in parotid acinar cells. *Proc. Natl. Acad. Sci. USA*. 86:167-171.
  20. Smolen, J. E., H. M. Korchak, and G. Weissmann. 1981. The roles of extracellular and intracellular calcium in lysosomal enzymes release and superoxide anion generation by human neutrophils. *Biochim. Biophys. Acta*. 477:512-520.
  21. Hidaka, H., Y. Sasaki, T. Tahaka, T. Endo, S. Ohno, Y. Fujii, and T. Nagata. 1981. N-(6-Aminoethyl)-5-chloro-1-naphthalenesulfonamide, a calmodulin antagonist, inhibits cell growth. *Proc. Natl. Acad. Sci. USA*. 78:4354-4357.
  22. Prozialek, W. C., and B. Weiss. 1982. Inhibition of calmodulin by phenothiazines and related drugs: structure-activity relationships. *J. Pharmacol. Exp. Ther.* 222:509-516.
  23. Windhager, E. E. 1983. Regulatory role of intracellular calcium ions in epithelial Na transport. *Annu. Rev. Physiol.* 45:519-532.
  24. Frindt, G., and E. E. Windhager. 1990. Ca<sup>2+</sup> dependent inhibitors of sodium transport in rabbit cortical collecting tubules. *Am. J. Physiol.* 258:F568-F582.
  25. Foskett, J. K., and J. E. Melvin. 1989. Activation of salivary secretion: coupling of cell volume and [Ca<sup>2+</sup>]; in single cells. *Science (Wash. DC)*. 244:1582-1585.
  26. Halevey, J., E. L. Boulpaep, M. E. Budinzer, H. J. Binder, and J. P. Hayslett. 1988. Glucocorticoids have a different action than aldosterone on target tissue. *Am. J. Physiol.* 254:F153-F158.
  27. Civan, M. M., T. Rubenstein, T. Mauro, and T. G. O'Brien. 1985. Effects of tumor promoters on sodium ion transport across frog skin. *Am. J. Physiol.* 248:C457-C465.
  28. Hays, S. R., M. Baum, and J. P. Kokko. 1987. Effects of protein kinase C activation on sodium, potassium, chloride, and total CO<sub>2</sub> transport in the rabbit cortical collection tubule. *J. Clin. Invest.* 80:561-570.
  29. Yanase, M., and J. S. Handler. 1986. Activators of protein kinase C inhibit sodium transport in A<sub>6</sub> epithelia. *Am. J. Physiol.* 250:C517-523.
  30. Mohrmann, M., H. F. Cantiello, and D. A. Ausiello. 1987. Inhibition of epithelial Na<sup>+</sup> transport by atriopeptin, protein kinase C, and pertussis toxin. *Am. J. Physiol.* 253:F372-F376.
  31. Garty, H., O. Veger, A. Yanovsky, and C. Asher. 1989. Guanosine nucleotide-dependent activation of the amiloride-blockable Na<sup>+</sup> channel. *Am. J. Physiol.* 256:F965-F969.
  32. Light, D. B., D. A. Ausiello, and B. A. Stanton. 1989. Guanine nucleotide-binding protein, α<sub>s</sub>, directly activates a cation channel in rat renal inner medullary collecting duct cells. *J. Clin. Invest.* 84:352-356.
  33. Cantiello, H. F., C. R. Patenaude, and D. A. Ausiello. 1989. G protein subunit, α<sub>s</sub>, activates a pertussis toxin-sensitive Na<sup>+</sup> channel from the epithelial cell line, A<sub>6</sub>. *J. Biol. Chem.* 264:20867-20870.
  34. Cantiello, H. F., C. R. Patenaude, J. Codina, L. Birnbaumer, and D. A. Ausiello. G α<sub>s</sub> regulates epithelial Na<sup>+</sup> channels by activation of phospholipase A<sub>2</sub> and lipoxygenase pathways. *J. Biol. Chem.* 264:21624-21628.
  35. Hamilton, K. L., and D. C. Eaton. 1989. Regulation of single sodium channels in renal tissues: a role in sodium homeostasis. *Fed. Proc.* 45:2713-2717.
  36. Fanestil, D. C., G. A. Porter, and I. S. Edelman. 1967. Aldosterone stimulation of sodium transport. *Biochim. Biophys. Acta*. 135:74-88.
  37. Fedelman, M. L., R. L. Duncan, and C. O. Watlington. 1988. Effect of aldosterone on <sup>86</sup>Rb fluxes in cultured kidney cells (A<sub>6</sub>). *Physiol. Chem. Phys. Med. NMR*. 20:43-53.
  38. Berridge, M. J., and A. Galione. 1988. Cytosolic calcium oscillations. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 2:3074-3082.
  39. Rasmussen, H., I. Kojima, W. Apfeldorf, and P. Barrett. 1986. Cellular mechanism of hormone action in the kidney: messenger function of calcium and cyclic AMP. *Kidney Int.* 29:90-97.
  40. Evans, R. M. 1988. The steroid and thyroid hormone receptor superfamily. *Science (Wash. DC)*. 240:889-895.