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Direct Inhibition of Epithelial Na^+ Channels by a pH-Dependent Interaction with Calcium, and by Other Divalent Ions

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Summary. Direct inhibitory effects of Ca^{2+} and other ions on the epithelial Na^+ channels were investigated by measuring the amiloride-blockable $^{22}Na^+$ fluxes in toad bladder vesicles containing defined amounts of mono- and divalent ions. In agreement with a previous report (H.S. Chase, Jr., and Q. Al-Awqati, *J. Gen. Physiol.* **81**:643–666, 1983) we found that the presence of micromolar concentrations of Ca^{2+} in the internal (cytoplasmic) compartment of the vesicles substantially lowered the channel-mediated fluxes. This inhibition, however, was incomplete and at least 30% of the amiloride-sensitive $^{22}Na^+$ uptake could not be blocked by Ca^{2+} (up to 1 mM). Inhibition of channels could also be induced by millimolar concentrations of Ba^{2+}, Sr^{2+}, or VO^{2+}, but not by Mg^{2+}. The Ca^{2+} inhibition constant was a strong function of pH, and varied from 0.04 μM at pH 7.8 to >10 μM at pH 7.0. Strong pH effects were also demonstrated by measuring the pH dependence of $^{22}Na^+$ uptake in vesicles that contained 0.5 μM Ca^{2+}. This Ca^{2+} activity produced a maximal inhibition of $^{22}Na^+$ uptake at pH $\geq$ 7.4 but had no effect at pH $\leq$ 7.0. The tracer fluxes measured in the absence of Ca^{2+} were pH independent over this range. The data is compatible with the model that Ca^{2+} blocks channels by binding to a site composed of several deprotonated groups. The protonation of any one of these groups prevents Ca^{2+} from binding to this site but does not by itself inhibit transport. The fact that the apical Na^+ conductance in vesicles, can effectively be modulated by minor variations of the internal pH near the physiological value, raises the possibility that channels are being regulated by pH changes which alter their apparent affinity to cytoplasmic Ca^{2+}, rather than, or in addition to changes in the cytoplasmic level of free Ca^{2+}.

Key Words amiloride · apical membrane · Ca^{2+} inhibition · epithelial transport · membrane vesicles · Na^+ channels · toad bladder

Introduction

Luminal Na^+ entry in several tight epithelia is mediated by amiloride-blockable Na^+-specific channels (Lindemann, 1984; Schultz, 1984). These channels are the main pathway influenced by mineralocorticoids and antidiuretic hormones (Li et al., 1982; Palmer et al., 1982; Taylor & Palmer, 1982; Helman, Cox & Van Driessche, 1983; Garty, 1986) and also appear to be controlled by an intracellular feedback mechanism the function of which is to prevent an acute accumulation of Na^+ in the cell (Schultz, 1981). Although the ability of epithelial cells to control their apical Na^+ permeability is well documented, the molecular mechanisms involved in these processes are mostly unknown. Many studies, however, indicate that an increase of the cytoplasmic Ca^{2+} activity brings about inhibition of Na^+ transport and this process can couple the apical Na^+ permeability to the basolateral Na^+ gradient (Grinstein & Erlij, 1978; Taylor & Windhager, 1979; Chase & Al-Awqati, 1981; Arruda, Sabatini & Westenfelder, 1982; Chase, 1984; Garty & Lindemann, 1984). In principle, Ca^{2+} ions can inhibit Na^+ channels either directly or indirectly. One possibility is that the increase of cell Ca^{2+} activates enzyme(s) (e.g. kinase, phospholipase, or protease), and initiates events which bring about closure or internalization of channels. Evidence for the existence of such a mechanism was recently provided by Garty and Asher (1985, 1986). In these studies it was shown that exposing whole, permeabilized toad bladder cells to increasing concentrations of Ca^{2+}, induces a stable inhibition of channels, which is preserved in apical membrane vesicles isolated from these cells, even when Ca^{2+} has been removed from the vesicle preparation. Another possibility is that Ca^{2+} ions block the Na^+ channel directly by means of an electrostatic interaction with the channel protein. This possibility is supported by the observation that including Ca^{2+} ions in isolated membrane vesicles lowers their amiloride-sensitive Na^+ conductance (Chase & Al-Awqati, 1983; Garty, 1984). This inhibition, unlike the previous effect, requires the presence of Ca^{2+} ions inside the vesicles during the transport assay.

In the present study we further examine this,

presumably direct, Ca^{2+} channel interaction, and study the effects of other ions as well. We find that including submicromolar concentrations of free Ca^{2+} in toad bladder apical vesicles substantially lowers their amiloride-blockable Na^+ conductance. The effect is fully reversible and can also be induced by higher concentrations of Ba^{2+}, Sr^{2+}, or VO^{2+}, but not by Mg^{2+}. The intravesicular Na^+ activity, on the other hand, does not affect the Na^+ permeability. The Ca^{2+} inhibition constant measured was strongly dependent on the intravesicular pH. 0.5 μM free Ca^{2+} was sufficient to produce maximal inhibition of Na^+ transport at pH $\geq$ 7.4, but the same concentration had no effect at pH $\leq$ 7.0. The data suggest that physiological control of Na^+ channels by Ca^{2+} ions may involve alkalization and an increase in the apparent affinity of the channels to Ca^{2+}, rather than the elevation of the Ca^{2+} concentration in the cells. This direct, pH-dependent inhibition of channels by internal Ca^{2+} is different from the Ca^{2+}-dependent, temperature-sensitive process studied before (Garty & Asher, 1985, 1986).

Abbreviations

EGTA, Ethylene glycol bis(β-aminoethyl ether)-N,N,N'N', tetraacetic acid; TES, N-Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid; Tris, Tris(hydroxymethyl)aminomethane.

Materials and Methods

Vesicle Preparation

Bufo marinus toads of either sex (Mexican origin, obtained from Lemberger, Oshkosh, Wis.) were doubly pithed and deblooded by transventricular perfusion with approximately 500 ml of Ringer's solution consisting of (mM): 110 NaCl, 1.0 $CaCl_2$, 0.5 $MgCl_2$, and 3.5 K_2HPO_4 plus KH_2PO_4 (pH 7.5). The urinary bladders were excised and rinsed well in an ice-cold incubating medium composed of (mM): 90 KCl, 45 sucrose, 5 $MgCl_2$, 10 EGTA, and 10 Tris · HCl (pH 7.8). The epithelium was scraped off the underlying connective tissue with a glass slide and the cells were dispersed in the incubating medium by rapidly drawing them in and out of a Pasteur pipette at least 10 times. The epithelial cells were washed twice in 15 ml incubating medium (at 0°C), by centrifugation at 1000 × *g*, and then incubated for 30 to 45 min at 25°C. The incubation at 25°C was required in order to assure maximal channel-mediated fluxes in vesicles (Garty & Asher, 1985). At the end of this period, the suspension was cooled to 0°C, divided into several aliquots (6 to 8 aliquots per toad), and the cells were sedimented by centrifugation at 1000 × *g* for 10 min. The cell pellets were suspended in 3-ml volumes of ice-cold homogenizing solutions, whose composition varied according to the experimental design, and were immediately homogenized by applying a shear force for 10 sec with a polytron type homogenizer (Ystral GmbH, Gottingen, FRG) at top speed. In most of the experiments, KCl or NaCl (required for establishing a membrane potential in vesicles) and the desired concentrations of divalent cations were included in the vesicles at this stage (i.e. during their formation). Accordingly, the various homogenizing solutions contained (mM): 90 KCl, 45 sucrose, 5 $MgCl_2$ (unless otherwise indicated) 10 buffer (TES or Tris at pH of 7.0 to 7.8) and either 1 EGTA or 10 EGTA ± various concentrations of divalent cations. In measuring the effects of internal Na^+ on Na^+ transport different homogenizing media were used. They all contained 5 $MgCl_2$, 10 Tris · HCl (pH 7.8), 10 EGTA, and one of the following mixtures: (1) 90 KCl + 45 sucrose; (2) 90 NaCl + 45 sucrose; (3) various KCl and NaCl mixtures whose combined concentration was 500 mM; (4) 50 NaCl + 657 sucrose.

The homogenates were centrifuged at 1000 × *g* for 5 min to precipitate intact cells, nuclei and debris and the supernatants were then centrifuged for one hour at 27,000 × *g*. The microsomal pellets were suspended in minimal volumes (200 to 400 μl) of different solutions (usually the same solutions used for the homogenization), and assayed for amiloride-blockable uptake shortly (1 to 5 hr) afterwards.

The Transport Assay

The rate of $^{22}Na^+$ uptake by the membrane vesicle was measured in the presence of a negative inside membrane potential (Garty, Rudy & Karlish, 1983*b*; Garty, 1984; Garty & Asher, 1985; Garty & Karlish, 1987). In most of the assays a valinomycin-induced K^+ diffusion potential was used to drive the tracer uptake. In experiments that tested the effects of internal Na^+ on the apical conductance, a Na^+ gradient was used. In principle, the transport assay consisted of 3 stages: (1) Establishing an electrical diffusion potential across the vesicle membrane by substituting Tris for all external cations and adding valinomycin (unless the vesicles were loaded with Na^+). (2) Mixing the charged vesicles with $^{22}NaCl$ in the presence or absence of amiloride (t = 0). (3) Sampling vesicle aliquots for $^{22}Na^+$ uptake at different time intervals. Dowex 50WX8 columns (Tris form, 50 to 100 mesh), poured in Pasteur pipettes plugged with glass wool, were used both for the substitution of external cations by Tris, and for the separation of internal and external radioactivity (Gasko et al., 1976). The columns used to establish the ion gradient were prewashed with 1.5 ml 175 mM sucrose and maintained at room temperature. The columns used for sampling were washed with 1.5 ml 175 mM sucrose + 10 mg/ml crude bovine serum albumin, and kept at 0°C. In the first stage, 200 μl vesicles were applied on a sucrose-washed Dowex column and eluted with 4 volumes of 300 μl 175 mM sucrose at room temperature. Only the last 900 μl, which contained nearly all the vesicles, were collected. The eluent's pH was adjusted to the desired value with a small volume of Tris base and a membrane potential was established by adding 3 μM valinomycin (if necessary). Two 300-μl aliquots were immediately removed and mixed with 60 μl volumes of isotonic reaction mixture containing: sucrose, $^{22}NaCl$ (final 4 μCi/ml, 0.2 to 0.4 μM) and either amiloride (final 1.5 μM) or water diluent. 150 μl samples were removed from the radioactive suspensions at times of 1.5 and 3.5 min, applied on Dowex columns, and eluted into counting vials with 1.5 ml ice-cold sucrose (175 mM). In measuring the full time course of $^{22}Na^+$ uptake (Fig. 1), a larger amount of vesicle was used, and the radioactive suspensions were sampled at various times, indicated in the Figure. The intravesicular (eluted) radioactivity was measured in a β counter after adding 15 ml scintillation fluid (xylene based). Na^+ uptake rates were calculated as pmol $^{22}Na^+$ · mg protein^{-1} · min^{-1}. As shown before (Garty, 1984; Garty & Asher, 1985), the amiloride

concentration used in the assay (1.5 μM) is sufficient to completely block the flux component with a high affinity to this diuretic. Accordingly, the uptake measured in the presence of 1.5 μM amiloride was considered to be an "amiloride-insensitive" flux, and the difference in uptake with and without 1.5 μM amiloride, is the "amiloride-blockable," or "channel-mediated" flux. Under normal conditions, the channel-mediated flux was 65 to 95% of the total tracer uptake.

Considerations in Choosing the Extravesicular pH and Ca^{2+} Activity During the Transport Assay

The application of vesicles pre-equilibrated with Ca^{2+}/EGTA buffers to a Dowex column, in the first stage of the transport assay, removes 40 to 60% of the total extravesicular Ca^{2+} (determined using $^{45}Ca^{2+}$). The rest is eluted, presumably chelated by EGTA. Since the eluant should contain nearly all the EGTA applied to the column the extravesicular free Ca^{2+} activity drops to less than 10^{-8} M. In most of the experiments readdition of $CaCl_2$ to the assay mixture was avoided, for the following reasons:

(1) Such an addition is not required since the inhibitory effect of Ca^{2+} is limited to the intravesicular compartment (Chase & Al-Awqati, 1983; Garty, 1984). This point was verified in the present study by comparing effects of Ca^{2+} present either on both sides of the membrane or inside the vesicles only (*cf.* Fig. 2).
(2) The negative inside membrane potential may drive Ca^{2+} uptake which, for a large enough external Ca^{2+}, can significantly increase its internal activity. On the other hand, substantial leakage of Ca^{2+} from the vesicles, due to the removal of external Ca^{2+}, is not likely to occur in the presence of this potential.
(3) The amount of EGTA eluted together with the vesicles is not accurately known. Accordingly it would not be possible to precisely determine the external free Ca^{2+} activity after such an addition.

The elution of vesicles through a Dowex column also acidified the external medium (due to the substitution of K^+ or Na^+ by protonated Tris). Normally, the extravesicular pH was readjusted to the previous (internal) pH value by adding a small volume of Tris base. This protocol, however, posed a problem in experiments that tested the pH dependence of the Ca^{2+} inhibition (Figs. 3 and 4), since the channel-mediated flux decreases sharply with the external (but not internal) pH (Garty, Civan & Civan, 1985). In one set of experiments, summarized in Fig. 3, the assay was carried out at $pH_{out} = pH_{in}$ in spite of the fact that the amiloride-blockable fluxes were quite low for pH_{out} of 7.0 to 7.2. In the following experiments (Fig. 4), the assay was carried out at $pH_{out} = 8.0$ irrespective of the internal pH, in order to obtain a pH-independent flux in Ca^{2+}-free solutions. Equilibration of the transmembrane pH gradient occurs quite slowly and variations of pH_{in} during an assay are not likely (Garty et al., 1985).

Ca^{2+}/EGTA Buffers

Media whose free Ca^{2+} concentration is buffered to different values were prepared by adding known amounts of $CaCl_2$ to 10 mM EGTA solutions. The amount of $CaCl_2$ to be added was calculated as described by Pershadsingh and McDonald (1980) assuming: $\log K_{H_1} = 9.58$; $\log K_{H_2} = 8.97$ and $\log K_{Ca} = 10.955$ (Fabiato, 1981). The pH of the homogenizing media and the vesicle suspensions was carefully monitored using a combined pH electrode which gives accurate readings in Tris solutions (Metrohm AG). The true free Ca^{2+} activities may, however, be slightly different from the calculated values, since the dissociation constants used were obtained for somewhat different temperature and ionic strength.

Statistics

Data are expressed as mean ± SEM with the number of measurement in brackets. The Ca^{2+} inhibition constant and the maximal inhibition, were calculated from a linear regression analysis of an Eadie-Hofstee plot of the data (% inhibition *vs.* % inhibition/free-Ca^{2+} concentration).

Materials

Bovine serum albumin (fraction V), TES, EGTA and valinomycin were obtained from Sigma Chemical Co.; A23187, from Calbiochem; and $^{22}NaCl$ (carrier-free 200 μCi/ml) from Amersham Radiochemicals. Dowex beads (50WX8 50 to 100 mesh H^+ form) were purchased from Fluka AG. The cation exchange resin was converted to the Tris form by incubating it for 24 hr with a large excess of Tris base (suspension pH > 9.5), and then removing the excess Tris by washing with water until the suspension pH dropped to 7.5. Amiloride was a gift from Merck, Sharp and Dohme (GmbH, Munich, FRG).

Results

Inhibition of $^{22}Na^+$ Uptake by Intravesicular Ca^{2+}

Table 1 summarizes the effects of 10^{-5} M free Ca^{2+}, included in the apical vesicles, on the amiloride blockable and amiloride insensitive $^{22}Na^+$ uptake. Two different protocols were used in order to include a defined amount of free Ca^{2+} in the particles. In the first, cells were homogenized and vesicles isolated in a medium that contained 10 mM EGTA + 10 mM $CaCl_2$ (10^{-5} M free Ca^{2+} at pH = 7.8)[1]. In the second, the vesicles were prepared in a Ca^{2+}-free medium containing a minimal amount of EGTA, and then incubated in a Ca^{2+}/EGTA mixture in the presence of the Ca^{2+} ionophore A23187. Advantages and disadvantages of each protocol are discussed in the Discussion. Irrespective of the method used to include Ca^{2+} in the vesicles, it was found that its presence during the transport assay

[1] The cells were suspended in the Ca^{2+}-containing solution only after being cooled to 0°C and less than 30 sec before the homogenization, to prevent the irreversible Ca^{2+}-dependent inhibition (Garty & Asher, 1985, 1986).

Table 1. Ca^{2+} effects on $^{22}Na^+$ uptake in vesicles[a]

Cell homogenizing solution	Vesicle suspending and incubating solution	$^{22}Na^+$ uptake	
		Amiloride blockable	Amiloride insensitive
A. EGTA	EGTA	100	100
B. 10^{-5} M Ca^{2+}	10^{-5} M Ca^{2+}	49 ± 4(8)	87 ± 6(8)
C. 10^{-5} M Ca^{2+}	EGTA + A23187	97 ± 2(4)	121 ± 10(4)
D. EGTA	10^{-5} M Ca^{2+} + A23187	41 ± 6(8)	87 ± 7(8)

[a] Cells were homogenized and vesicles isolated in media buffered to pH 7.8 containing either 1 mM EGTA (A and D) or 10 mM EGTA + 10 mM $CaCl_2$ (10^{-5} M free Ca^{2+} at pH 7.8) (C and D). The vesicle pellets were suspended in similar media containing either EGTA (A and C) or a 10 mM EGTA + 10 mM $CaCl_2$ (B and D). Aliquots C and D received 4 μM A23187 and aliquots A and B an equal volume of diluent (DMSO). The vesicle suspensions were incubated for 2 hr at 25°C and then assayed at pH 7.8 for amiloride-blockable and amiloride-insensitive $^{22}Na^+$ uptake. Data are expressed as % of the control value (condition A) ± SEM and the number of experiments is given in brackets. The amiloride blockable and amiloride-insensitive fluxes under condition A, were 10.7 ± 1.6(8) and 3.3 ± 0.65(8) pmol $^{22}Na^+ \cdot mg^{-1} \cdot min^{-1}$, respectively.

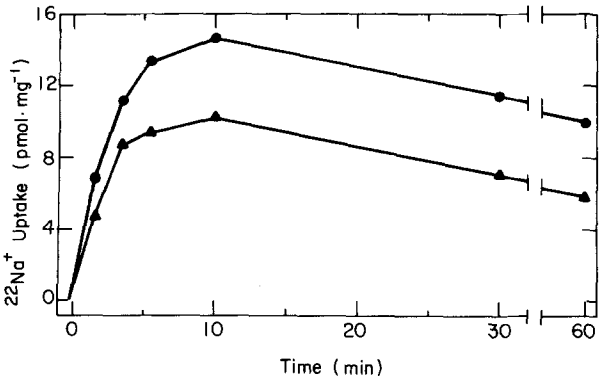

Fig. 1. Time course of $^{22}Na^+$ uptake in the presence and absence of Ca^{2+}. Cells were homogenized in media buffered to pH 7.8, that contained either 10 mM EGTA (●--●), or 10 mM EGTA + 9.09 mM $CaCl_2$ (10^{-7} M free Ca^{2+}, ▲--▲). Vesicles were isolated, suspended in the homogenizing solutions, and assayed for $^{22}Na^+$ uptake, as described in Materials and Methods. The Figure illustrates the amiloride-sensitive uptake, i.e. the difference between values measured with and without 1.5 μM amiloride, as a function of time

lowered the amiloride-sensitive $^{22}Na^+$ uptake by more than 50% without influencing the amiloride insensitive flux (Table 1). Ca^{2+} had no effect on the amiloride dose-response relationships; thus the decrease in $^{22}Na^+$ uptake is the result of a direct inhibition of channels rather than their desensitization to amiloride. This inhibition was fully reversible. Incubating vesicles, prepared to contain 10^{-5} M free Ca^{2+}, in a Ca^{2+}-free EGTA medium (plus A23187), restored the control (maximal) levels of Na^+ uptake (Table 1*C*). The fact that inhibition of transport can both be induced and reversed by incubating isolated vesicles with and without Ca^{2+}, respectively, indicates a direct Ca^{2+}-channel interaction (*see* Discussion).

The fluxes in Table 1 were calculated from the initial rates of $^{22}Na^+$ uptake. The full time course of the amiloride-sensitive tracer uptake in the presence and absence of internal Ca^{2+}, is depicted in Fig. 1. In order to be able to measure Ca^{2+} effects on the kinetics of the channel-mediated $^{22}Na^+$ uptake, a submaximal concentration of Ca^{2+} which lowers the amiloride-blockable flux by less than 50%, was used in this experiment. It was found that the presence of the divalent ion in the vesicles markedly lowered the initial rate and the maximal level of the amiloride-blockable $^{22}Na^+$ accumulation, but did not influence the time at which the internal radioactivity reached its maximal value or the rate of the following $^{22}Na^+$ efflux[2]. As discussed before (Garty, 1984; Garty & Asher, 1986; Garty & Karlish, 1987), this behavior is indicative of Ca^{2+} effects on the Na^+ permeability, and argues against the possibility that the divalent ion lowers $^{22}Na^+$

[2] The peak value at $t \sim 10$ min represents a steady state whose magnitude is determined by the relative permeabilities and concentrations of all ions, as well as the intravesicular volume (Garty et al., 1983*b*). Thus, the fact that the maximal uptake was smaller in vesicles prepared to contain Ca^{2+}, does not necessarily mean that their internal volume was smaller than that of the control vesicles. At equilibrium ($t > 3$ hr) the intravesicular radioactivity was the same for the two preparations.

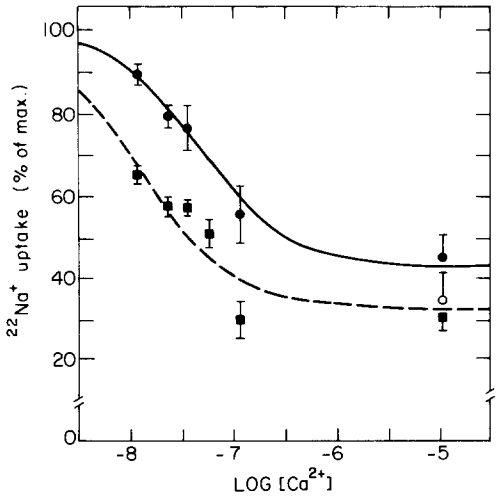

Fig. 2. The Ca^{2+} dose-response relationships. Vesicles were prepared using media buffered to pH 7.8 that contained either 1 mM EGTA (■) or 10 mM EGTA + various concentrations of $CaCl_2$ (●). The vesicles were suspended either in the homogenizing media (●) or in media that contained 10 mM EGTA, various concentrations of $CaCl_2$, and 4 μM A23187 (■). $^{22}Na^+$ uptake was measured as described in Materials and Methods after a 2-hr incubation at 25°C. The amiloride-sensitive uptake by the various vesicle preparations, expressed as % of the values measured for Ca^{2+}-free solutions, is plotted against the free Ca^{2+} concentration of the incubating medium. In each case the mean ± SEM of three vesicle preparations is presented. The open circle represents a group of four experiments in which the vesicles were prepared to contain 10^{-5} M Ca^{2+}, and in addition $\geq 10^{-5}$ M Ca^{2+} was present in the final assay mixture. The continuous lines are the best fits of the experimental data with Michaelis-Menten kinetics

uptake by depolarizing the membrane potential (i.e. increasing the permeability to other ions)[3]. This conclusion is also supported by the observation that similar Ca^{2+} effects were observed in vesicles that were prepared in K_2SO_4 instead of KCl solutions (*data not shown*).

The Ca^{2+} Dose-Response Relationships

The concentration dependence of the Ca^{2+} effects on Na^+ transport was evaluated by measuring the amiloride-sensitive fluxes in vesicles prepared to contain different amounts of Ca^{2+} (either by adding Ca^{2+} to the cell homogenizing medium or by incubating the vesicles with various Ca^{2+}/EGTA mixtures in the presence of A23187). In both cases inhibition curves which could be fitted to a Michaelis-Menten kinetics were obtained (Fig. 2). Both curves appeared, however, to be displaced. This extra, Ca^{2+}-independent inhibition, induced by A23187 may reflect unspecific effects of the ionophore accumulated in the membrane. The kinetic parameters of the best fits are listed in Table 2. As observed before, using fast flow measurements (Chase & Al-Awqati, 1983) inhibition of the channel-mediated Na^+ flux can be induced by submicromolar concentrations of ionized Ca^{2+}. However, the data of Fig. 2 and Table 2 differ from the previously reported dose-response relationships in two aspects. First, the inhibition constants obtained are at least an order of magnitude lower than the value measured before (0.5 μM). Second, in our hands the inhibition of transport was never complete. The curves extrapolate to a maximal inhibition of less than 70%; about 30% of the amiloride-sensitive uptake remained, even if vesicles were prepared to contain 1 mM free Ca^{2+} (*data not shown*). The differences in sensitivity to Ca^{2+} can be explained by the different pH's used in the two studies (*see* next section). The fact that in our measurements the Ca^{2+}-dependent inhibition was incomplete may in principle reflect the existence of an 'inside-out' oriented vesicle whose channels are not blocked when Ca^{2+} is present only in the internal compartment (*see* Materials and Methods). This possibility is, however, excluded by showing that more than 30% of the amiloride-sensitive uptake is Ca^{2+} insensitive even when the divalent ion is present both inside and outside the vesicles during the transport assay

Table 2. The kinetic parameters of the Ca^{2+}-induced inhibition of Na^+ transport[a]

	Ca^{2+} introduced during the homogenization	Ca^{2+} introduced by incubation with A23187
Inhibition constant (M)	$(4.09 \pm 0.9) \times 10^{-8}$	$(1.1 \pm 0.3) \times 10^{-8}$
Maximal inhibition (%)	56.8 ± 6.6	66.4 ± 5.6
Correlation coefficient	0.935	0.869

[a] The experimental data of Fig. 2 were fitted with a Michaelis-Menten kinetics by a linear regression of the corresponding Eadie-Hofstee plots (% inhibition *vs.* % inhibition/Ca^{2+} concentration). The slopes, intercepts, and correlation coefficients of the best fits are listed.

[3] In contrast to previous findings (Garty, 1984; Garty & Karlish, 1987) the decrease in Na^+ permeability did not cause a shift of the maximal uptake level to longer times. The reason is that in the present study the membrane potential was established by imposing a K^+ gradient rather than a Na^+ gradient, and was therefore independent of the Na^+ permeability (at the nearly zero external Na^+ activity).

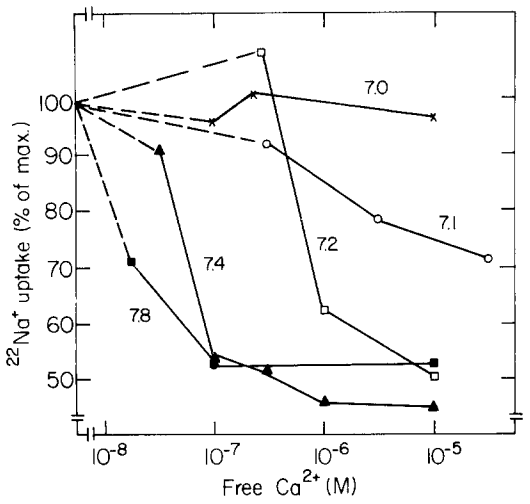

Fig. 3. The Ca^{2+} dose-response relationships at various pH's. Vesicles were prepared and suspended in homogenizing solutions that contained various concentrations of free Ca^{2+} (0 to 5×10^{-5} M) and buffered to different pH values (7.0 to 7.8). The amiloride-blockable $^{22}Na^+$ uptake was measured in the various vesicle preparations as described in Materials and Methods. The external pH during the transport assay was always adjusted to the internal pH value. The amiloride-blockable fluxes obtained are expressed as % of the value measured for the same pH in the absence of Ca^{2+}

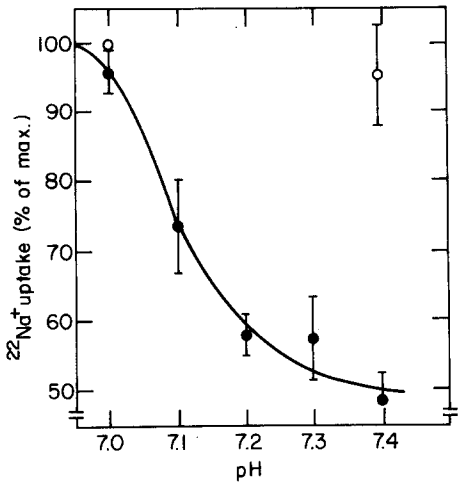

Fig. 4. pH effects in the presence and absence of Ca^{2+}. Vesicles were prepared and suspended in homogenizing solutions buffered to various pH's. Each solution contained either 10 mM EGTA (open circles) or 10 mM EGTA plus a corresponding amount of $CaCl_2$ that buffered the free Ca^{2+} to 0.5 μM, at the experimental pH (closed circles). The amiloride-blockable uptake was measured as described in Materials and Methods. In this case the external pH of the eluted vesicles was adjusted to 8.0 irrespective of the internal value. The amiloride-sensitive $^{22}Na^+$ uptake, expressed as % of the value measured at pH_{in} = 7.0 in the absence of Ca^{2+}, is plotted versus the internal pH. The means ± SEM of four experiments are presented. The open circles represent the relative fluxes in the absence of Ca^{2+} at pH 7.0 (100% by definition) and 7.4

(open circle in Fig. 2). Thus, other reasons must account for the only partial inhibition observed.

pH Dependence of the Ca^{2+} Channel Interaction

Chelation of Ca^{2+} by organic compounds such as EGTA, EDTA and CDTA occurs in competition with protons and the apparent Ca^{2+}-chelator association constant is a strong function of pH (Durham, 1983). This fact led us to explore the possibility that the association of Ca^{2+} with the apical membrane, involved in the inhibition of transport, is pH dependent too. To test this hypothesis, Ca^{2+} dose-response relationships were measured in vesicles buffered to different pH's. Naturally, due to the pH dependence of the apparent Ca^{2+}-EGTA association constant, a different set of Ca^{2+}/EGTA mixtures was used at each pH. The results of these experiments, depicted in Fig. 3, clearly show that the inhibition of channel-mediated $^{22}Na^+$ uptake by Ca^{2+} ions is pH dependent. Lowering the vesicular pH, shifted the inhibition curve to higher Ca^{2+} values. The strongest pH effects are observed at 7.2 to 7.4, i.e. in the vicinity of the physiological intracellular pH. This behavior can be accounted for by assuming a Ca^{2+}-H^+ competition on a binding site (*see below*).

In a second set of experiments, the inhibition induced by a single concentration of Ca^{2+} (0.5 μM) at various pH's was evaluated. In this case, $^{22}Na^+$ uptake was measured in vesicles prepared to contain either 0.5 μM or no free Ca^{2+} at pH's ranging from 7.0 to 7.4 (Fig. 4). As reported before (Garty et al., 1985) the channel-mediated uptake in the absence of Ca^{2+} is independent of the internal pH at this range, and similar amiloride-sensitive fluxes are obtained at pH's of 7.0 and 7.4 (open circles in Fig. 4). Including 0.5 μM free Ca^{2+} in the particles induced a steep pH dependence. At pH 7.4, 0.5 μM Ca^{2+} lowered the amiloride-sensitive flux by about 50%, i.e. produced a near maximal inhibition. Reducing the intravesicular pH desensitized the channels to Ca^{2+} and at pH 7.0 $^{22}Na^+$ uptake was hardly affected by 0.5 μM free Ca^{2+}. These data are in full agreement with the Ca^{2+} dose-response relationships measured in Fig. 3. The steepness of the pH dependence of Ca^{2+} inhibition seems to indicate a cooperative Ca^{2+}-H^+ interaction. One possibility to account for such a cooperativity would be to assume that the Ca^{2+} binding site is composed of several deprotonated groups. The protonation of any one of these groups would be sufficient to prevent

Table 3. Effects of divalent ions on $^{22}Na^+$ uptake[a]

Internal divalent ions	$^{22}Na^+$ uptake	
	Amiloride blockable	Amiloride insensitive
5 mM Mg^{2+}	100	100
—	77 ± 11(5)	60 ± 6(5)
5 mM Mg^{2+} + 1 mM Ba^{2+}	59 ± 4(7)	92 ± 5(7)
5 mM Mg^{2+} + 1 mM Ba^{2+} followed by incubation of vesicles in Ba^{2+}-free solution	108 ± 11(3)	109 ± 5(3)
5 mM Mg^{2+} + 1 mM Ba^{2+} + 10 μM Ca^{2+}	62 ± 8(8)	101 ± 14(8)
5 mM Mg^{2+} + 1 mM Sr^{2+}	57 ± 8(6)	96 ± 9(6)

[a] Vesicles were prepared and suspended in homogenizing solutions buffered to pH 7.8 that contained 10 mM EGTA and the divalent cations indicated in the first column. (A free Ca^{2+} concentration of 10 μM was achieved by adding 10 mM $CaCl_2$ to the 10 mM EGTA solution.) In one set of experiments vesicles prepared to contain 1 mM Ba^{2+} were incubated for 2 hr in a Ba^{2+}-free solution prior to the assay. $^{22}Na^+$ uptake was measured as described in Materials and Methods. The fluxes are expressed as % of the uptake measured in a matched preparation which contained only 5 mM $MgCl_2$. The amiloride-blockable and amiloride-insensitive fluxes under these conditions were 6.0 ± 1.7(13) and 2.4 ± 0.3(13) pmol · mg^{-1} · min^{-1}, respectively.

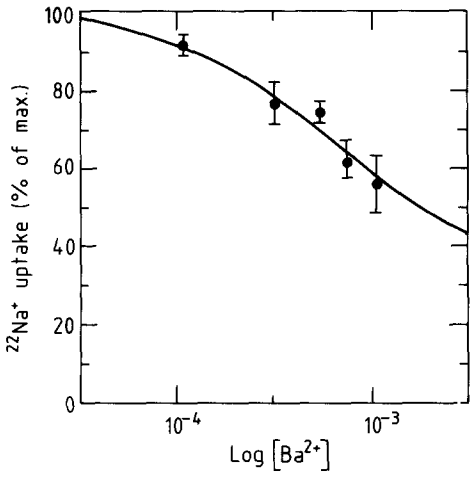

Fig. 5. Ba^{2+} dose-response relationships. Vesicles were prepared and suspended in media that contained 1 mM EGTA and 0 to 1 mM $BaCl_2$ (pH 7.8). $^{22}Na^+$ uptake was measured as described in Materials and Methods. The channel-mediated flux, expressed as % of the value obtained in the absence of Ba^{2+}, is plotted against the intravesicular Ba^{2+} activity. The means ± SEM of four experiments are presented. The continuous line is the best fit of the data with Michaelis-Menten kinetics. Its parameters are: inhibition constant = 6.5 ± 2.2 × 10^{-4} M Ba^{2+}; Maximal inhibition = 70 ± 14.6% of the total amiloride-sensitive uptake; and a correlation coefficient of 0.862

the association of Ca^{2+} (but will not by itself inhibit transport). In the experiments of Fig. 3, the external pH was always the same as the internal pH. In the measurements of Fig. 4 pH_{out} was constant irrespective of pH_{in}. The fact that both protocols gave comparable results proves that the modulation in the sensitivity to Ca^{2+} is induced by the internal and not external pH.

Effects of Other Divalent Ions on $^{22}Na^+$ Uptake

To examine the specificity of the above-described inhibition by Ca^{2+} ions, we studied the potential effects of other divalent ions as well. Aqueous salt solutions are normally contaminated by at least 10 μM Ca^{2+}, thus EGTA has to be included in all solutions. Therefore, we were limited to the study of cations which do not bind with a high affinity to this chelator. For others, distinction between a direct inhibition of channels and an indirect effect, as a result of the displacement of Ca^{2+} from EGTA, could not be made.

Most of the above-described experiments were carried out in media that contained 5 mM $MgCl_2$. Eliminating Mg^{2+} from the intravesicular solution caused a decrease in both the amiloride-blockable and amiloride-insensitive fluxes (Table 3). Thus, Mg^{2+} at a concentration of up to 5 mM does not produce a Ca^{2+}-like inhibition. Ba^{2+} and Sr^{2+} on the other hand, induced a partial inhibition of transport quite similar to the effect observed with Ca^{2+}. Both of them lowered the amiloride-sensitive uptake to half of its control value, without affecting the amiloride-insensitive flux component. Incubating Ba^{2+}-loaded vesicles in a Ba^{2+}-free medium for 2 hr before the transport assay, reversed the inhibition. Including both 1 mM Ba^{2+} and 10^{-5} M Ca^{2+} in the vesicles lowered the channel-mediated flux by less than 50%, i.e. the combined effect of Ca^{2+} and Ba^{2+} is not larger than the maximal inhibition by each of them alone.

Figure 5 depicts the relative inhibition induced by increasing concentrations of $BaCl_2$ included in the vesicles during the homogenization of cells. The best-fitted line was characterized by an inhibition constant of (6.5 ± 2.2) × 10^{-4} M Ba^{2+} and a maximal inhibition of 70 ± 14.6% of the amiloride-sensitive tracer uptake; i.e. like Ca^{2+}, Ba^{2+} can block only part of the amiloride-sensitive $^{22}Na^+$ uptake, but the inhibition is produced only by much higher concentrations of this ion. Ba^{2+} concentrations higher than 1 mM were not used in the above experiments in order to avoid potential nonspecific effects by the divalent ion. Sr^{2+} also inhibited Na^+ transport only in the millimolar range.

Table 4. Effects of VO^{2+} on $^{22}Na^+$ uptake[a]

Cell homogenizing solution	Vesicle incubating solution	$^{22}Na^+$ uptake	
		Amiloride blockable	Amiloride insensitive
A. —	—	100	100
B. 1 mM VO^{2+}	1 mM VO^{2+}	18.4 ± 5.8(8)	44.3 ± 12.4(8)
C. 1 mM VO^{2+}	—	47.1 ± 13.7(8)	66.9 ± 8.1(8)
D. —	1 mM VO^{2+}	55.5 ± 15.8(8)	95.1 ± 12.3(8)

[a] Cells were homogenized either in the usual medium (A and D), or in a modified medium that contained also 1 mM $VOSO_4$ (B and C). In each experiment a freshly made $VOSO_4$ solution was used, and cells were exposed to it for less than 30 sec (at 0°C) prior to their disruption. Vesicles were isolated as usual, incubated for 2 hr at 25°C in the presence (B and D) or absence (A and C) of 1 mM $VOSO_4$, and assayed for amiloride-blockable and amiloride-insensitive $^{22}Na^+$ fluxes. Data are expressed as % of the uptake measured under control conditions (A).

Another divalent ion tested was VO^{2+} (Table 4). Including VO^{2+} in the vesicles by homogenizing cells in the presence of 1 mM $VOSO_4$, blocked more than 80% of the channel-mediated flux (Table 4B). Similar but smaller inhibition (45%) was measured if vesicles prepared in the absence of VO^{2+} were incubated for 2 hr with 1 mM $VOSO_4$ (Table 4D). If, however, vanadyl was added to the vesicle suspension only shortly (<5 min) before the transport assay, it had no effect on the $^{22}Na^+$ uptake (*data not shown*). Part of the inhibition induced by trapping VO^{2+} in the vesicles during their formation was reversed upon incubating the isolated membrane in vanadyl-free solution for 2 hr (Table 4C). Unlike Ca^{2+}, Ba^{2+} and Sr^{2+}, VO^{2+} seems to substantially lower the amiloride-insensitive uptake as well. This result raises the possibility that at least part of the vanadyl effect is a nonspecific one.

Effects of the Internal Na^+ Activity on Na^+ Transport

One of the mechanisms proposed for the regulation of luminal Na^+ entry is a direct "feedback" inhibition of channels by cellular Na^+ (Lewis, Eaton & Diamond, 1976; Frizzell & Schultz, 1978). Although previous electrophysiological measurements did not support this view (Palmer, Edelman & Lindemann, 1980; Garty, Edelman & Lindemann, 1983*a*), it was of interest to examine whether the amiloride-blockable flux in vesicles is influenced by the intravesicular Na^+ activity. The quantitative determination of Na^+ permeability for different internal Na^+ activities, using our transport assay, is complicated by the fact that the intravesicular Na^+ and K^+ activities determine the magnitude of the membrane potential that drives the tracer flux (Garty et al., 1983*b*; Garty, 1984). Thus, possible permeability changes induced by increasing internal Na^+ activities, could be masked by the expected increase in membrane potential. Nevertheless, it was possible to design experiments, which excluded the possibility that the internal Na^+ activity had a major, direct, effect on the apical permeability in vesicles. First, we compared tracer fluxes driven by equal K^+ and Na^+ gradients. In both cases a similar Na^+ uptake was measured and their paired ratio did not significantly differ from 1 (Table 5*A*). Thus, unless the diffusion potential induced by the Na^+ gradient (due to the native Na^+ permeability of the channel-containing membrane) is much larger than the potential induced by an equal K^+ gradient in the presence of 3 μM valinomycin, loading the vesicles with 90 mM Na^+ does not block the channels. In a second group of experiments (Table 5*B*) vesicles were prepared in K^+-free media that contained either 500 mM NaCl or 50 mM NaCl + 675 mM sucrose, and assayed in the presence of either 50 or 500 μM NaCl in the external solution. In this way we could compare the channel-mediated fluxes for two very different internal concentrations of Na^+, but for similar chemical gradients of Na^+ and the same osmolarity (adjusted by the addition of sucrose). The disadvantage of this protocol, however, is that vesicles prepared in solutions whose ionic strengths were very different, had to be compared. Here too similar amiloride-sensitive fluxes were measured and the very high internal Na^+ activity did not prevent the channel-mediated tracer uptake. The fact that $^{22}Na^+$ uptake was not lowered by including 500 mM Na^+ in the vesicles, indicates that the channel is not blocked or saturated even by this very high internal Na^+. The fluxes given in Table 5*B* are smaller than

Table 5. The influence of internal Na^+ on the amiloride-sensitive $^{22}Na^+$ flux[a]

Internal composition	Amiloride-sensitive $^{22}Na^+$ uptake pmol · mg^{-1} · min^{-1} ± SEM (*n*)	Paired ratio
A. 90 mM KCl	3.34 ± 0.75(6)	
90 mM NaCl	3.77 ± 0.67(6)	
		1.15 ± 0.12(6)
B. 50 mM NaCl + 675 mM sucrose	1.33 ± 0.12(3)	
500 mM NaCl	1.8 ± 0.35(3)	
		1.36 ± 0.4(3)

[a] Vesicles were prepared and suspended in media that contained (in mM) EGTA 10, $MgCl_2$ 5, Tris-HCl 10 (pH 7.8) and either KCl or NaCl at the concentrations indicated in the first column. The amiloride-sensitive $^{22}Na^+$ uptake was measured as described in Materials and Methods. In the experiments of part B the transport assay was performed using 700 mM sucrose (rather than 175 mM). In addition, 50 and 500 μM NaCl were added to the assay mixtures of vesicles containing 50 and 500 mM NaCl, respectively.

those measured under the conditions of the experiments in Table 5A. This, however, could result from the fact that in these experiments cells are homogenized and vesicles isolated in solutions whose osmolarity is much higher than usual. Finally, we measured the amiloride-sensitive uptake in vesicles which contained various KCl and NaCl mixtures whose combined concentration is 500 mM (Fig. 6). In this case, too, no major dependence of the channel-mediated uptake on the internal Na^+ activity could be detected. Thus, the data of Table 5 and Fig. 6 argue against the possibility that cellular Na^+ can directly block the channels, and indicate that the channels do not saturate even at 500 mM Na^+, i.e. the dissociation rate of Na^+ ions from the internal side of the channel should be very high.

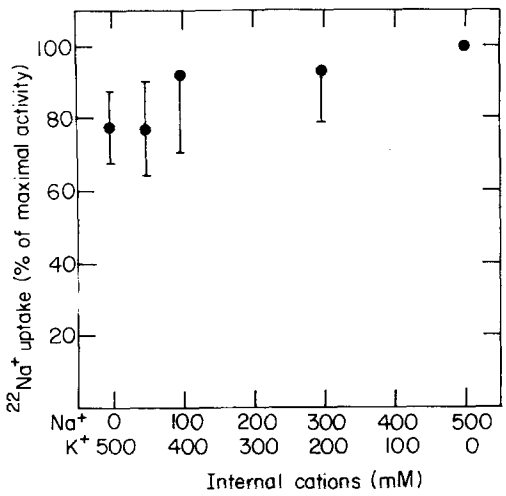

Fig. 6. Effects of internal Na^+ on ^{22}Na uptake. Vesicles were prepared and suspended in media that contained (in mM): EGTA 10, $MgCl_2$ 5, Tris · HCl 10 (pH 7.8), and various NaCl/KCl mixtures as indicated at the bottom of the Figure. The amiloride-blockable fluxes measured are expressed as % of the values measured for 500 mM NaCl and no KCl (the data point with no error bars). The means ± SEM of three preparations are presented

Discussion

The current paper describes effects of Ca^{2+} and other divalent ions on the amiloride-blockable Na^+ flux in toad bladder membrane vesicles. In agreement with a previous report (Chase & Al-Awqati, 1983) we found that including submicromolar concentrations of free Ca^{2+} in the vesicles, substantially lowers the channel-mediated flux. These results appear, however, to be different from those obtained recently using rat collecting tubules (Palmer & Frindt, 1986). The inhibition measured by us was Ca^{2+} specific. It could be induced only by much higher concentrations of Ba^{2+} or Sr^{2+}, and was not observed at all with Mg^{2+} (5 mM). It is interesting, however, that both Ba^{2+} and Sr^{2+} mimicked Ca^{2+} effects. $^{22}Na^+$ uptake could also be inhibited by including $VOSO_4$ in the vesicles. The interpretation of this result is, however, complicated by the complex chemistry of vanadyl ions. At the experimental pH (7.8) an aqueous solution of $VOSO_4$ will mostly contain $VO(OH)^+$ and $VO(OH)_2$ (Rubinson, 1981). In addition, vanadyl can be oxidized to VO_3^-, and might also be, in part, chelated by EGTA.

Two different protocols were employed in order to include defined amounts of ionized Ca^{2+} in the particles during the transport assay. In the first, cells were homogenized and membranes isolated in a media which contained various Ca^{2+}/EGTA mixtures. This procedure assures that the intravesicular

space is buffered to the desired Ca^{2+} activity; however, Ca^{2+} effects on the formation of vesicles, rather than on the Na^+ conductance of the isolated membrane, are possible. In the second procedure, vesicles were first isolated in a Ca^{2+}-free medium and then incubated with various Ca^{2+}/EGTA mixtures in the presence of A23187. This protocol rules out Ca^{2+} effects on the formation or isolation of vesicles, but it is not certain that the vesicle interior is buffered to the external Ca^{2+} activity. Moreover, the ionophore-mediated Ca^{2+}/H^+ exchange may alter the intravesicular pH. These uncertainties are, however, excluded by the fact that both procedures yielded very similar inhibition curves, and the finding that incubating vesicles prepared to contain Ca^{2+}, in EGTA + A23187 reversed the inhibition. Thus the decrease in transport cannot reflect Ca^{2+} effects on the formation of vesicles, and the inhibition constants calculated from Fig. 2 are probably close to the true value under the conditions of this experiment.

In principle, Ca^{2+} ions may alter Na^+ transport either directly, e.g. by binding to the channel protein, or indirectly, by activating an enzyme which in turn acts on the channel protein or its surrounding lipids. Such an indirect effect could even be an unspecific action of a Ca^{2+}-activated protease or phospholipase which may have contaminated the vesicle preparation. A Ca^{2+}-mediated enzymatic process is, however, unlikely due to the complete reversibility of the inhibition shown in Table 1. Even if a Ca^{2+}-activated enzyme, whose substrate is a membrane protein or lipid, is present in the vesicle preparation, it is inconceivable that the enzyme(s) and substrate(s) required for the opposite reaction will be present there, too. Thus, the fact that transport changes can either be induced or reversed by incubating the isolated membrane with Ca^{2+} or EGTA, respectively, implied that they are caused by a direct, electrostatic, Ca^{2+}-apical membrane interaction. Such an interaction would involve binding of Ca^{2+} to a membrane protein, presumably the channel itself, or to its surrounding lipids.

The inhibition of the channel-mediated flux measured was incomplete and at least 30% of the amiloride-sensitive uptake could not be blocked by Ca^{2+} ions. This only partial inhibition was observed even when Ca^{2+} was present on both sides of the membrane, i.e., it does not result from a mixed orientation of the particles. There are three possible explanations for this phenomenon: (1) A "non-competitive" Ca^{2+} effect which reduces either the single channel current or the mean channel open time without being able to lower them to zero. (2) The existence of two types of channels with similar affinities to amiloride but very different affinities to Ca^{2+}. (3) Partial impairment of the ability of Ca^{2+} to block channels by the disruption of cells or by the specific conditions used to assay Na^+ uptake (e.g., the large membrane potential or low Na^+ activity).

In addition to measuring the effects of divalent ions on the amiloride-sensitive fluxes, we tested the hypothesis that channels are directly blocked by high internal Na^+ activity. The expected Na^+ effect on the membrane potential that drives the tracer uptake, and the need to homogenize cells in media of different compositions in order to include defined amounts of Na^+ in the vesicles, complicated these measurements. Nevertheless, the data of Table 5 and Fig. 6 clearly indicate that channels are not blocked even by a Na^+ concentration which is much higher than the physiological cytoplasmic activity. This somewhat surprising insensitivity to the very high internal Na^+ activities tested suggests that the dissociation of Na^+ ions from the channel on the cytoplasmic side is very fast.

An interesting observation of potential significance to the physiological regulation of Na^+ channels is the pH dependence of the Ca^{2+} effect. This dependence was demonstrated both by measuring the Ca^{2+} dose-response relationships at different pH's, and by measuring the Na^+ permeability as a function of pH at a constant internal Ca^{2+} activity of 0.5 μM. The experimental data can be accounted for by assuming that Ca^{2+} interacts with a protein binding site in a manner similar to its interaction with chelators such as EGTA or EDTA; i.e., it forms a coordinated bonding with several deprotonated groups. The protonation of any one of the coordinating groups is sufficient to prevent the Ca^{2+} attachment, but does not by itself inhibit transport; (the uptake is pH independent in the absence of free Ca^{2+}). Such an interaction can be described by a simple Michaelis-Menten kinetics with an apparent Ca^{2+}-association constant K_{app}, equal to $K_o/(1 + K_1[H^+] + K_1 \cdot K_2 \cdot [H^+]^2 + \ldots + K_1 \ldots K_n \cdot [H^+]^n)$, when K_o is the association constant of Ca^{2+} with the fully deprotonated site, and $K_1 \ldots K_n$ the protonation constants of the n groups involved in its chelation (*cf.* Pershadsingh & McDonald, 1980). Accordingly, the apparent Ca^{2+} association constant will be a function of the pH, and for $n > 1$ a steep pH dependence is expected. The occupancy degree of the Ca^{2+} binding site measured at a constant Ca^{2+} activity, will also be pH dependent. If the pK of two or more groups interacting with the divalent cation is between 7.0 and 7.4, the sharp pH effects observed in Fig. 4 are expected.

The importance of the above pH effect is twofold. Firstly, it seems to provide some preliminary information on the structure of a Ca^{2+}-specific binding site involved in the function of the apical Na^+

channel. Secondly, it implies that the modulation of apical Na^+ conductance can be induced by intracellular pH changes which determine the apparent affinity of the channels to Ca^{2+} ions rather than changes of cell Ca^{2+}. The fact that the sharpest change in amiloride-blockable fluxes, measured for 0.5 μM Ca^{2+}, was observed at the vicinity of the physiological pH in tight amphibian epithelia (Leaf, Keller & Dempsey, 1964; Roos & Boron, 1981; Civan et al., 1984), supports this view. Moreover, since the Ca^{2+} inhibition curve is not cooperative, a half-maximal decrease in Na^+ conductance would require at least a 10-fold increase in cell Ca^{2+}. A change of that magnitude was not observed experimentally (Crutch & Taylor, 1983; Burch & Halushka, 1984; Chase & Wong, 1984). On the other hand, a similar decrease in conductance can be achieved by increasing the internal pH by 0.2 units (at constant Ca^{2+} activity of 0.5 μM), a value which is well within the physiological fluctuations of cytoplasmic pH. Thus pH changes could be, in principle, a more sensitive means for regulating the Na^+ permeability than changes in Ca^{2+} activity. Testing this hypothesis will, however, require the parallel determination of Na^+ permeability, cellular pH, and the cellular Ca^{2+} activity under a variety of conditions. It is also not clear yet, if and how the cellular pH varies in response to stimuli which are thought to affect Na^+ transport.

The above-discussed Ca^{2+}-H^+ interaction may also serve as a general mechanism for inducing large cellular responses by minor pH changes. Many cellular events are believed to be directly triggered by pH changes, but usually the intracellular pH does not vary by more than 0.2 units (Roos & Boron, 1981; Shuldiner & Rosengurt, 1982). Such a minor pH change will induce only a slight shift in the protonation degree of a group, and cannot account for a large response, unless cooperative effects take place. One possible mechanism for such a cooperative pH effect is provided in this study, i.e. coordinated interaction between Ca^{2+} and several groups whose pK is around the physiological pH. Minor pH changes would then be sufficient to markedly change the occupancy degree of such a Ca^{2+} binding site and either trigger or terminate cellular events.

In previous recent publications, we provided evidence for the existence of a Ca^{2+}-dependent, temperature-sensitive process, which down regulates Na^+ channels (Garty & Asher, 1985, 1986). This process differs from the currently studied Ca^{2+} effects in at least three aspects: (1) It could be induced in whole cells only, but produced a stable modification of the isolated membrane, preserved in the complete absence of Ca^{2+} ions. In the current study, inhibition was obtained only if Ca^{2+} was present inside the particles during the transport assay, and similar inhibition was obtained when Ca^{2+} was introduced during or after the isolation of membranes. (2) The previous Ca^{2+} effect was highly cooperative (Hill coefficient ~4), whereas the inhibition curves in Fig. 2 could be fitted to a Michaelis-Menten kinetics, i.e. it has a Hill coefficient of 1. (3) The irreversible Ca^{2+}-dependent temperature-sensitive effect could not be induced by 1 mM Ba^{2+}. In the present paper, Ba^{2+} could mimic Ca^{2+} effects. Thus, experiments in vesicles seem to indicate the presence of two different and presumably independent processes. One is a Ca^{2+}-activated reaction which can be triggered only in whole cells but covalently modifies the channel protein or the lipids around it. The other is a direct, noncovalent, Ca^{2+}-apical membrane interaction. Although both processes should down regulate channels in response to an increase in cell Ca^{2+}, each one of them is apparently controlled by other means, too (i.e. the unexplained shift in the Ca^{2+} dose-response reported by Garty and Asher (1986), and the pH dependence described in this paper). Thus, the fact that both of them are Ca^{2+} dependent does not necessarily couple them. It is possible that a change in Ca^{2+} will affect one of them but not the other, or that the apical Na^+ permeability will vary at a constant Ca^{2+} activity. The existence of two different channel-activating mechanisms in toad bladder cells was suggested on the basis of the differential effects of apical trypsinization on the aldosterone and vasopressin-induced short-circuit current changes (Garty & Edelman, 1983). It is possible that the two-channel regulating mechanisms identified in vesicles are those responsible for the augmentation of Na^+ transport by aldosterone and vasopressin. So far, however, we have not been able to demonstrate differences in transport rates between vesicles derived from control and hormone-treated cells (Garty & Edelman, 1984), and more work is needed in order to relate the above Ca^{2+}-dependent processes to the hormonal or intracellular regulation of epithelial transport.

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