Regulation of $^{22}\text{Na}^+$ Transport by Calcium in an Established Kidney Epithelial Cell Line*

(Received for publication, November 27, 1978, and in revised form, April 18, 1979)

Mary Taubj and Milton H. Saier, Jr.§

From the Department of Biology, The University of California at San Diego, La Jolla, California 92039

The role of calcium in regulating the Na$^+$ channel in an established kidney epithelial cell line has been examined. Extracellular calcium was inhibitory to Na$^+$ uptake, and a Dixon plot of the initial Na$^+$ uptake rate in the presence of Ca$^{2+}$ was nonlinear, suggesting a mixed pattern of inhibition. Similar patterns of inhibition were also observed for other divalent cations, including Ba$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$. In contrast, elevated concentrations of intracellular calcium resulted in a stimulation of Na$^+$ entry. This intracellular effect was specific to calcium, with Mg$^{2+}$ and Mn$^{2+}$ appearing much less effective. Lineweaver-Burk plots of Na$^+$ influx in calcium-loaded and unloaded cells were linear, suggesting that under both conditions a single system transported Na$^+$. Although Na$^+$ entry was stimulated by intracellular Ca$^{2+}$, the cells did not exhibit other counter transport phenomena reported with cell types in which a Na$^+$/Ca$^{2+}$ exchange system is operative. Thus, the results indicate that calcium acts as an allosteric regulator of Na$^+$ transport by the Na$^+$ channel.

Calcium has been implicated as a modulator of Na$^+$ transport in several cell types. In the gated Na$^+$ channel of the nerve, extracellular calcium increases the critical depolarization necessary to reach the firing threshold (1). Extracellular calcium also inhibits Na$^+$ transport in frog skin (2), presumably by acting as a “plug” to the Na$^+$ channel. These effects of Ca$^{2+}$ have been attributed to the screening of negatively charged molecules on the external surfaces of the plasma membranes (3, 4). On the other hand, intracellular calcium may influence the rate of monovalent cation transport in a positive sense. A transient increase in cytoplasmic calcium reportedly precedes the increased Na$^+$ transport rates through the Na$^+$ channel in toad bladder after treatment with antidiuretic hormone (5), and in the sea urchin egg following fertilization (6). Whether or not cell calcium modulates Na$^+$ uptake in kidney epithelial cells has not been determined.

An excellent model system for the investigation of differentiated kidney epithelial transport functions is provided by the dog kidney epithelial cell line, MDCK. The MDCK cells have been shown to transport fluids vescially as first indicated by the observation that hemicysts or blisters form in confluent monolayer cultures of these cells (8). The hemicysts (groups of cells in the monolayer slightly raised from the dish surface) presumably form as a result of vectorial transport of salt and water from the mucosal surface of the cells (facing the medium) through their serosal surface (facing the culture dish), which results in the trapping of these substrates between the cell monolayer and dish and the buildup of hydrostatic pressure. More recently, transepithelial cation and water fluxes were demonstrated directly employing MDCK monolayers grown on semipermeable supports and placed in a Ussing chamber (9, 10).

The first phase of the transepithelial Na$^+$ flux, transport of Na$^+$ into the epithelial cells through the mucosal surface, may be examined by measuring $^{22}\text{Na}^+$ influx in the presence of ouabain which inhibits the (Na$^+$/K$^+$)-ATPase (11). In the $^{22}\text{Na}^+$ flux studies presented below the effects of both intracellular and extracellular calcium on Na$^+$ transport are examined. These studies show that 1) extracellular calcium inhibits mucosal Na$^+$ influx, and 2) cytoplasmic calcium increases the Na$^+$ influx. Thus, Ca$^{2+}$ may function as a second messenger of hormone action in kidney epithelial cells, controlling the rates of transepithelial salt transport.

MATERIALS AND METHODS

Cells and Maintenance—The dog kidney epithelial cell line MDCK, obtained from Dr. John Holland at the University of California, San Diego, was maintained as monolayer cultures in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 1.9 x 10$^{-5}$ IU/ml of penicillin, 0.2 mg/ml of streptomycin, and 25 µg/ml of ampicillin.

Transport Studies—Uptake of $^{22}\text{Na}^+$ was studied in confluent monolayer cultures of MDCK cells in 30-mm tissue culture dishes at 24°C using 10 mM Tris buffer, pH 7.3, made isotonic to 110 mM NaCl with sucrose. The presence of sucrose in the uptake buffer permitted the monolayers to remain attached to the dishes over extended time periods (1 or more), although the sucrose itself had no significant effect on the initial rate of Na$^+$ influx. The cells were routinely washed three times with 100 mM Tris-HCl buffer, pH 7.3, immediately before and after the uptake period. After the final washing, the intracellular label was extracted into deionized water for at least 1 h, and the label in the cell extract was determined by scintillation counting using Triton/toluene/3,5-diphenyloxazole/1,4-bis[2-(5-phenyloxazolyl)]benzene scintillation fluid. All uptake determinations were made in duplicate (or in triplicate, when determining Michaelis-Menten constants) and were corrected for zero time uptake (label not removed by the washing procedure). Duplicate determinations were reproducible within 10%. Na$^+$ uptake was measured in the presence of 10$^{-5}$ M ouabain. Prior to Na$^+$ uptake measurements, MDCK cells were preincubated for at least 1 h in Dulbecco’s modified Eagle’s medium containing 10$^{-5}$ M ouabain at 37°C. These conditions were shown to permit maximum inhibition of (Na$^+$/K$^+$)-ATPase activity, as suggested by the fact that the initial rate of Rb$^+$ uptake was maximally inhibited after a 1-h preincubation with 10$^{-5}$ M ouabain.

The initial rate of Na$^+$ uptake was determined using a 5-min time interval in cells not preloaded with calcium, while a 1-min time interval was used with calcium-loaded cells. The rate was constant with time within this time interval over the entire Na$^+$ concentration...
The inhibitory effects of calcium on Na+ transport were found to be resolvable into two components, a noncompetitive component (between 0 and 1.8 mM CaCl₂) with a Ki of 0.7 mM and a competitive component (between 1.8 and 18 mM CaCl₂) with a Ki of 17 mM. The latter component suggested the possibility that calcium might be transported by the Na+ channel. However, inhibition studies using lanthanum indicated that calcium was not transported exclusively by the Na+ channel. While 5 mM lanthanum inhibited calcium influx by 95% when the Ca2+ concentration was 1.8 mM, influx of Na+ (at 1.1 mM) was inhibited by only 50%.

The nonlinear Dixon plot (Fig. 1) would also be anticipated if calcium inhibited Na+ influx by means of a surface charge effect (4). The Gouy-Chapman or diffuse double layer theory concerning such a surface charge effect predicts that Na+ influx would be inhibited to a similar extent by equimolar concentrations of divalent cations, whereas trivalent cations were sometimes enhanced by as much as 50%. The increase in Na+ uptake in cells exposed to Ca2+ and the ionophore correlated with increased levels of intracellular calcium (Fig. 3B). Cells preincubated with A23187 + 1.8 mM calcium had intracellular calcium levels 3-fold higher than control cells at the range studied. To determine Michaelis-Menten constants for Na+ uptake, the reciprocal uptake rates were graphed versus the reciprocal Na+ concentrations after correction for diffusion (12). To determine the diffusion coefficient of Na+, the intracellular space was estimated to be 75% of the total cell size which was determined using a Coulter Counter.

Chemicals—Radiochemicals (\(^{32}\)Na, \(^{86}\)Rb, and \(^{45}\)Ca) were obtained from New England Nuclear. A23187, a gift from Eli Lilly, was dissolved in dimethyl sulfoxide at 1 mg/ml before use.

RESULTS

The Effect of Extracellular Calcium on Na+ Influx—The inhibitory effects of extracellular calcium on the initial rate of Na+ influx are illustrated in Fig. 1. The Dixon plots were nonlinear at each Na+ concentration studied, and the inhibition of Na+ influx by calcium was incomplete, even at 18 mM CaCl₂. Such nonlinear plots could be explained if Na+ enters the MDCK cells via two distinct transport systems. However, inhibition studies using lanthanum indicated that calcium inhibited Na+ influx by means of a surface charge effect (4). The Gouy-Chapman or diffuse double layer theory concerning such a surface charge effect predicts that Na+ influx would be inhibited to a similar extent by equimolar concentrations of divalent cations, whereas trivalent cations were all found to exert similar inhibitory effects, but La3+ was no more inhibitory than the divalent cations (Fig. 2).

The Effect of Intracellular Calcium on Na+ Influx—The effect of intracellular calcium on Na+ influx is illustrated in Fig. 3A. To increase the cytoplasmic concentration of calcium, MDCK cells were preincubated in buffer containing 1.8 mM calcium and the divalent cation ionophore A23187. The initial rate of Na+ uptake in the presence of Ca2+ was enhanced 10-fold by treatment with the ionophore. Cells preincubated with ionophore in the absence of calcium did not show an equivalent increase in the Na+ transport rate, although Na+ uptake was sometimes enhanced by as much as 50%. The increase in Na+ uptake in cells exposed to Ca2+ and the ionophore correlated with increased levels of intracellular calcium (Fig. 3B). Cells preincubated with A23187 + 1.8 mM calcium had intracellular calcium levels 3-fold higher than control cells at the

Fig. 2. Specificity of the inhibition of Na+ uptake by extracellular polyvalent cations. The dependence of the initial rate of 1.1 mM Na+ uptake on extracellular polyvalent cations (CaCl₂, ○; BaCl₂, □; LaCl₃, ▲) examined as described in Fig. 1. The total Na+ uptake remaining in the presence of these cations (% standard Na+ uptake) was plotted as a function of the extracellular polyvalent cation concentration present during the Na+ uptake period.

Fig. 3. The effect of preincubation with calcium and A23187 on Na+ and Ca2+ uptake. The effect of preincubation with CaCl₂ and A23187 on intracellular Na+ and Ca2+ concentrations was examined. Quinbain-treated MDCK cells were preincubated 20 min in 1 mM Tris buffer, pH 7.3, containing 6% sucrose and either 1.8 mM CaCl₂ + 10 μg/ml of A23187 (○, A23187 treated cells), 1.8 mM CaCl₂ (●, control cells pretreated with calcium), or no added CaCl₂ or A23187 (□, control cells). MDCK cells then were incubated for a 20 min time interval with 1.1 mM NaCl (i) in the presence of 1.8 mM CaCl₂ + 10 μg/ml of A23187 (●, A23187-treated cells), (ii) in the presence of 1.8 mM CaCl₂ (□, control cells), or (iii) in the absence of both CaCl₂ and A23187 (○, control cells). During this latter incubation period \(^{22}\)Na+ uptake was examined (i) in the presence of 1.8 mM CaCl₂ in A23187-treated cells and in control cells or (ii) in the absence of CaCl₂ (control cells). In parallel experiments, MDCK cells were incubated during both 20-min periods with \(^{45}\)Ca2+ (●, A23187-treated cells, and ○, control cells pretreated with calcium).
In the experiments described above, cells were loaded with Ca" employing the divalent ion carrier A23187. As an alternative method to increase cytoplasmic calcium, cells were preincubated with relatively high concentrations of extracellular calcium in the absence of the ionophore (Fig. 4). The rate of Na" influx was enhanced by preincubation with calcium under these conditions. The net increase in the Na" influx rate was shown to be a saturable phenomenon, preincubation with 10 mM CaCl giving half maximal stimulation.

The effects of other divalent cations on Na" influx were also examined. To compare the effects of intracellular Mg2+, Mn', and Ca" on Na" transport, MDCK cells were preincubated with these cations at 0 to 16 mM in the presence of A23187. Subsequently, the divalent cations and the ionophore were removed, and the initial rates of Na' uptake were measured (Fig. 5). Under these conditions, Na" influx was not appreciably stimulated by Mg2+ or Mn' above control values. The ionophore alone sometimes stimulated Na" uptake, presumably due to the release of mitochondrial calcium into the cytoplasmic compartment.

The intracellular Ca", Mg", and Mn" concentrations were not directly assayed in this study. However, A23187 has been shown to have an equivalent capacity to shuttle Mg2+ and Ca" across membranes and to shuttle Mn" with a 2-fold higher affinity (14). Thus, the ionophore should equilibrate Ca", Mg", and Mn" to equivalent intracellular concentrations. That Mg" and Mn" do compete with Ca" for the ionophore was indicated by the observation that the increased rate of Na" uptake which followed preincubation with 1.8 mM CaCl2 + A23187 was not observed if either MgCl2 or MnCl2 was also present during the preincubation period at minimal concentrations of 1.8 and 0.9 mM, respectively.

Divalent cations of certain heavy metals (Cd2+ or Hg2+) also enhanced the Na" influx rate. Na" uptake was stimulated when either of these cations (18 µM) was added simultaneously with 1.1 mM NaCl at the onset of the influx measurement (data not shown). The mechanism of this effect is not known, but Hg2+ and Cd2+ may react with critical sulfhydryl groups.

The Effects of Intracellular Calcium on the Kinetics of Na" Transport—Intracellular calcium might enhance the rate of Na" influx by any one of several mechanisms: by affecting the diffusion of Na" through the plasma membrane, by increasing Na" uptake via the Na" channel, or by activating an alternative transport system. To distinguish between these possibilities, the effect of intracellular calcium on the kinetics of Na" uptake was examined.

The initial rates of Na" uptake were measured over a 5-min time interval in control cells and in calcium-loaded cells. The initial rate of Na" uptake was examined over a 5-min time interval in control cells, and a 1-min time interval in calcium-loaded cells. The initial rate of Na" uptake was corrected for zero time uptake and diffusion was described under "Materials and Methods."
of Na⁺ influx were studied. Lineweaver-Burk plots of Na⁺ influx in calcium-loaded and unloaded cells were linear (Fig. 6); suggesting that, under both conditions, a single system transported Na⁺. In unloaded cells the $K_m$ and $V_{max}$ were 50 mM and 20 nmol/mg of protein/min, respectively. In calcium-loaded cells the $V_{max}$ was estimated to be relatively high (250 nmol of Na⁺/mg of protein/min).

The inhibitory effects of monovalent cations on Na⁺ transport were also examined. In control cells 1.1 nmol Na⁺/mg of protein/min). In calcium-loaded cells Na⁺, Rb⁺, and guanidine, while 11 mM NaCl and RbCl had no significant inhibitory effects (Table I). In calcium-loaded cells Na⁺, Rb⁺, amiloride, and guanidine were all inhibitory to Na⁺ uptake (Table I). This result is consistent with the hypothesis that in calcium-loaded cells the affinity of the Na⁺ channel for these cations is altered.

The possibility that increased Na⁺ uptake was due to a Na⁺/Ca²⁺ exchange process was examined. Contrary to results reported with cell types in which a Na⁺/Ca²⁺ exchange system is operative (15, 16), extracellular Na⁺ did not have stimulatory effect on calcium efflux from MDCK cells. Moreover, elevated concentrations of intracellular Na⁺ were inhibitory rather than stimulatory to calcium influx, and extracellular Ca²⁺ did not stimulate Na⁺ efflux from Na⁺-preloaded cells. Thus, no supportive evidence for a Na⁺/Ca²⁺ exchange process was obtained. However, Na⁺ efflux was enhanced by intracellular calcium, while the Na⁺ efflux rate was inhibited by extracellular calcium (Fig. 7). Thus, intracellular Ca²⁺ stimulated and extracellular calcium inhibited Na⁺ transport regardless of whether Na⁺ entry or Na⁺ efflux was observed.

Reversibility of the Calcium Stimulatory Effect—To examine the reversibility of the uptake stimulation caused by calcium, cells were loaded with calcium for 20 min using A23187. The Na⁺ entry rate was significantly enhanced immediately following this treatment. However, if a 20-min incubation period in the absence of these reagents followed the preincubation period no stimulation of Na⁺ uptake is observed. This observation indicates that the stimulation of Na⁺ uptake is a fully reversible effect.

**Discussion**

Calcium appears to play two direct roles in regulating the Na⁺ channel in the cultured kidney epithelial cell line MDCK. Intracellular calcium was stimulatory to Na⁺ transport, while extracellular calcium was inhibitory. The inhibitory effect of extracellular calcium on Na⁺ entry has been observed in other cell types, and Na⁺ absorption by dog kidney has been shown to be inhibited by calcium (17). Stimulatory effects of intracellular calcium on Na⁺ influx have not been reported previously in transporting epithelial cells. However, high cell calcium has been reported to lower the entrance of Na⁺ across the mucosal surface of some epithelial cells (18, 19).

The presented results indicate that extracellular calcium and intracellular calcium affect Na⁺ influx by different mechanisms. The inhibition of Na⁺ influx by extracellular calcium may be explained (i) by both competitive and noncompetitive mechanisms or (ii) by a surface charge effect (4). Consistent with the latter hypothesis divalent cations (Mg²⁺, Mn²⁺, and Ba²⁺) were similarly inhibitory to Na⁺ uptake. However, the stimulatory effect of extracellular calcium on Na⁺ uptake was highly calcium specific, which distinguishes the mechanism of the intracellular effect from the extracellular effect of calcium on Na⁺ influx.

Several possible effects of intracellular calcium on Na⁺ transport were considered. The possibility seems unlikely that increased Na⁺ transport resulted from (i) a surface charge effect, as the intracellular affect is highly calcium specific, (ii) transport by another Na⁺ channel, as Lineweaver-Burk plots of Na⁺ influx were linear, (iii) Na⁺/Ca²⁺ countertransport, as extracellular calcium was inhibitory, rather than stimulatory to Na⁺ efflux, or (iv) loss of inhibitory cations from the cells as the stimulation caused by extracellular calcium is a reversible phenomenon. However, the data presented here are consistent with the hypothesis that intracellular calcium stimulates Na⁺ transport by specifically altering the activity of the Na⁺ channel. For instance calcium may bind to a regulatory site on the Na⁺ channel protein.

While the physiological significance of these observations concerning calcium and Na⁺ transport cannot presently be assessed, an inhibitory effect of extracellular Ca²⁺ on renal Na⁺ absorption has been reported (17). Moreover, while cytoplasmic calcium is generally maintained at micromolar concentrations, cells have the potential for dramatic increases in these levels (20). An earlier report showing that extracellular calcium enhances transepithelial water flux in the frog urinary bladder (21) suggests that intracellular calcium may regulate the rates of transepithelial sodium and water transport in a number of cell types. A connection between the regulation of salt and fluid transport by cytoplasmic Ca²⁺ and the mechanism of action of specific hormonal agents has yet to be investigated.

**Acknowledgments**—We thank Lorraine Chuman for technical assistance, K. Walsh for preparation of the manuscript, and Dr. G. Fortes for valuable discussion.
REFERENCEs

Regulation of 22Na+ transport by calcium in an established kidney epithelial cell line.
M Taub and M H Saier, Jr


Access the most updated version of this article at http://www.jbc.org/content/254/22/11440

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/254/22/11440.full.html#ref-list-1