Ca\(^{2+}\)-induced Down-regulation of Na\(^{+}\) Channels in Toad Bladder Epithelium*

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Regulation of epithelial Na\(^{+}\) channels was investigated by measuring the amiloride-blockable \(^{22}\)Na\(^{+}\) fluxes in apical membrane vesicles, derived from cells exposed to various treatments. Maximal amiloride-blockable \(^{22}\)Na\(^{+}\) uptake into vesicles was obtained if the cells were precultured at 25 °C in a Ca\(^{2+}\)-free [ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA)] solution. Including 10\(^{-5}\) M Ca\(^{2+}\) in the cell incubating medium blocked nearly all of the amiloride-sensitive flux in vesicles, even though the Ca\(^{2+}\) was removed before homogenization of the cells. This Ca\(^{2+}\)-dependent inhibition of Na\(^{+}\) channels could be induced in whole cells only; incubating cell homogenates with Ca\(^{2+}\) had no effect on the transport in vesicles. The dose-response relationships of this effect were measured by equilibrating cell aliquots with various Ca\(^{2+}\)-EGTA buffers, preparing membrane vesicles (in the absence of Ca\(^{2+}\) ions), and assaying them for amiloride-sensitive Na\(^{+}\) permeability. It was found that the Ca\(^{2+}\) blockage is highly cooperative (Hill coefficient of nearly 4) and is characterized by an inhibition constant which varies between 6.4 \times 10^{-8} to 8.15 \times 10^{-4} M Ca\(^{2+}\). Thus, it is likely that the above process is involved in the physiological control of Na\(^{+}\) transport. The Ca\(^{2+}\)-dependent transport changes were not affected by the calmodulin inhibitor trifluoperasine, vanadate (VO\(^{3+}\)), phorbol ester, colchicine, cytochalasin B, 3-deazaadenosine, and 8-bromo-CAMP. Vanadyl (VO\(^{2+}\)) ions, on the other hand, produced a "Ca\(^{2+}\)-like" inhibition of transport.

Luminal Na\(^{+}\) entry in many tight epithelial tissues is mediated by amiloride-blockable Na\(^{+}\)-specific channels (1–5). These channels are the main target to the mineralocorticoid action of aldosterone and are also regulated by antidiuretic hormones such as vasopressin (3, 6, 7). In addition to the hormonal control, Na\(^{+}\) channels appear to be subject to an intracellular feedback mechanism, whose function is to prevent acute changes in cell Na\(^{+}\) activity that would otherwise accompany variations in transepithelial transport rate (8). Although the ability of tight epithelia to control their apical Na\(^{+}\) permeability is well documented, the molecular mechanisms involved are not clear yet. Many studies, however, suggest that increases in the cytoplasmic Ca\(^{2+}\) activity can bring about a decrease in the apical conductance and that the function of this process is to couple the apical Na\(^{+}\) permeability to the basolateral Na\(^{+}\) gradient (9–13).

Chase and Al-Awqati (14) reported that incubating apical vesicles derived from toad bladder mucosal cells with Ca\(^{2+}\) ions decreases their amiloride-sensitive Na\(^{+}\) permeability. The Ca\(^{2+}\) effects were restricted to the cytoplasmic phase of the membrane and half-maximal inhibition was induced by 0.5 \mu M free Ca\(^{2+}\). The ability of Ca\(^{2+}\) ions to inhibit Na\(^{+}\) transport in isolated membrane vesicles suggests that this inhibition is mediated by an apo Ca\(^{2+}\)-specific membrane interaction. In a recent work (15), we provided evidence for a different type of Ca\(^{2+}\)-dependent down-regulation of channels. It was found that the amiloride-sensitive \(^{22}\)Na\(^{+}\) uptake by apical vesicles strongly depends on the conditions at which the cells were incubated prior to their homogenization. Accordingly, maximal channel activity in vesicles was obtained if the epithelial cells were preincubated in a Ca\(^{2+}\)-free EGTA\(^1\) solution for at least 30 min at 25 °C. Including Ca\(^{2+}\) (1 mM) in the cell incubation medium almost completely abolished the amiloride-sensitive uptake into vesicles, in spite of the fact that the membranes were isolated and assayed in Ca\(^{2+}\)-free solutions.

In the current paper we further characterize this Ca\(^{2+}\)-dependent temperature-sensitive regulation of Na\(^{+}\) channels. The Ca\(^{2+}\)-induced inhibition was found to be a highly cooperative process (Hill coefficient of nearly 4) with a K\(_{d}\) that varied, in different vesicle preparations, between 6.4 \times 10^{-8} and 8.15 \times 10^{-4} M Ca\(^{2+}\). Reagents known to influence several Ca\(^{2+}\)-dependent cellular events such as calmodulin inhibitors, phorbol ester, and cycoskeleton disrupting agents had no effect on this process, CAMP, which mediates the antidiuretic hormone-induced activation of Na\(^{+}\) channels and the methylidy inhibitor 3-deazaadenosine were also ineffective. Vanadyl (VO\(^{2+}\)) a potent inhibitor of alkaline phosphatase could mimic the Ca\(^{2+}\) effects, but vanadate (VO\(^{3+}\)) had no effect.

MATERIALS AND METHODS

Vesicle Preparation—Toads (Bufo marinus, Mexican origin, obtained from Lemberger, Oshkosh, WI) were doubly pithed and de-blooded by transventricular perfusion with 300–600 ml of Ringer's solution containing (in mM): NaCl, 110.0; CaCl\(_{2}\), 1.0; MgCl\(_{2}\), 0.5; and K-phosphate buffer, 3.5 (pH = 7.5). The urinary bladders were excised and rinsed several times in an ice-cold Ca\(^{2+}\)-free medium composed of (in mM): KCl, 90; sucrose, 45; MgCl\(_{2}\), 5; EGTA, 10; and a pH buffer, 10. The buffer used varied according to the experimental design and was Tris-HCl (pH = 7.8), TES (pH = 7.3), or PIPES (pH = 6.8). The epithelium was scraped off the underlying connective tissue with a glass slide, and the cells were dispersed in the above medium by rapidly drawing them in and out of a Pasteur pipette at least 10 times. The epithelial cells derived from a single animal were divided among 6–8 test tubes and pelleted by centrifugation at 1000 × g.

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X g for 5 min at 2°C. The cell pellets were washed twice by resuspension and centrifugation and then resuspended in either one of the above media or similar media containing various concentrations of CaCl₂ in addition to EGTA (see below). Different reagents were added according to the experimental design, and the suspensions were incubated for 30 min at 25°C. At the end of this period they were cooled back to 0°C, filtered 5-fold in homogenizing solution containing (in mM): KC₁, 90; sucrose, 45; MgCl₂, 5; EGTA, 10; and Tris- HCl, 10 (pH = 7.8), pelleted, suspended in 3 ml of this (Ca²⁺-free) medium, and immediately sheared by a single 10-s burst of a Polytron homogenizer (Ystral GmbH, Dottingen, Federal Republic of Germany) at highest speed. In one set of experiments the cells were suspended and homogenized in medium containing 90 mM K₂SO₄ instead of KCl. Intact cells, nuclei, and debris were removed by centrifuging the homogenates at 1000 × g for 5 min. The supernatants were then centrifuged for another hour at 27,000 × g to pellet the membrane fraction. The microsomal pellets were suspended in minimal volumes (200–400 μl) of the homogenizing medium, maintained at 6°C, and used the same day.

Transport Assay—Na⁺ uptake into toad bladder vesicles was measured in the presence of a membrane potential induced by a KCl gradient + valinomycin, as described previously (15–17). Microsomal preparations in Tris solutions. The suspensions were filtered through short Dowex (50W-X8, 30-50 mesh, Tris form) columns with 0.9 ml of 175 mM sucrose to exchange the extravesicular K⁺ by Tris. The pH of the eluted suspension was increased to 8.2 to maximize the tracer uptake (17), and valinomycin (5 μM) was added. Two 300-μl aliquots of the eluted vesicles were therefore added to reaction mixtures containing 80 μl of 175 mM sucrose, 5 μl of carrier-free ²²NaCl (final concentration 4 μCi/ml, 0.2–0.4 μM Na⁺) and 6 μl of either amiloride (final concentration 1.5 μM) or water diluent. The tracer uptake was measured by removing 150-μl aliquots (4–15 μg of protein) at 1.5 and 3.5 min from the radioactive suspensions and eluting them through Dowex columns into counting vials with 1.5 ml of ice-cold 175 mM sucrose. Usually the uptake was linear for at least 3.5 min. The amount of radioactivity associated with the particles was insensitive to the time the vesicles were on the column (20–60 s) or to the time delay between the formation of membrane potential and the addition of Na⁺ (0.5–2 min). This advantage is a consequence of the fact that the absence of membrane permeable cations in the external medium, the membrane potential dissipates very slowly, and internal Na⁺ does not leak after the removal of external radioactivity. The channel-mediated flux was calculated from the difference in internal radioactivity between the aliquots incubated with and without amiloride, and expressed as pmol of ²²Na⁺·mg of protein⁻¹·min⁻¹. In vesicles prepared from cells incubated in Ca²⁺-free solution, the amiloride-sensitive flux accounted for 10 mM EGTA. A high vacuum pump which gave a filtration rate of about 4 ml/s was used in order to minimize loss of internal radioactivity during the washing. The filters were immersed in scintillation fluid (xylene based) and counted in a β scintillation counter. The background radioactivity estimated from the sampling of a cell-free solution was less than 1% of the maximal Ca²⁺ uptake.

Ref. 18, assuming the following values: log Kₐ = 9.58; log Kₐ = 9.87; and log Kₐ = 10.955 (19). Very similar Ca²⁺-EGTA dissociation constants were obtained by taking the value measured by Harafushi and Ogawa (20) and correcting it for the different pH, temperature, and ionic strength of our experiments, using the equations suggested by them.

4²²Ca Flux Measurements in Whole Cells—Scraped epithelial cells were suspended in EGTA-free, homogenizing medium to a final concentration of 4.5 mg of protein/ml. The cells were either incubated with ⁴⁴CaCl₂ (100 nM, ~1 μM Ca²⁺) for different periods of time (influx assay) or incubated with the tracer for 10 min, followed by addition of 10 mM EGTA and incubation for additional timed periods (efflux assay). Sampling was done by filtering 100-μl aliquots through polycarbonate membrane filters (0.6 μm, Nucleapore Corp., Pleasonton, CA) and washing the cells with 4 ml of homogenizing medium containing 10 mM EGTA. To reduce the lipolysis which resulted from the washing procedure, 0.5 ml of 0.1% BSA (w/v) was added to the homogenizing medium. The EGTA concentration was chosen to minimize binding of the tracer to the medium while maintaining the EGTA concentration of the cells at 10 mM. The washing procedure was repeated until washing fluids contained less than 1% of the total radioactivity. The rate of efflux was determined by incubating the cells with tracer for 10 min, filtering the cells, and washing them five times with homogenizing medium devoid of additional CaCl₂. The radioactivity incorporated into the cells was measured by counting cell pellets (100 μl) in 2 ml of scintillation fluid.

Materials—Valinomycin, Trizma (Tris base), PIPES, TES, 8-(β-D-gluco-6-aminohexyl)-amino-3-methyl-chroman-4-one, phorbol 12-myristate 13-acetate, colchicine, and cytochalasin B were obtained from Sigma; tri-fluoperasine from Theraplix, France; daunomycin from the National Cancer Institute, Birmingham, AL; EGTA from Sigma or Fluka AG, and Dowex beads from Fluka AG (Buchs, Switzerland). NaCl (carrier-free, 1.66 ml/Ci/ml), CaCl₂ (17.4 ml/mg), and RbCl (1 ml/mg; 507 μg of Rb/ml) were purchased from Amersham Radiochemicals (Buckinghamshire, United Kingdom). Amiloride was a gift from Merck, Sharp and Dohme (GmbH, Munich, Federal Republic of Germany). All conventional chemicals were analytical grade.

RESULTS

Inhibition of Na⁺ Transport by Ca²⁺—As was recently reported (15), incubating scraped toad bladder epithelial cells for 30 min at 25°C in a Ca²⁺-free solution increases by more than 4-fold the amiloride-sensitive ²²Na⁺ flux measured in membrane vesicles derived from them. This activation was prevented if Ca²⁺ ions (≥1 μM) were present in the cell incubating media (even though removed before the homogenization) or if cells were maintained at 0°C. On the basis of these data it was concluded that the toad bladder Na⁺ channels are down-regulated by a temperature-sensitive Ca²⁺-dependent reaction which takes place in whole cells and induces a stable change in the membrane lipids and/or proteins preserved by the isolated vesicles.

The ability of Ca²⁺ ions to prevent the activation of channels was further examined in the experiments summarized in Table I. Vesicles prepared from cells incubated under the control conditions (i.e. for 30 min at 25°C in a Ca²⁺-free solution were added to the vesicle suspension. The mean amiloride-blockable and amiloride-sensitive ²²Na⁺ accumulation in vesicles prepared from cells incubated in Ca²⁺-free solution contained 10 mM EGTA + 10 mM CaCl₂ (10⁻⁵ M free Ca²⁺ at pH = 7.8); C, for 30 min in a solution containing 1 mM EGTA + 1 mM BaCl₂; D, as for B plus an additional 30-min incubation in a Ca²⁺-free 10 mM EGTA medium; E, as for A. The incubations were terminated by cooling the suspensions to 0°C and diluting them 1-fold with the Ca²⁺-free homogenizing medium. Cells were pelleted, suspended in the same medium, and homogenized. To aliquot E, Ca²⁺-containing solution was added after the homogenization to a final concentration of 10 mM EGTA + 10 mM CaCl₂ (10⁻⁶ M free Ca²⁺), and this was incubated for an additional 15 min at 25°C. Vesicles were isolated and ²²Na⁺ uptake measured as described under "Materials and Methods." The mean amiloride-blockable and amiloride-insensitive fluxes, expressed as a fraction of the control values (condition A), are presented. The mean amiloride-sensitive and amiloride-insensitive fluxes under condition A were 11.4 ± 1.5 [51] and 3.4 ± 0.3 [51] pmol of ²²Na⁺·mg of protein⁻¹·min⁻¹, respectively.

Table I

| Vesicle Preparation | ²²Na⁺ Uptake | ²²Na⁺ Uptake |
|---------------------|-------------|-------------|
| Cell incubating conditions | Amiloride-sensitive | Amiloride-insensitive |
| A. EGTA | 1.00 | 1.00 |
| B. 10⁻⁵ M Ca²⁺ | 0.19 ± 0.02 [31] | 0.92 ± 0.05 [31] |
| C. 10⁻⁶ M BaCl₂ | 1.00 ± 0.05 [49] | 0.98 ± 0.06 [49] |
| D. 10⁻⁵ M Ca²⁺ followed by an incubation in EGTA | 0.22 ± 0.07 [3] | 0.98 ± 0.02 [3] |
| E. EGTA, followed by an incubation of the broken cells in 10⁻⁵ M Ca²⁺ | 1.08 ± 0.09 [5] | 1.04 ± 0.05 [5] |
EGTA solution) had mean amiloride blockable and amiloride-insensitive initial rates of 11.4 ± 1.5 [31] and 3.4 ± 0.3 [31] pmol of $^{22}$Na$^+$ mg of protein$^{-1}$ min$^{-1}$, respectively. 2 Including 10$^{-5}$ M free Ca$^{2+}$ in the cell incubating medium lowered the amiloride-insensitive uptake to 2.24 ± 0.12 pmol of $^{22}$Na$^+$ mg of protein$^{-1}$ min$^{-1}$ (19.7 ± 2.5% of the control value) and hardly affected the amiloride-insensitive flux (3.11 ± 0.16 pmol of $^{22}$Na$^+$ mg of protein$^{-1}$ min$^{-1}$ or 91.6 ± 4.9% of the control value). This inhibition was not observed by incubating cells with Ba$^{2+}$ (1 mM) which is known to mimic some of the Ca$^{2+}$ effects in adrenal medulla cells (21, 22). The decrease in Na$^+$ uptake induced by Ca$^{2+}$ was irreversible; incubating Ca$^{2+}$ treated cells for an additional period of 30 min in a Ca$^{2+}$-free EGTA solution did not increase the Na$^+$ flux in vesicles. In another set of experiments Ca$^{2+}$ was applied to homogenates of cells preactivated in EGTA. Addition of Ca$^{2+}$ ions to a broken cell suspension followed by their removal before the transport assay had no effect on the Na$^+$ permeability in vesicles, i.e. the above inhibition can be induced in whole cells only. It therefore either involves a structural component which is destroyed by the homogenization or requires a cytoplasmic factor which is largely diluted during the shearing of the cells.

Possible Sources of the Ca$^{2+}$ Effect—The observed Ca$^{2+}$-induced decrease in amiloride-sensitive Na$^+$ uptake may in principle result from one of three events. 1) A decrease in the apical Na$^+$ permeability, i.e. closure or internalization of Na$^+$ channels. 2) An increase in the apical permeability to other ion, in particular C1$^-$ (23) and therefore depolarization of the driving potential. 3) A decrease in the yield or dimensions of the channel containing particles. Elimination of the two last, less interesting, possibilities was made by substituting SO$^-$ for Cl$^-$, looking at the details of the full time course of $^{22}$Na$^+$ uptake, and comparing the uptake of $^{86}$Rb$^+$ into control and Ca$^{2+}$-inhibited vesicles. First, the tracer fluxes were measured in vesicles prepared to contain 90 mM K$_2$SO$_4$ instead of KCl.$^3$ It was found that pretreatment of cells with 10$^{-3}$ M Ca$^{2+}$ lowered the amiloride-sensitive uptake into these vesicles to 13 ± 2% [3] of the control value, i.e. the Ca$^{2+}$-induced inhibition of transport can be demonstrated in the complete absence of Cl$^-$ and therefore cannot stem from an increased permeability of this ion. The second protocol used to exclude the possibility that Ca$^{2+}$ acts on the permeability of other ions, was to measure the full time course, i.e. both the initial accumulation and the following efflux of $^{22}$Na$^+$ (Fig. 1). As shown previously, both experimentally and by computer simulations, a decrease in the initial uptake induced by an increase in the permeability to other ions, is accompanied by a faster efflux due to the enhanced dissipation of membrane potential (16, 24). The data of Fig. 1 clearly show that the inhibition of $^{22}$Na$^+$ uptake is not accompanied by an earlier and faster loss of internal radioactivity, i.e. the time course of $^{22}$Na$^+$ fluxes measured in vesicles derived from Ca$^{2+}$-treated cells is as expected from Ca$^{2+}$ affecting the Na$^+$ permeability and is different from the kinetic behavior predicted by an increased leakiness of the particles.$^4$

$^2$ Note that the assay mixture contained 1.5 μM amiloride only, i.e. the "amiloride-blockable" component in this study is the "high affinity phase" of (15, 16).

$^3$ Since the activity coefficient of K$^+$ in SO$_4$ solutions is about 0.55, the K$^+$ activity of 90 mM K$_2$SO$_4$ is only slightly higher than of 90 mM KCl.

$^4$ Unlike before (16, 24), lowering the Na$^+$ permeability did not considerably slow down $^{22}$Na$^+$ efflux. The reason for this difference is that in the present study the potential was induced by a KCl gradient and not by a NaCl gradient. Thus, the decrease of Na$^+$ permeability had no direct effect on the potential dissipation rate.

Possible effects of Ca$^{2+}$ on the yield or dimensions of the channel containing vesicles can be assessed by comparing the intravesicular spaces in membrane preparations derived from control and Ca$^{2+}$-treated cells. The common methods for measuring an intravesicular volume (e.g. comparing the space accessible to $^3$H water and a nonpermeable solute) will estimate the overall intravesicular volume of our crude membrane preparation and may therefore overlook Ca$^{2+}$-induced changes in a subpopulation of the particles. An alternative measurement which could be more sensitive to a change of the apical volume is evaluating the steady state distribution of $^{86}$Rb$^+$ across the vesicle membrane. In the presence of valinomycin $^{86}$Rb$^+$ will equilibrate according to the membrane potential (Δψ) and its steady state distribution (cpm$\text{in}/$cpm$\text{out}$) will be equal to (V$\text{in}$/V$\text{out}$)$^e^{Δψ/RT}$ (when V$\text{in}$ and V$\text{out}$ are the intra- and extravesicular volumes). The $^{86}$Rb$^+$ accumulation will therefore preferentially measure the internal volume of "tight" (maximal Δψ) vesicles. Moreover, by comparing the steady state $^{86}$Rb$^+$ uptake in the presence and absence of external Na$^+$, it is possible to estimate the volume of a vesicle subpopulation which is impermeable to Cl$^-$ and permeable to Na$^+$, i.e. the apical particles. Table II summarizes measure-
ments of $^{86}$Rb$^+$ and $^{22}$Na$^+$ uptake in vesicles derived under matched conditions from control and Ca$^{2+}$-treated cells. Unlike $^{22}$Na$^+$ fluxes which were relatively slow, $^{86}$Rb$^+$ readily equilibrated across the membrane of valinomycin-treated vesicles, and the internal activity measured after a 2-min incubation represents its equilibrium distribution. As seen from Table II, $^{86}$Rb$^+$ was taken up to a large extent only in the presence of valinomycin. This accumulation was completely prevented if K$^+$ (25 mM) was present in the external solution, by entering through amiloride-insensitive electrogenic pathways representing its equilibrium distribution. As seen from two measurements. 

$\text{Ca}^{2+}$ Dose-Response Relationships—Valuable information on the nature of the above Ca$^{2+}$ effect and its possible involvement in the physiological control of Na$^+$ transport can be obtained from measurements of the Ca$^{2+}$ dose-response relationships. Such measurements are meaningful only if the cell membrane is sufficiently permeable to Ca$^{2+}$ ions, i.e. setting the external Ca$^{2+}$ activity at a given value will buffer the cytoplasm at the same value. Normally the toad bladder plasma membrane is Ca$^{2+}$-impermeable, and a substantial influx of Ca$^{2+}$ into intact cells can only be induced with an ionophore or a favorable basolateral Na$^+$ gradient (11,13,25). However, it is possible that the scraping and dispersion of epithelial cells induces a limited mechanical damage of the plasma membrane and permeabilizes them to Ca$^{2+}$ ions (15, 26). The Ca$^{2+}$ permeability of whole cells was evaluated by measuring $^{45}$Ca$^{2+}$ influx under conditions similar to those used to pretreat them with Ca$^{2+}$/EGTA mixtures (Fig. 2). Indeed, saturating $^{45}$Ca$^{2+}$ influx and efflux in a time scale of a few minutes could be observed. Thus, it is feasible that buffering the external Ca$^{2+}$ activity at a given value will soon clamp the cytoplasmic free Ca$^{2+}$ to the same value.

The Ca$^{2+}$ dose-response relationships were measured by assaying the amiloride-blockable fluxes in vesicles derived from cell aliquots incubated with different Ca$^{2+}$/EGTA mixtures. In one series of 8 experiments carried out during November 1984–January 1985, the amiloride-sensitive $^{22}$Na$^+$ uptake was almost completely blocked by $10^{-6}$ M Ca$^{2+}$. The Ca$^{2+}$-inhibition curve was measured in 6 of these preparations by incubating cell aliquots at pH = 7.8. This pH was chosen in order to match the effective buffering range of EGTA (apparent Ca$^{2+}$/EGTA dissociation constant of $9.5 \times 10^{-6}$ M$^{-1}$ at pH = 7.8) to the inhibitory concentrations of free Ca$^{2+}$. The experimental data, depicted in Fig. 3A, could be fitted to an inhibition curve with a $K_i$ value of $6.4 \times 10^{-6}$ M Ca$^{2+}$ and a Hill coefficient of 3.9 ± 0.4. In another series of 8 experiments carried out during September–November 1985, using the same protocols, $10^{-3}$ M Ca$^{2+}$ had no effect, but the uptake could still be inhibited with $10^{-4}$ M Ca$^{2+}$. The Ca$^{2+}$ inhibition curve was then remeasured in another five preparations in which the cells were incubated at pH = 6.8 (apparent Ca$^{2+}$/EGTA dissociation constant of $1.0 \times 10^{-6}$ M$^{-1}$). The results of these measurements, shown in Fig. 3B, could be fitted to an inhibition curve with a $K_i$ value of $8.15 \times 10^{-6}$ M Ca$^{2+}$ and a Hill coefficient of 3.7 ± 0.6. Moreover, incubating cells with low concentrations of Ca$^{2+}$ (1–4 μM) seemed to produce a slight activation of transport. It should be emphasized that the shift in the Ca$^{2+}$ inhibition curve observed was not the result of a different experimental protocol or different batch of EGTA (27). We attribute this shift to physiological changes in the intracellular activities of factors which modulate the Ca$^{2+}$-induced reaction. This behavior is in fact reminiscent of the changes observed in the affinity of protein kinase C to Ca$^{2+}$ ions in the presence of different lipids (28, 29). However, including the phorbol ester 4-phorbol 12-myristate 13-acetate
(100 ng/ml) in the cell incubating medium failed to shift the inhibition curve shown in Fig. 3B to lower values. Such a shift is expected if Ca⁺⁺ inhibits Na⁺ transport by activating protein kinase C. Consequently, some other physiological change must be involved.

Possible Mechanisms of the Ca⁺⁺-induced Inhibition of Channels—In an attempt to shed light on the molecular events that mediate the permeability changes, we studied effects of several reagents on the activation of channels in EGTA solutions and their inhibition by 10⁻⁵ M Ca⁺⁺ (Table III). The preparations used in this set of experiments had a Ca⁺⁺ inhibition constant in the micromolar range, i.e. at 10⁻⁵ M Ca⁺⁺ most of the channels were closed, but the divalent ion was not present in a large excess. Preincubating cells with the lipid-soluble calmodulin inhibitor trifluoperasine had no significant effect on the base-line channel-mediated uptake or its inhibition by Ca⁺⁺. Thus, Ca⁺⁺-calmodulin interaction does not seem to play a role in the regulation of Na⁺ channels. Vanadate (VO₄²⁻), known to inhibit many phosphate transfer reactions (30), did not influence the Na⁺ permeability changes. On the other hand, vanadyl (VO₅²⁻) strongly inhibited Na⁺ uptake into vesicles derived from cells incubated in EGTA and increased the inhibition observed with 10⁻⁵ M Ca⁺⁺. These effects were observed only with freshly made vanadyl solutions presumably due to its atmospheric oxidation to vanadate. No major effect on transport in vesicles was observed upon incubating scraped cells and whole bladders with the cytoskeleton-disrupting agents colchicin and cyto-

**TABLE III**

*Effects of various reagents on the amiloride-sensitive flux in vesicles*

Scraped cells were suspended at 0 °C either in a Ca⁺⁺-free EGTA solution or in solution buffered to 10⁻⁵ M Ca⁺⁺ (pH = 7.3). Half of each suspension received one of the above listed reagents at the indicated concentration and the other half an equal volume of diluent. After an initial period of 5 min at 0 °C (to enable the penetration of the tested drug), the cells were incubated at 25 °C for 30 min. The incubation was terminated by cooling the cells to 0 °C and diluting them into a Ca²⁺-free EGTA solution. Cells were pelleted, suspended in the Ca⁺⁺-free medium, and homogenized. Membrane vesicles were prepared and assayed for amiloride-sensitive uptake as described under "Materials and Methods." The amiloride-blockable fluxes, expressed as a fraction of the control values (no added reagents, Ca⁺⁺-free solution) in the same experiment, are presented.

| Additions            | Amiloride-sensitive ²²Na⁺ uptake |
|----------------------|---------------------------------|
|                      | Cells incubated in Ca⁺⁺-free solution | Cells incubated with 10⁻⁵ M Ca⁺⁺ |
| A. No additions      | 1.00 ± 0.02 [4]                  | 0.21 ± 0.05 [4]                  |
| Trifluoperasine (10⁻⁴ M) | 0.73 ± 0.13 [4]                  | 0.17 ± 0.04 [4]                  |
| B. No additions      | 1.00 ± 0.04 [4]                  | 0.18 ± 0.04 [4]                  |
| VO₄²⁻ (10⁻⁵ M)       | 0.85 ± 0.09 [5]                  | 0.21 ± 0.05 [4]                  |
| VO₅²⁻ (10⁻³ M)       | 0.05 ± 0.02 [3]                  | 0.05 ± 0.03 [4]                  |
| C. No additions      | 1.00 ± 0.05 [3]                  | 0.15 ± 0.05 [3]                  |
| Colchicine (10⁻⁴ M)   | 0.70 ± 0.04 [4]                  | 0.16 ± 0.05 [4]                  |
| Cytoskeleton B (20 µg/ml) | 0.90 ± 0.07 [3]                  | 0.09 ± 0.03 [3]                  |
| 3-Deazaadenosine (2 x 10⁻⁵ M) | 0.91 ± 0.04 [5]                  |

*To avoid the atmospheric oxidation of VO₂⁺ to VO₃⁻, VO₂SO₃ was dissolved in the incubating medium shortly before the experiment.

**TABLE IV**

*Effects of 8Br-cAMP on the amiloride-sensitive ²²Na⁺ uptake*

Scraped cells were divided into 6 aliquots and incubated under various conditions in the presence of either 8Br-cAMP (1 mM) + 3-isobutyl-1-methylxanthine (IBMX) (0.1 mM) (treated) or water diluent (control). At the end of the incubation period the aliquots were cooled to 0 °C, pelleted, resuspended in the standard Ca⁺⁺-free medium, and immediately homogenized. Vesicles were isolated and ²²Na⁺ fluxes measured as described under "Materials and Methods." The amiloride-blockable fluxes presented are expressed as fractions of the fluxes measured in vesicles isolated from cells incubated for 30 min at 25 °C in Ca⁺⁺-free solutions.

| Cell incubation conditions | Amiloride-blockable ²²Na⁺ uptake |
|---------------------------|---------------------------------|
|                          | Control | Treated (8Br-cAMP + IBMX) |
| 30 min at 25 °C in a Ca⁺⁺-free solution | 1.00 | 1.08 ± 0.13 [4] |
| 30 min at 25 °C in the presence of 7 x 10⁻⁵ M Ca⁺⁺ | 0.50 ± 0.06 [4] | 0.57 ± 0.15 [4] |
| 10 min at 25 °C in a Ca⁺⁺-free solution | 0.47 ± 0.11 [3] | 0.40 ± 0.08 [3] |

**DISCUSSION**

The present paper examines the effects of Ca⁺⁺ ions interacting with whole, permeabilized, toad bladder cells on the channel-mediated ²²Na⁺ fluxes assayed in isolated membrane vesicles. As before (15) we observed that the amiloride-sensitive Na⁺ flux in vesicles is large if cells are preincubated at 25 °C in a Ca⁺⁺-free EGTA solution and is greatly reduced if the cells are exposed to 10⁻⁶ M Ca⁺⁺. By monitoring the full time course of tracer uptake and measuring the steady state ⁹⁹ᵐ⁺ distribution, we were able to exclude the possibilities that inhibition of transport in vesicles results from depolariza-
zation of the membrane or a decrease in the vesicular volume. The data of Table II do not totally exclude the possibility that Ca\(^{2+}\) ions act by preventing the vesiculation of a membrane fraction which is so minor as not to be detected by \(^{22}\)Na\(^{+}\) uptake, but happens to contain all the Na\(^{+}\) channels. However, such an effect induced by a transient exposure of whole cells to 10\(^{-6}\) M Ca\(^{2+}\) seems unlikely. The likely remaining possibility is that Ca\(^{2+}\) induces a reaction which lowers the Na\(^{+}\) permeability of the apical membrane. This putative closure of channels should not be confused with the inhibition observed by interaction of the isolated membrane with Ca\(^{2+}\) ions (14, 16). We too observed a direct inhibition of channels by including Ca\(^{2+}\) ions in the isolated vesicles, and this process is different from the currently discussed effect in many aspects.

The inhibition of transport could not be reversed by transferring cells from the Ca\(^{2+}\)-induced incubation medium to a Ca\(^{2+}\)-free EGTA solution (Table I). One explanation for this irreversibility is that the prolonged incubation of permeabilized cells in the homogenizing buffer depletes them of a factor required for the activation of channels upon the removal of Ca\(^{2+}\). Similarly we observed that Ca\(^{2+}\) ions applied to cells preincubated for 30 min in EGTA induced only a partial inhibition of \(^{22}\)Na\(^{+}\) flux (cf. Table IIIID in Ref. 15). An alternative explanation is that the recovery from an increase in the cytoplasmic Ca\(^{2+}\) activity involves relatively slow processes such as cannot be detected within 1/2 h. Prolonged effects induced by a transient increase of cell Ca\(^{2+}\) were observed in other systems too (35). The apparent irreversibility of the inhibition also raises the possibility that the permeability change reflects unspecific damage to the apical surface by a Ca\(^{2+}\)-activated lipase or protease, released to the external medium from lysed cells. This potential artifact is excluded by the observation that the Ca\(^{2+}\)-dependent down-regulation of channels cannot be induced in broken cell suspensions. The fact that the inhibition of transport depends on the cell integrity indicates that it either involves structural components which are destroyed by the shearing of cells (e.g. membrane-cytoskeleton or plasma-membrane-internal vesicles interactions) or requires a soluble component which is diluted too much during the homogenization.

The Ca\(^{2+}\) dose-response relationships were measured by incubating aliquots of scraped, permeabilized cells with different Ca\(^{2+}\)/EGTA mixtures. This procedure yielded inhibition curves characterized by an Hill coefficient of 3.5-4.0 and two different values of \(K_i\). The variability in the measured \(K_i\) is too large to be accounted for by EGTA impurities (27) or experimental errors. It was also independent of the source of chemicals used or the cell incubating pH. The most likely explanation for the different \(K_i\) values obtained in different experiments is spontaneous variations in the concentration of cellular Ca\(^{2+}\) binding proteins or in their affinity to this ion. One example for such behavior is the dramatic effect of the lipid composition on the activation of protein kinase C by Ca\(^{2+}\) (28). The possibility that Ca\(^{2+}\) blocks channels by inducing protein phosphorylation is supported by the inhibitory effect of VO\(^{2+}\). On the other hand, the fact that VO\(^{2+}\) and 4-phorbol 12-myristate 13-acetate had no effect on the Ca\(^{2+}\) inhibition curve argues against this view. Regardless of the measured value of the inhibition constant, it is clear from the data that closure of Na\(^{+}\) channels is induced by Ca\(^{2+}\) concentrations which are within the physiological range of intracellular Ca\(^{2+}\) and the process is highly cooperative. The strong dependence of Na\(^{+}\) transport on cell Ca\(^{2+}\) means that large permeability changes can be induced by small variations in cell Ca\(^{2+}\). This point is of special importance since recent estimations of the intracellular Ca\(^{2+}\) in toad bladder cells failed to indicate large changes in its activity in response to changes in the external osmolarity, Na\(^{+}\) activity, or the presence of vasopressin (58, 36, 37).

In an attempt to identify the Ca\(^{2+}\)-activated reaction which down-regulates Na\(^{+}\) channels we examined the influence of various reagents, added to the cell incubating medium, on the transport in vesicles. Most of the reagents tested should readily permeate the cell membrane and reach their potential intracellular target site within minutes. Two others, VO\(^{2+}\) and colchicine, are less permeable but may enter the scraped, permeabilized cells. In addition, the bladders were exposed to colchicine for 4 h, a period that was sufficient to evoke the inhibitory effect of this drug on the hydroosmotic response (38). None of the tested reagents besides vanadyl appeared to have an effect on the base-line channel-mediated flux or on the ability of Ca\(^{2+}\) ions to inhibit it. Thus, the current data do not support the possibilities that the transport changes measured in this study require a Ca\(^{2+}\)-calmodulin interaction, involve methyl transfer reaction, or are mediated by Ca\(^{2+}\)-dependent membrane-cytoskeleton interactions. In addition, 8Br-CAMP failed to enhance the \(^{22}\)Na\(^{+}\) uptake in vesicles or reduce its inhibition by Ca\(^{2+}\) ions, in spite of the well established augmentation in Na\(^{+}\) transport induced by this cyclic nucleotide in intact bladders. This result suggests that the mechanism we have identified is different from the one involved in the natriuretic response to antidiuretic hormones. Alternatively, it is possible that CAMP mediates its action on Na\(^{+}\) permeability by lowering Ca\(^{2+}\) and, thus, is ineffective once the cell Ca\(^{2+}\) had been buffered to a given value, by suspending permeabilized cells in the Ca\(^{2+}\)/EGTA mixture.

Complete inhibition of the channel-mediated flux in vesicles was also induced by incubating cells with 1 mM VO\(^{2+}\), and vanadyl also abolished transport in vesicles derived from cells that were incubated with submaximal concentration of Ca\(^{2+}\). From the fact that an equivalent amount of vanadate had no effect on \(^{22}\)Na\(^{+}\) uptake, one may conclude that the active ion is indeed VO\(^{2+}\) and not one of its oxidation products (39). The observed effects of vanadyl may be accounted for by at least 3 mechanisms. One possibility is that VO\(^{2+}\) acts as a Ca\(^{2+}\) analog and activates the same process triggered by Ca\(^{2+}\) ions. A second possibility is that VO\(^{2+}\) causes a transient increase of the intracellular level of free Ca\(^{2+}\), either by blocking Ca\(^{2+}\)-ATPase or by displacing bound Ca\(^{2+}\). The possibility that VO\(^{2+}\) displaces Ca\(^{2+}\) bound to EGTA can, however, be excluded since full inhibition is observed upon incubation of cells with 10 mM EGTA, 1 mM VO\(^{2+}\), and no added Ca\(^{2+}\). Finally it is possible that vanadyl may act by inhibiting a phosphatase (40). According to this interpretation Na\(^{+}\) channels are activated by protein dephosphorylation and can be down-regulated either by inhibiting this process with VO\(^{2+}\) or activating phosphorylation with Ca\(^{2+}\).

In summary, the above data establish the finding that Ca\(^{2+}\) can down-regulate Na\(^{+}\) channels by activating a process which takes place in whole cells only but induces a stable modification of a membrane component preserved by the isolated vesicles. This process is presumably involved in the physiological control of Na\(^{+}\) transport, but the molecular events which mediate it are as yet unknown.

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