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Sodium-Calcium Exchange in Renal Epithelial Cells: Dependence on Cell Sodium and Competitive Inhibition by Magnesium

Rong-Ming Lyu, Lucinda Smith, and Jeffrey Bingham Smith
Department of Pharmacology, Schools of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, Alabama 35294

Summary. Kinetic properties of Na^+-Ca^{2+} exchange in a renal epithelial cell line (LLC-MK_2) were assessed by measuring cytosolic free Ca^{2+} with fura-2 and $^{45}Ca^{2+}$ influx. Replacing external Na^+ with K^+ produced relatively small increases in free Ca^{2+} and $^{45}Ca^{2+}$ uptake unless the cells were incubated with ouabain. Ouabain markedly increased cell Na^+ and strongly potentiated the effect of replacing external Na^+ with K^+ on free Ca^{2+} and $^{45}Ca^{2+}$ uptake. $^{45}Ca^{2+}$ influx in 140 mM K^+ or N-methyl-D-glucamine minus influx in 140 mM Na^+ was used to quantify Na^+-Ca^{2+} exchange activity of Na^+-loaded cells. The dependence of exchange on cell Na^+ was sigmoidal; the $K_{0.5}$ was 26 ± 3 mmol/liter cell water space, and the Hill coefficient was 3.1 ± 0.2. The kinetic features of the dependence of exchange on cell Na^+ partly account for the small increase in Ca^{2+} influx when all external Na^+ is replaced by K^+. Besides raising cell Na^+ ouabain appears to activate the exchanger. Magnesium competitively inhibited exchange activity. The potency of Mg^{2+} was 8.2-fold lower with potassium instead of N-methyl-D-glucamine or choline as the replacement for external Na^+. Potassium also increased the V_{max} of exchange by 86% and had no effect on the K_m for Ca^{2+}. The exchanger does not cause detectable $^{22}Na^+$-Mg^{2+} exchange and does not appear to require K^+ or transport $^{86}Rb^+$. Although exchange activity was plentiful in the epithelial cells from monkey kidney, others from amphibian, canine, opossum, and porcine kidney had no detectable exchange activity. All of the measured kinetic properties of Na^+-Ca^{2+} exchange in the renal epithelial cells are very similar to those of the exchanger in rat aortic myocytes.

Key Words transport · calcium absorption · magnesium · smooth muscle · kidney

Introduction

The Na^+-Ca^{2+} exchange mechanism makes a prominent contribution to Ca^{2+} regulation in heart and brain, as well as certain vascular smooth muscle cells (Benos, Warnock & Smith, 1991). Gmaj, Murer and Kinne (1979) demonstrated Na^+-Ca^{2+} exchange activity in basolateral membranes from rat renal cortex. Talor and Arruda (1985) solubilized the Na^+-Ca^{2+} exchanger from bovine kidney cortex, reconstituted it in liposomes, and obtained a stoichiometry of 3 Na^+ : 1 Ca^{2+}. Recently Ramachandran and Brunette (1989) isolated basolateral membranes from rabbit proximal and distal convoluted tubules and found Na^+-Ca^{2+} exchange activity only in membranes from the distal tubule. There seems to be a reciprocal relationship between Na^+ reabsorption and intracellular Ca^{2+} in the proximal tubule (Yang, Lee & Windhager, 1988). The specific activities of Na^+-Ca^{2+} exchange in the renal membranes is very small (references cited above), however, and a substantial portion of Ca^{2+} reabsorption apparently occurs by a paracellular mechanism (Bourdeau, 1986). Therefore, it is unclear what role Na^+-Ca^{2+} exchange plays in transcellular Ca^{2+} reabsorption in the nephron (Bronner, 1989).

Snowdowne and Borle (1985) observed that the replacement of external Na^+ with tetramethylammonium (TMA)[1] ion increased $[Ca^{2+}]_i$ from 60 to 700 nM in a line of kidney cells (LLC-MK_2) from *Macacca mulatta*. They measured $[Ca^{2+}]_i$ with the photoprotein, aequorin, which was introduced by scrape loading. Decreasing external Na^+, however, evokes the release of stored Ca^{2+} in some mammalian cells including human skin and lung fibroblasts, neuroblastoma, intestinal and aortic myocytes, and canine coronary endothelial cells (Smith, Dwyer & Smith, 1989*a*; Dwyer, Zhuang & Smith, 1991). Therefore, we have examined LLC-MK_2 cells to clarify whether Ca^{2+} influx via the plasma mem-

[1] *Abbreviations:* $[Ca^{2+}]_i$ = the free calcium concentration in the cytosol; DMEM = Dulbecco's modified Eagle's medium; FBS = fetal bovine serum; HEPES = N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; IC_{50} = the concentration that produces 50% inhibition; MDCK = Madin-Darby canine kidney cells; NMG = N-methyl-D-glucamine; OK = opossum kidney cells; PSS = physiological salts solution; SE = standard error; and TMA = tetramethylammonium.

brane Na^+-Ca^{2+} exchanger or the release of stored Ca^{2+} increases $[Ca^{2+}]_i$ in response to a decrement in external Na^+. We show here that Na^+-Ca^{2+} exchange is plentiful in LLC-MK_2 cells and that it shares several kinetic properties with the exchanger in aortic myocytes. In contrast to the findings of Snowdowne and Borle (1985), however, we found that replacing external Na^+ with TMA or K^+ strikingly increased $[Ca^{2+}]_i$ only if cell Na^+ was increased by incubating the cells with ouabain. $^{45}Ca^{2+}$ influx data presented here agree well with our $[Ca^{2+}]_i$ data and confirm that Na^+-Ca^{2+} exchange is abundant in LLC-MK_2 cells, although other lines of renal epithelial cells had no detectable exchange activity. These results were presented at the 35th Annual Meeting of the Biophysical Society (Lyu, Smith & Smith, 1991*b*).

Materials and Methods

Cell Culture

LLC-MK_2, MDCK, and OK cells were grown at 37°C in DMEM containing 5 to 10% (vol/vol) FBS. DMEM contained 4.5 g/liter D-glucose, 0.11 g sodium pyruvate, 0.37 g/liter $NaHCO_3$, and 10 mM HEPES. A6 cells were grown at 27°C in DMEM containing 10% FBS and 100 U/ml penicillin G and 0.1 mg/ml streptomycin. LLC-PK_1 cells were grown at 37°C in medium 199 (Earle's salts) containing 10 mM HEPES and 10% FBS.

Stock cultures in 80-cm^2 culture flasks were rinsed twice with Dulbecco's phosphate buffered saline minus Ca^{2+} and Mg^{2+}, and 1 ml of this saline containing 0.6 mM ethylenediaminetetraacetic acid and 0.05% trypsin (2.5% solution of trypsin, porcine papavirus and mycoplasma tested) was added. The cells rounded up, usually in less than a minute, were detached by slapping the flask against the palm of the hand, and 9 ml of culture medium was added to the flask to stop the trypsin. Usually the cells from three flasks were diluted to 120 ml with culture medium and used to seed sixty 35-mm (diameter) culture dishes. Confluent cultures were usually used two days after plating for $^{45}Ca^{2+}$ influx experiments. Stock cultures were passaged 1:2 three times per week. All cells were grown in an humidified atmosphere of 5% CO_2-95% air.

Cytosolic Free Calcium

LLC-MK_2 cells were grown on cover glasses and incubated with fura-2 acetoxymethyl ester one day after plating as previously described (Smith & Smith, 1987; Smith et al., 1989*a*). Briefly, confluent cover glasses were rinsed with DMEM and incubated for 30 min in 1 ml of DMEM containing 5 μM fura-2 acetoxymethyl ester and 0.25 mM sulfinpyrazone in a humidified atmosphere of 5% CO_2-95% air. Then they were rinsed with a PSS which contained (in mM): 120 NaCl, 20 $NaHCO_3$, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, and 20 HEPES/Tris, pH 7.4. The cover glasses were incubated for 30 min in PSS containing 10 mM glucose and 0.25 mM sulfinpyrazone and rinsed again with PSS. The cover glass was placed inside of a triangular cuvette (NSG Precision Cells, Farmingdale, NY), on the diagonal face with the cell side of the cover glass facing the solution. Fluorescence was measured with a dual-excitation spectrofluorometer (Deltascan I, Photon Technology International, Princeton, NJ) as described previously (Smith & Smith, 1987; Smith, Zheng & Smith, 1989*b*). Free Ca^{2+} was calculated as indicated previously (Grynkiewicz, Poenie & Tsien, 1985). Sulfinpyrazone, which is more potent than probenicid as an inhibitor of organic anion transport in the kidney (Weiner, 1990), increased the retention of fura-2 by the LLC-MK_2 cells as previously reported for PC12 and N2A cells (Di Virgilio, Fasolato & Steinberg, 1988). The fluorescence of a cover glass of cells that were not incubated with fura-2 acetoxymethyl ester was subtracted from the fluorescence of the dye-loaded cells. Net fluorescence was at least equal to autofluorescence at both wavelengths. The experimental treatments had no effect on autofluorescence.

Na^+-Ca^{2+} Exchange

$^{45}Ca^{2+}$ uptake was measured as previously described (Smith & Smith, 1987). Briefly, 0.1 mM ouabain (100 μl of 2 mM) was added to the culture medium (2 ml/dish) two days after plating the cells. After 0.5 or 1 hr (as indicated) the cultures were rinsed three times with a solution containing 140 mM NaCl, KCl, NMG chloride, or choline chloride (as indicated) and 20 mM HEPES/Tris, pH 7.4. The cultures were then incubated for the indicated time interval with the rinse solution which also contained 0.06 to 1 mM $CaCl_2$ (as indicated), 0.1 mM ouabain, and 2 μCi $^{45}Ca^{2+}$. Uptake was stopped by rinsing the cultures eight times with 0.1 M $MgCl_2$ containing 10 mM $LaCl_3$ and 10 mM HEPES/Tris, pH 7.4. $^{45}Ca^{2+}$ was extracted with 0.1 N nitric acid and measured by liquid scintillation spectrometry.

Cell Sodium, Potassium, and Water Space

The ions were measured by atomic absorption and water space by equilibration with [^{14}C]urea as previously described (Smith et al., 1989*b*). Briefly, for Na^+ and K^+ determinations the cells were incubated as described above for assaying exchange activity, rinsed eight times with 0.1 M $MgCl_2$ containing 10 mM HEPES/Tris, pH 7.4, and extracted with 0.1 N nitric acid. For cell water space the cells were incubated for 30 min with 0.1 mM urea containing 1 μCi [^{14}C]urea and rinsed three times with ice-cold 0.1 M $MgCl_2$ containing 10 mM HEPES/Tris, pH 7.4.

Materials

LLC-MK_2 (ATCC CCL 7.1, passage 11), LLC-PK_1 (ATCC CRL 1392, CL 101, passage 196), and MDCK (ATCC CCL 34, passage 52) cells were obtained from the American Type Culture Collection (Rockville, MD). A6 and OK cells were provided by Drs. James Schaeffer and David G. Warnock, respectively, of the Departments of Physiology and Biophysics and Medicine, University of Alabama at Birmingham. FBS was from Hyclone Laboratories, Logan UT. Culture media, trypsin, and antibiotics were from Gibco BRL, Grand Island, NY. Sulfinpyrazone was from Sigma Chemical, St. Louis, MO. Fura-2 and fura-2 acetoxymethyl ester were from Molecular Probes, Eugene, OR. $^{45}CaCl_2$ (20–30 Ci/g), [^{14}C]urea (55 Ci/mol), and ^{86}RbCl (5 Ci/g) were from Du Pont, NEN Research Products, Boston, MA.

All incubations were at 37°C unless otherwise indicated. Error bars in the figures indicate one SE value. If no error bars

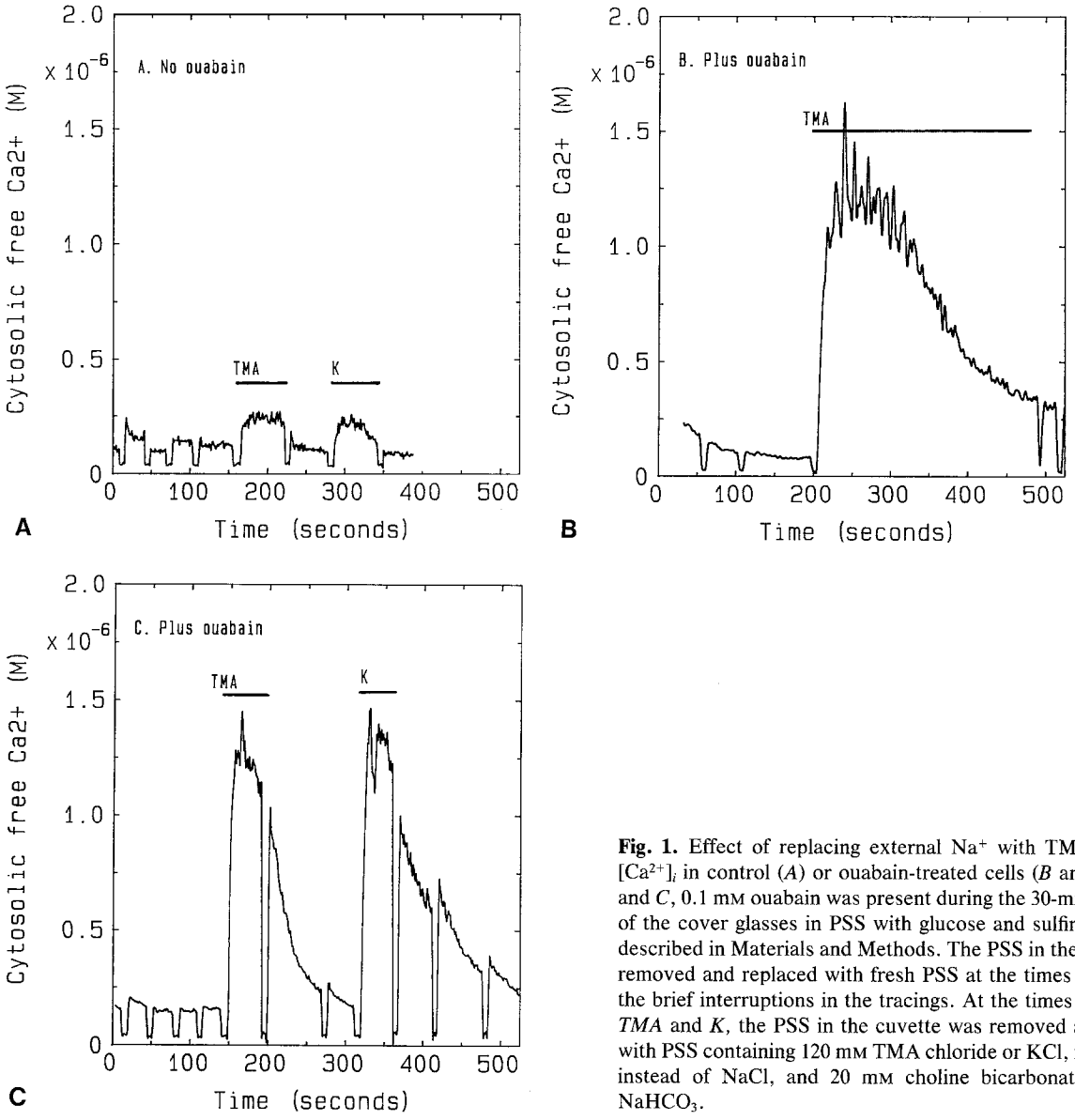

Fig. 1. Effect of replacing external Na^+ with TMA or K^+ on $[Ca^{2+}]_i$ in control (*A*) or ouabain-treated cells (*B* and *C*). For *B* and *C*, 0.1 mM ouabain was present during the 30-min incubation of the cover glasses in PSS with glucose and sulfinpyrazone as described in Materials and Methods. The PSS in the cuvette was removed and replaced with fresh PSS at the times indicated by the brief interruptions in the tracings. At the times indicated by *TMA* and *K*, the PSS in the cuvette was removed and replaced with PSS containing 120 mM TMA chloride or KCl, respectively, instead of NaCl, and 20 mM choline bicarbonate instead of $NaHCO_3$.

are shown, SE was smaller than half the diameter of the marker used to indicate the mean. The data in the tables were compared by two-way analysis of variance, and means were compared by the Scheffe *F* test.

Results

Raising Intracellular Na^+ Potentiates the Effect of Removing External Na^+ on $[Ca^{2+}]_i$

To detect small $[Ca^{2+}]_i$ increases it was necessary to remove and replace the PSS in the cuvette a few times to be sure that this manipulation caused no change in basal $[Ca^{2+}]_i$ (Fig. 1). Aspirating the PSS from the cuvette which contained the cover glass of cells and adding 1 ml of PSS which contained TMA chloride or KCl, instead of NaCl, produced a small increase in $[Ca^{2+}]_i$ (Figs. 1*A* and 2). Replacing external Na^+ with TMA or K^+ increased $[Ca^{2+}]_i$ from 86 ± 5 to 226 ± 18 or 265 ± 36 nM, respectively, in cells that were not treated with ouabain (Fig. 2). By contrast, in cells that were incubated with 0.1 mM ouabain for 30 min, replacing external Na^+ with TMA or K^+ increased $[Ca^{2+}]_i$ from 132 ± 6 to 1188 ± 117 or 1194 ± 112 nM, respectively (Fig. 2). $[Ca^{2+}]_i$ rose immediately to a peak, which was sustained for about 50 sec, and then gradually decreased over a 5-min period (Fig. 1*B*). The addition of Na^+ shortly after its removal from ouabain-treated cells markedly accelerated the return of $[Ca^{2+}]_i$ to the basal level (Fig. 1*C* compared to *B*).

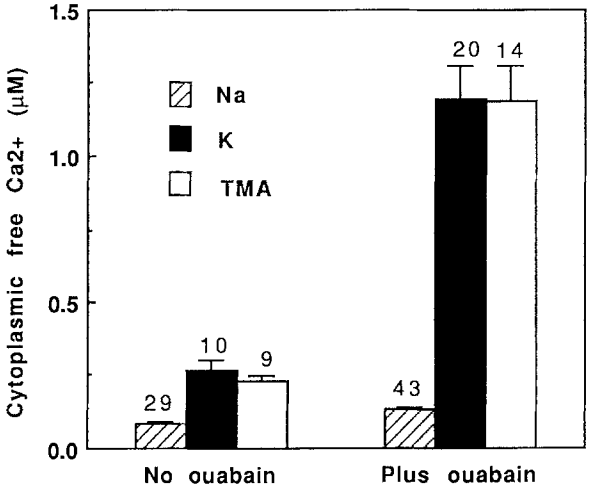

Fig. 2. Summary of the effects of ouabain treatment and the replacement of external Na^+ with TMA or K^+ on $[Ca^{2+}]_i$. Peak levels of $[Ca^{2+}]_i$ evoked by the replacement of external Na^+ with K^+ or TMA are shown for control (*No ouabain*) or Na^+-loaded (*Plus ouabain*) cells. The data were collected as indicated in Fig. 1. Peak $[Ca^{2+}]_i$ was taken as the highest value that was sustained for at least 20 sec. For example, the peak value for Fig. 1*B* was about 1.25 μM. Values are mean ± SE with the number of cover glasses indicated above each bar.

These data show that replacing external Na^+ with TMA or K^+, respectively, maximally increased $[Ca^{2+}]_i$ by 2.63- and 3.08-fold in cells that were not treated with ouabain compared to 9.00- and 9.04-fold in ouabain-treated cells (Fig. 2). Additionally, the data show that the ouabain treatment increased basal $[Ca^{2+}]_i$ by 1.54-fold.

Increasing Cell Na^+ Greatly Potentiates the Effect of Removing External Na^+ on $^{45}Ca^+$ Influx

Figure 3 shows the time courses of $^{45}Ca^{2+}$ uptake in the presence of Na^+ or K^+. Uptake was measured in the presence of 1 mM Ca^{2+} and no Mg^{2+} because it is a competitive inhibitor of exchange activity in aortic myocytes (Smith, Cragoe & Smith, 1987) and these cells (*see* Fig. 6). Replacing external Na^+ with K^+ only slightly increased $^{45}Ca^{2+}$ uptake in cells that were not treated with ouabain (Fig. 3*A*). Treating the cells with 0.1 mM ouabain for 0.5 hr greatly increased $^{45}Ca^{2+}$ uptake in K^+ and slightly decreased uptake in the solution containing Na^+ as the principle cation (Fig. 3). Hence, the initial rate of net $^{45}Ca^{2+}$ uptake (i.e., uptake in the solution containing 140 mM KCl minus that in 140 mM NaCl) was 10.4 times greater in the ouabain-treated cells

Table 1. Effect of replacing Na^+ with K^+, NMG, or choline on $^{45}Ca^{2+}$ uptake by Na^+-loaded cells

External cation	$^{45}Ca^{2+}$ uptake (nmol/min mg)	Na^+-Ca^{2+} exchange (nmol/min mg)	(% control)*
Na^+	3.5 ± 0.5 (12)	0	0
K^+	14.8 ± 1.5 (12)	11.3 ± 1.1 (12)	100[a]
NMG	10.4 ± 1.4 (9)	6.9 ± 0.9 (9)	61[b]
Choline	8.1 ± 0.8 (3)	4.6 ± 0.3 (3)	41[b]

* Values with different superscripts are statistically different at 95% significance. Values are mean ± SE with the number of experiments indicated in parentheses. Duplicate cultures were incubated for 1 hr with 0.1 mM ouabain as described in Materials and Methods and rinsed three times with a solution containing 140 mM of the chloride salt of the indicated cation and 20 mM HEPES/Tris, pH 7.4. Then they were incubated for 30 or 60 sec (depending on the experiment) in the rinse solution which also contained 0.1 mM $CaCl_2$, 0.1 mM ouabain, and 2 μCi $^{45}CaCl_2$. Each experiment compared uptake in Na^+ and K^+ to that in either NMG or choline. Exchange activity was determined as the difference between the initial rate of $^{45}Ca^{2+}$ uptake in the K^+, NMG, or choline solution and that in the Na^+ solution.

(Fig. 3). High external Na^+ would be expected to inhibit Na^+-Ca^{2+} exchange by competing for its Ca^{2+} site as shown in cardiac sarcolemmal vesicles (Slaughter, Sutko & Reeves, 1983) and decreasing the driving force for Ca^{2+} influx.

The half-hour incubation with ouabain increased total cell Na^+ by 55 mmol/liter cell water space and decreased cell K^+ by 52 mmol/liter cell water space. Cell Na^+ increased from 30 ± 3 to 85 ± 4 mmol/liter (n = 5 experiments on quadruplicate cultures), and K^+ decreased from 114 ± 5 to 62 ± 3 mmol/liter (n = 6 experiments on quadruplicate cultures). The ouabain treatment had no effect on cell water space, which was 9.8 ± 0.5 and 9.8 ± 0.4 μl/mg protein (n = 6 experiments on quadruplicate cultures).

Additionally, replacing external Na^+ with NMG or choline instead of K^+ markedly increased $^{45}Ca^{2+}$ influx by the ouabain-treated cells (Table 1). In these experiments, which were done at 0.1 mM external Ca^{2+}, Na^+-Ca^{2+} exchange activity was 6.9, 4.6, and 11.3 nmol/min mg in the presence of NMG, choline, or K^+, respectively (Table 1). External K^+ increased the V_{max} of Na^+-Ca^{2+} exchange by 86% compared to the V_{max} in NMG as discussed below (Table 2). Because replacing external Na^+ with K^+ only slightly increased $^{45}Ca^{2+}$ uptake in cells that were not Na^+-loaded (Fig. 3), raising cell Na^+ *and* substantially decreasing the concentration of external Na^+, appears to be necessary to markedly increase $^{45}Ca^{2+}$ influx.

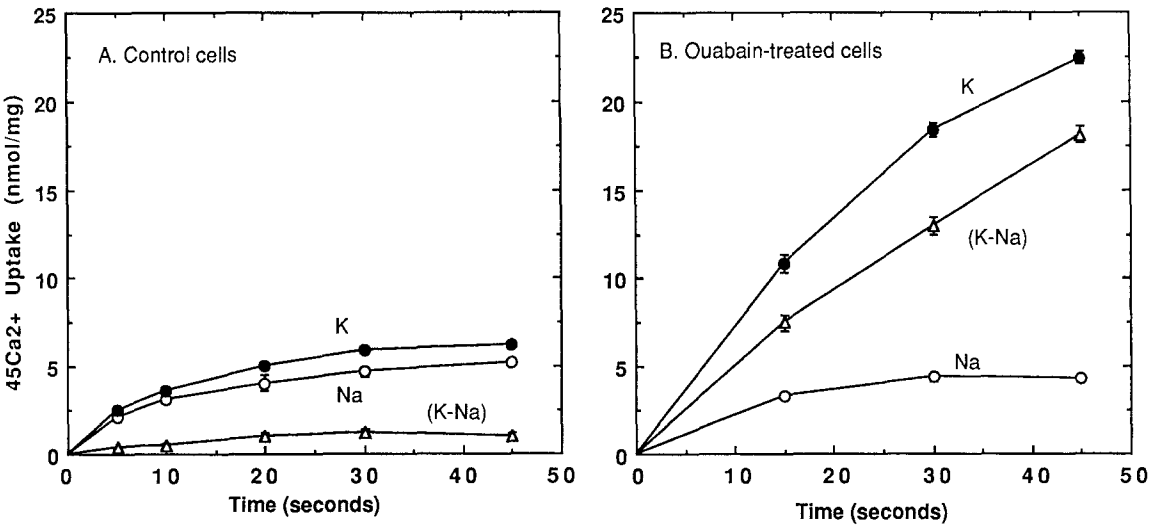

Fig. 3. Effect of treating the cells with ouabain on the time course of $^{45}Ca^{2+}$ uptake in the presence of external Na^+ or K^+. Some cultures received 100 μl water (*A*) and others (*B*) received 100 μl 2 mM ouabain per 2 ml of conditioned culture medium 0.5 hr before assaying $^{45}Ca^{2+}$ uptake in the presence of 140 mM NaCl (*Na*) or KCl (*K*). The triangles indicate the difference between uptake in NaCl and KCl. The cultures were rinsed three times with 140 mM NaCl containing 20 mM HEPES/Tris, pH 7.4, and $^{45}Ca^{2+}$ uptake was measured in 1 ml of the NaCl or a solution which contained 140 mM KCl instead of NaCl. The uptake solution also contained 1 mM $CaCl_2$, 2 μCi $^{45}CaCl_2$, and, for *B*, 0.1 mM ouabain. Open circles indicate uptake by cultures rinsed and incubated with NaCl, and filled symbols indicate uptake by cultures rinsed with NaCl and incubated with KCl. The initial portion (0 to 30 sec) of the net uptake curves were fitted by linear regression: for control cells, $y = 0.077 + 0.0417\,x$, $r^2 = 0.97$; for ouabain-treated cells, $y = 0.333 + 0.433\,x$, $r^2 = 0.99$. Values are mean ± SE (n = 9 to 12 cultures, four experiments for *A* and three for *B*).

Table 2. Summary of kinetic parameters of Na^+-Ca^{2+} exchange in LLC-MK_2 cells

Assay condition	K_m for Ca^{2+} (mM)	V_{max} (nmol/min mg)	K_i for Mg^{2+} (mM)	IC_{50} for Mg^{2+} (mM)	$K_{0.5}$ for Na^+ (mM)	Hill coefficient for Na^+
High K^+	0.20 ± 0.02 (6)[a]	38.0 ± 6.8 (4)[a]	ND	1.56 ± 0.18 (5)[a]	26 ± 3	3.1 ± 0.2
No K^+ (NMG)	0.23 ± 0.03 (8)[a]	20.4 ± 1.9 (8)[b]	0.08 ± 0.01 (4)	0.19 ± 0.04 (3)[b]	ND	ND
No K^+ (choline)	ND	ND	ND	0.19 ± 0.05 (2)[b]	ND	ND

Values within a column that have different superscripts are statistically different at ≥95% significance. Values are mean ± SE with the number of experiments in parentheses. Initial rates of exchange activity were used to estimate K_m and V_{max} as indicated in Fig. 5, K_i as indicated in Fig. 6, IC_{50} as indicated in Fig. 7, and $K_{0.5}$ and Hill coefficient as indicated in Fig. 4. ND = not done.

Dependence of Na^+-Ca^{2+} Exchange on Intracellular Na^+

Cell Na^+ was varied from 5.7 to 67.6 mmol/liter cell water space by incubating the cells for 30 min with 0.1 mM ouabain and 5 to 140 mM extracellular Na^+ (Fig. 4). $^{45}Ca^{2+}$ influx at 0.25 mM external Ca^{2+} was measured in the presence of K^+ or Na^+ to estimate Na^+-Ca^{2+} exchange activity. The dependence of exchange activity on intracellular Na^+ was sigmoidal (Fig. 4). The data, which were fitted by nonlinear regression to the Hill equation, gave a Hill coefficient of 3.1 ± 0.2 and a $K_{0.5}$ of 26 ± 3 mmol/liter cell water space.

Note that the data point for cells with basal Na^+ (i.e., cells that were incubated at 140 mM external Na^+ without ouabain) is well below the curve for the ouabain-treated cells (Fig. 4). Therefore, it appears that ouabain increased exchange activity by some mechanism that is independent of cell Na^+.

Dependence of Na^+-Ca^{2+} Exchange on External Ca^{2+}

Figure 5 shows the dependence of exchange activity on extracellular Ca^{2+}. Exchange activity was higher at all Ca^{2+} concentrations when assayed with K^+ instead of NMG as the major external cation (Fig.

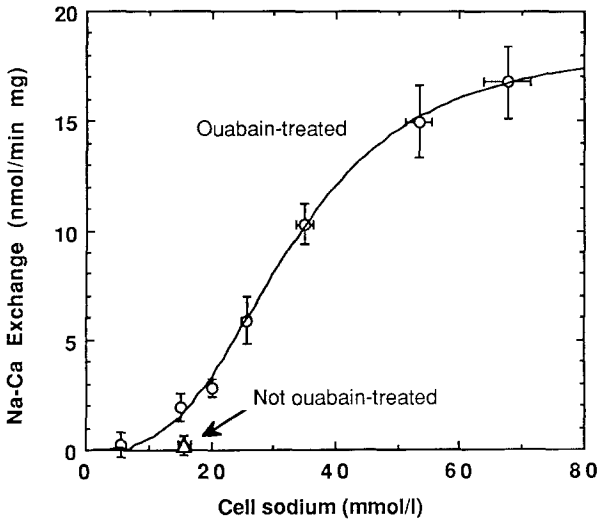

Fig. 4. Dependence of Na^+-Ca^{2+} exchange on intracellular Na^+. The cultures were rinsed and incubated for 30 min with 0.1 mM ouabain in solutions containing 10 mM glucose, 1 mM $MgCl_2$, 20 mM HEPES/Tris, pH 7.4, and 5, 20, 30, 40, 60, 100, or 140 mM NaCl. NaCl plus choline chloride always equaled 140 mM. The cultures indicated by the triangle were incubated for 30 min without ouabain in a solution containing (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, and 20 HEPES/Tris, pH 7.4. Some cultures were rinsed with ice-cold 0.1 M $MgCl_2$ and extracted with nitric acid for measurement of total cell Na^+. Some cultures also received 0.1 mM urea and 1 μCi [^{14}C]urea for measurement of intracellular water space during the 30-min incubation with (circles) or without (triangle) ouabain. Other cultures were rinsed three times with 140 mM NaCl or KCl solution containing 0.1 mM ouabain and 20 mM HEPES/Tris, pH 7.4, and incubated for 1 min with 1 ml of the rinse solution which also contained 0.25 mM $CaCl_2$, and 2 μCi $^{45}Ca^{2+}$. Na^+-Ca^{2+} exchange activity (nmol/min mg protein) is the difference between $^{45}Ca^{2+}$ influx in the K^+ and Na^+ solutions. Values are mean ± SE for three experiments on duplicate cultures.

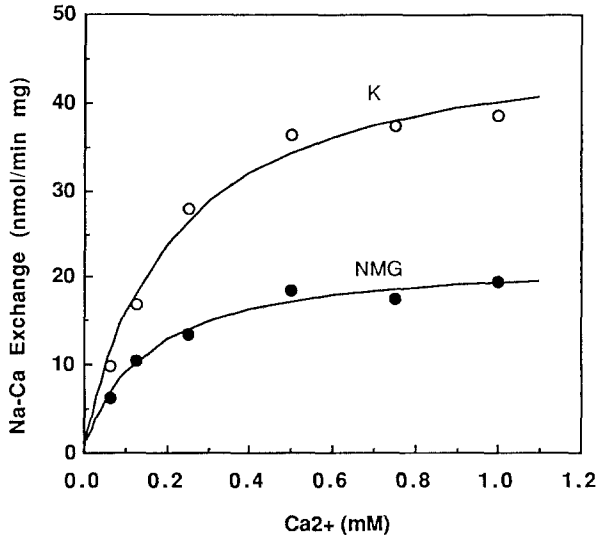

Fig. 5. Dependence of Na^+-Ca^{2+} exchange on Ca^{2+} concentration with K^+ or NMG as the principal external cation. The cultures were incubated with 0.1 mM ouabain for 1 hr as described in Materials and Methods. Then they were rinsed three times with a solution containing 140 mM NMG chloride (filled circles) or KCl (open circles) and 20 mM HEPES/Tris, pH 7.4, and incubated for 0.5 min with 1 ml of the same solution which also contained 0.1 mM ouabain, 2 μCi $^{45}Ca^{2+}$, and the indicated concentration of $CaCl_2$. $^{45}Ca^{2+}$ uptake in the presence of 140 mM NaCl and the indicated concentration of $CaCl_2$ was subtracted from uptakes in NMG chloride or KCl to obtain exchange activity. The data were fitted to the Michaelis-Menten equation by nonlinear regression (Marquart) with Enzfitter (Elsevier-BIOSOFT, Cambridge, UK). The curve for the data with K^+ as the principal external cation has a K_m of 0.21 ± 0.03 mM and a V_{max} of 48.3 ± 2.5 nmol/min mg. The curve for the data with NMG has a K_m of 0.15 ± 0.03 mM and a V_{max} of 22.2 ± 1.1 nmol/min mg. Values are means of duplicates for one of four experiments (Table 2) that gave similar results.

5). The K_m for Ca^{2+} was 0.20 ± 0.02 mM in the presence of K^+ and 0.23 ± 0.03 mM in the presence of NMG (Table 2). The V_{max} of exchange was 1.86-fold higher when exchange activity was assayed in the presence of K^+ instead of NMG (Fig. 5, Table 2).

Mg^{2+} Inhibition of Na^+-Ca^{2+} Exchange

Magnesium strikingly inhibited $^{45}Ca^{2+}$ influx that depended on inverting the Na^+ gradient (Figs. 6 and 7). The inhibition by Mg^{2+} was competitive with Ca^{2+} (Fig. 6). The K_i of Mg^{2+} was 80 ± 10 μM, which is about one-third the K_m for Ca^{2+} determined under the same conditions, i.e., with NMG as the principal external cation (Table 3). When exchange activity was measured in the presence of K^+ instead of NMG, Mg^{2+} was much less potent, but almost completely inhibited Na^+ gradient-dependent $^{45}Ca^{2+}$ uptake at 5 mM (Fig. 7). The IC_{50} of Mg^{2+} was 0.19 mM with either NMG or choline as the principal external cation. The IC_{50} of Mg^{2+} increased 8.2-fold when exchange was assayed in the presence of K^+ instead of NMG. Potassium clearly decreases the potency of Mg^{2+} as a competitive inhibitor of the exchanger because Mg^{2+} was similarly potent whether Na^+-Ca^{2+} exchange was assayed with choline or NMG as the principal external cation (Table 2).

Effects of Calcium and Magnesium on $^{22}Na^+$ Efflux

Ca^{2+} (0.25 or 2 mM) stimulated by ~twofold the initial rate of $^{22}Na^+$ efflux from Na^+-loaded cells (Table 3). By contrast to Ca^{2+}, Mg^{2+} (0.25 or 2 mM) decreased basal $^{22}Na^+$ efflux by 27 and 38%, respectively (Table 3). Mg^{2+} (2 mM) also abolished Ca^{2+}-

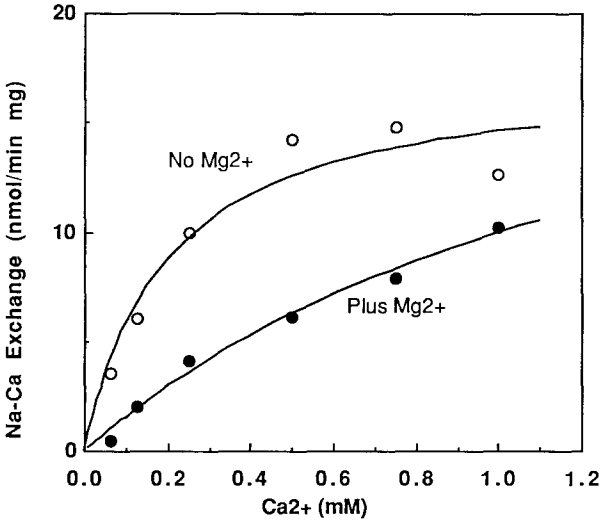

Fig. 6. Ca^{2+} concentration dependence of the inhibition of Na^+-Ca^{2+} exchange by Mg^{2+}. The cultures were incubated for 1 hr with 0.1 mM ouabain as described in Materials and Methods. Then they were rinsed three times with a solution containing 140 mM NMG chloride or NaCl and 20 mM HEPES/Tris, pH 7.4, and incubated for 0.5 min with 1 ml of the same solution which also contained 0.1 mM ouabain, 2 μCi $^{45}Ca^{2+}$, and the indicated concentration of $CaCl_2$. The cultures indicated by the filled circles also received 0.2 mM $MgCl_2$ during the incubation with $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ uptake in the presence of 140 mM NaCl (or 140 mM NaCl plus 0.2 mM $MgCl_2$) and the indicated concentration of $CaCl_2$ was subtracted from uptake in NMG chloride (or NMG chloride plus 0.2 mM $MgCl_2$) at each Ca^{2+} concentration to obtain exchange activity. The data were fitted to the Michaelis-Menten equation by nonlinear regression (Marquart) with Enzfitter (Elsevier-BIOSOFT, Cambridge, UK). The curve for the data without Mg^{2+} has a K_m of 0.20 ± 0.08 mM and a V_{max} of 17.5 ± 2.1 nmol/min mg. The curve for the data with Mg^{2+} has a $K_{0.5}$ of 1.49 ± 0.53 mM and a V_{max} of 24.8 ± 6.0 nmol/min mg. A K_i of 0.064 was obtained from these data using Dose-Effect Analysis with Microcomputers (Elsevier-BIOSOFT, Cambridge, UK). Values are means of duplicates for one of four experiments (Table 2) that gave similar results.

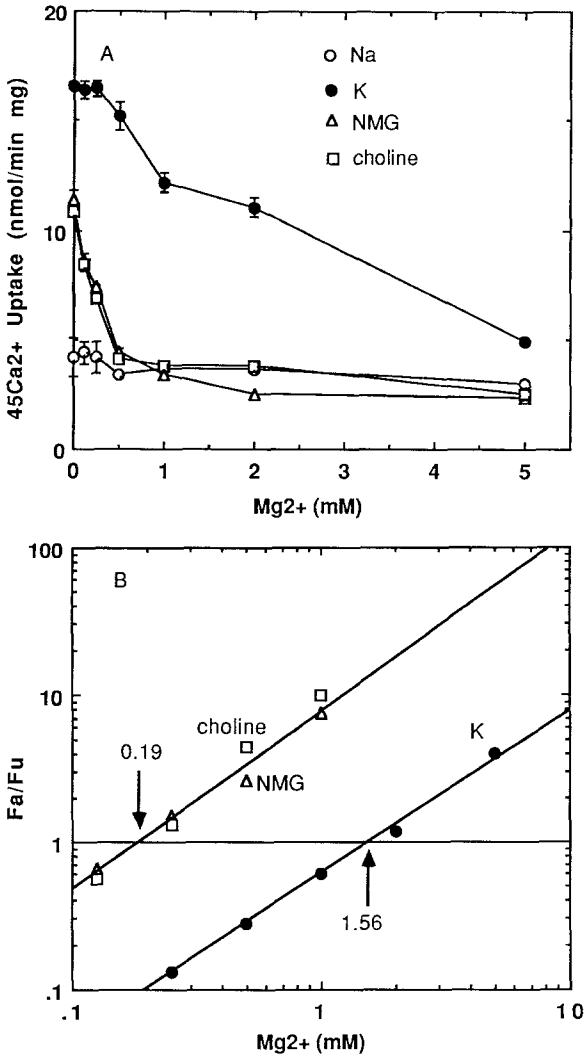

Fig. 7. High K^+ decreases the potency of Mg^{2+} as an inhibitor of Na^+-Ca^{2+} exchange. The cultures were incubated for 1 hr with 0.1 mM ouabain (*see* Materials and Methods) and rinsed three times with 140 mM KCl (filled circles), NMG chloride (triangles), choline chloride (squares), or NaCl (open circles) containing 20 mM HEPES/Tris, pH 7.4. Then they were incubated for 1 min with the same solutions which also contained 0.1 mM $CaCl_2$, 0.1 mM ouabain, 1 μCi $^{45}Ca^{2+}$, and the indicated concentration of $MgCl_2$. Exchange activity was calculated from the data in *A* by subtracting $^{45}Ca^{2+}$ uptake in the Na^+ solutions from the uptakes in the NMG or K^+ solutions. Data are mean ± SE (n = 2). To obtain the IC_{50} values (*B*), the log of the fraction of the uptake affected by Mg^{2+} (Fa) divided by the fraction unaffected by Mg^{2+} (Fu) was plotted against the log of the Mg^{2+} concentration using Dose-Effect Analysis with Microcomputers (Elsevier-BIOSOFT, Cambridge, UK). Data are means of two, four, and five experiments on duplicates for the concentration dependence of Mg^{2+} in the presence of choline, NMG, and K^+, respectively.

stimulated $^{22}Na^+$ (Table 3). The $^{22}Na^+$ efflux data confirm the presence of Na^+-Ca^{2+} exchange activity which is inhibited by Mg^{2+}. Mg^{2+}-$^{22}Na^+$ exchange was not detected.

Evidence that Potassium Is Not Required or Transported by the Na^+-Ca^{2+} Exchanger

The Na^+-Ca^{2+} exchanger in rod outer segments requires and transports K^+ in the same direction as Ca^{2+} (Cervetto et al., 1989; Schnetkamp, Basu & Szerencsei, 1989). The following evidence indicates that the exchanger in LLC-MK_2 cells does not require or transport K^+. Na^+-Ca^{2+} exchange activity was measured in ouabain-treated cells as the difference in $^{45}Ca^{2+}$ uptake with NMG or Na^+ as the principal external cation (as in Table 1). The addition of 5 mM KCl to the assay solution had no effect on $^{45}Ca^{2+}$ uptake in the presence of Na^+ or NMG (*data*

not shown). Because Rb^+ is known to substitute for K^+ in biological K^+ transporters, $^{86}Rb^+$ was used as tracer for K^+. $^{86}Rb^+$ uptake was measured by control and Na^+-loaded cells in the presence and absence of external Ca^{2+} and Na^+ (Table 4). Ouabain and bumetanide were used to block $^{86}Rb^+$ uptake by the Na^+-K^+ pump and the Na^+-K^+-Cl^- contransporter, respectively. Under these conditions loading the cells with Na^+ strongly increased $^{45}Ca^{2+}$ uptake via the exchanger (Table 4). The presence of external Ca^{2+} or loading the cells with Na^+ and replacing external Na^+ with NMG did not affect $^{86}Rb^+$ uptake in a manner consistent with uptake via the Na^+-Ca^{2+} exchanger (Table 4).

Table 3. Effects of Ca^{2+} and Mg^{2+} on $^{22}Na^+$ efflux

Ca^{2+} (mM)	Mg^{2+} (mM)	$^{22}Na^+$ efflux ($k \times 10^3$) sec^{-1}	% Control*
0	0	2.08 ± 0.13 (7)	100[a]
0.25	0	4.50 ± 0.11 (7)	216[b]
0	0.25	1.52 ± 0.14 (6)	73[a,c]
2	0	3.84 ± 0.64 (6)	185[b]
0	2	1.30 ± 0.14 (6)	62[c]
0.25	2	1.76 ± 0.73 (6)	85[a,c]

* Values with different superscripts are statistically different at 99% significance. The cultures were rinsed three times with PSS and incubated for 40 min with 0.8 ml PSS containing 10 mM glucose, 0.1 mM ouabain, and 2.5 μCi $^{22}Na^+$. To start efflux the cultures were rinsed five times with a solution which contained 140 mM NMG chloride and 20 mM HEPES/Tris, pH 7.4. Efflux (10-sec intervals from 0 to 180 sec) was assayed in this solution which contained 0.1 mM ouabain and the indicated concentrations of $CaCl_2$ or $MgCl_2$. First-order rate constants were calculated from the data between 20 and 80 sec with an HP-11C calculator programmed for exponential curve fitting by linear regression. Regression coefficients were all ≥0.99. Values are mean ± SE with the number of cultures indicated in the parentheses.

Lack of Na^+-Ca^{2+} Exchange Activity in Other Renal Epithelial Cell Lines

No exchange activity was detected in the following continuous epithelial cell lines: A6 kidney cells from the aquatic toad *Xenopus laevis*, MDCK cells, OK cells, or porcine kidney ($LLC\text{-}PK_1$) cells. The cells were incubated with 0.1 mM ouabain for 1 hr (*see* Materials and Methods). $^{45}Ca^{2+}$ uptake was assayed in the presence of 1 mM $CaCl_2$, 0.1 mM ouabain, and 140 mM NaCl or KCl as indicated (Fig. 1*B* legend). $^{45}Ca^{2+}$ influx in KCl was not significantly different from uptake in the presence of NaCl (*data not shown*).

Discussion

Cell Sodium Dependence of Na^+-Ca^{2+} Exchange

The present data show that raising cell Na^+ by a brief incubation of $LLC\text{-}MK_2$ cells with ouabain markedly potentiates Ca^{2+} influx evoked by the replacement of extracellular Na^+ with K^+, TMA, or NMG. The low level of cell Na^+ under basal conditions probably limits the capacity of the Na^+-Ca^{2+} exchanger to catalyze Ca^{2+} influx when extracellular Na^+ is acutely replaced with another cation. This interpretation is supported by the finding that the $K_{0.5}$ of the exchanger for Na^+ is about 26 mmol/liter cell water space which is about 65% greater than basal cell Na^+ (Fig. 4, not ouabain

Table 4. Effect of replacing external Na^+ with NMG on $^{86}Rb^+$ and $^{45}Ca^{2+}$ uptake by control and Na^+-loaded cells in the presence and absence of external Ca^{2+}

	$^{86}Rb^+$ uptake (nmol/min mg)			$^{45}Ca^{2+}$ uptake (nmol/min mg)		
	NMG	Na^+	NMG-Na^+	NMG	Na^+	NMG-Na^+
Control cells						
No Ca^{2+}	14.6 ± 0.6	11.2 ± 0.1	3.4 ± 0.6	ND	ND	ND
Plus Ca^{2+}	16.6 ± 0.5	15.4 ± 1.7	1.2 ± 2.0	1.9 ± 0.2	2.2 ± 0.3	−0.3 ± 0.1
Ouabain-treated cells						
No Ca^{2+}	4.3 ± 0.2	5.0 ± 0.1	−0.7 ± 0.1	ND	ND	ND
Plus Ca^{2+}	8.3 ± 0.8	7.7 ± 0.8	0.6 ± 0.3	12.5 ± 0.7	1.8 ± 0.1	10.8 ± 0.6

$^{86}Rb^+$ and $^{45}Ca^{2+}$ uptakes were determined under the same conditions. Some cultures were incubated with 0.1 mM ouabain for 30 min as described in Materials and Methods. The cultures were rinsed three times with a solution containing 140 mM NMG chloride or NaCl and 20 mM HEPES/Tris, pH 7.4. Then they were incubated for 1 min in the same solution which also contained 5 mM KCl, 0.1 mM ouabain, 10 μM bumetanide, and either 4 μCi $^{86}Rb^+$ or 2 μCi $^{45}Ca^{2+}$ as indicated. "Plus Ca^{2+}" indicates that the uptake solution also contained 0.25 $CaCl_2$. ND = not done. Values are mean ± SE (n = 3).

treated). Moreover, exchange activity exhibits a sigmoid dependence on cell Na^+ (Fig. 4). The Hill coefficient was 3.1 ± 0.2 which is consistent with the stoichiometry of 3 Na^+ : 1 Ca^{2+} of exchange activity in reconstituted basolateral vesicles from kidney cortex (Taylor & Arruda, 1985) and cardiac sarcolemma (Reeves & Hale, 1984), as well as exchange currents in cardiac myocytes (Bridge, Smolley & Spitzer, 1990; Crespo, Grantham & Cannell, 1990). The outward Na^+-Ca^{2+} exchange current in single, perfused ventricular myocytes has a $K_{0.5}$ for internal Na^+ of 20 ± 7 mM (Miura & Kimura, 1989). The Hill coefficient and $K_{0.5}$ for Na^+ of the exchanger in immortalized aortic myocytes are 2.8 ± 0.3 and 28 mmol/cell water space, respectively (Lyu, Smith & Smith, 1991*a*). Thus, the exchanger in LLC-MK_2 cells is similar to those in aortic and cardiac mycocytes with respect to the dependence on internal Na^+.

Activation of Na^+-Ca^{2+} Exchange by Ouabain

Incubating the cells with ouabain appears to activate the Ca^{2+} influx mode of exchange in addition to increasing the concentration of the intracellular substrate, Na^+. Hence, cells that contained 15 mmol Na^+/liter, which were not incubated with ouabain, had much lower exchange activity than ouabain-treated cells with the same Na^+ level (Fig. 4). Additionally, immediately after removing the culture medium the cells contained 30 mmol Na^+/liter and had an exchange activity of 1.8 nmol $^{45}Ca^{2+}$/min mg protein when assayed at 1 mM external Ca^{2+} (Fig. 3). By contrast, exchange activity would be 12.1 nmol/min/mg at 30 mmol Na^+/liter cell water space and 1.0 mM external Ca^{2+} in ouabain-treated cells[2]. These data suggest that ouabain activates the exchanger by shifting its dependence on cell Na^+. Perhaps the $[Ca^{2+}]_i$ produced by ouabain (Fig. 2) activated the exchanger. Intracellular Ca^{2+} allosterically activates exchanger in the Ca^{2+} influx mode in squid axon (DiPolo & Beauge, 1987) and cardiac cells (Kimura, Noma & Irisawa, 1986). However, in cardiac myocytes ~50 nM $[Ca^{2+}]_i$ was sufficient to maximally activate exchange (Miura & Kimura, 1989), which is well below free Ca^{2+} in LLC-MK_2 cells that were not incubated with ouabain (Fig. 2). Additionally, for determining the dependence of exchange activity on cell Na^+ the cells were incubated with ouabain in a solution which contained no added Ca^{2+} (Fig. 4). Therefore, it seems unlikely that ouabain activates the exchanger by raising $[Ca^{2+}]_i$, although we have no direct evidence to exclude this possibility.

[2]Exchange activity was 8.0 nmol/min mg at 30 nmol Na^+/liter when assayed at 0.25 mM Ca^{2+} (Fig. 4). Because exchange activity increased by 51% when external Ca^{2+} was raised from 0.25 to 1 mM (Fig. 5), the activity at 30 mmol Na^+/liter would be 12.1 nmol/min mg at 1 mM Ca^{2+}.

Physiological Role of Na^+-Ca^{2+} Exchange

From our previous studies of the role of Na^+-Ca^{2+} exchange in Ca^{2+} regulation by angiotensin in aortic myocytes (Smith & Smith, 1987; Smith et al., 1989*b*) and the similarity of the kinetic properties of the exchangers in aortic myocytes and LLC-MK_2 cells, it seems likely that the exchanger in both cell types predominantly operates in the Ca^{2+} efflux mode, for example, to expel Ca^{2+} after it is released from intracellular stores. Snowdowne and Borle (1985), however, suggested the Na^+-Ca^{2+} exchanger in LLC-MK_2 cells operates predominantly in the Ca^{2+} influx mode. They suggested that only in exceptional and unlikely conditions, such as hyperpolarization (membrane potentials greater than −60 mV) or very low intracellular Na^+ (<10 mM), could the exchanger operate as a Ca^{2+} efflux pathway. The main difference between the present data and those of Snowdowne and Borle (1985) is that treating the cells with ouabain markedly potentiated the $[Ca^{2+}]_i$ response to replacing extracellular Na^+ with TMA (Fig. 1). In contrast to the present data, Snowdowne and Borle (1985) found that replacing external Na^+ with TMA increased $[Ca^{2+}]_i$ from about 60 to 700 nM in aequorin-loaded cells that were not treated with ouabain. They also observed that treating the cells with 5 mM ouabain for 10 or 60 min increased the peak $[Ca^{2+}]_i$ response to replacing Na^+ with TMA by only 12 and 20%, respectively. We have shown here, however, that replacing external Na^+ with TMA evoked a ninefold $[Ca^{2+}]_i$ increase in ouabain-treated cells compared to only a 2.6-fold increase in control cells (Fig. 2). Moreover, $^{45}Ca^{2+}$ influx evoked by replacing external Na^+ with K^+ was 10.4-fold greater in ouabain-treated compared to control cells (Fig. 3). The data on $^{45}Ca^{2+}$ influx are in excellent agreement with the fura-2 data (Figs. 1 to 3) which excludes the possibility that the present findings are peculiar to fura-2-loaded cells. Perhaps the scrape-loading method used to introduce aequorin, which is a 21-kDa protein, raised cell Na^+. The possibility that scrape loading increased cell Na^+ is consistent with the finding that replacing external Na^+ with choline increased $[Ca^{2+}]_i$ by only twofold in cells that were loaded with aequorin by centrifugation compared to ~ninefold in scrape-loaded cells (Borle, Freudenrich & Snowdowne, 1986).

Extracellular Magnesium Competitively Inhibits the Ca^{2+} Influx Mode of Na^+-Ca^{2+} Exchange

Extracellular magnesium was previously shown to competitively inhibit Ca^{2+} influx via the Na^+-Ca^{2+} exchanger in aortic myocytes (Smith et al., 1987). The kinetic properties of Mg^{2+} inhibition in the renal epithelial cells (Table 2) are similar to those in aortic myocytes. The K_i was 80 μM in the epithelial cells compared to 100 μM in the myocytes. The K_m for Ca^{2+} was also 100 μM in the myocytes (Smith et al., 1987) compared to about 200 μM in the epithelial cells (Table 2) and 140 μM in cardiac myocytes (Miura & Kimura, 1989). The V_{max} with K^+ as the principal external cation was also similar in both cell types, about 40 nmol/min mg protein (Table 2; Smith & Smith, 1990). The exchanger does not appear to transport Mg^{2+} because it produces no detectable stimulation of $^{22}Na^+$ efflux from Na^+-loaded cells (Table 3). Moreover, Mg^{2+} strongly inhibited Ca^{2+}-stimulated $^{22}Na^+$ efflux in both cell types (Table 3; Smith et al., 1987). Because external Ca^{2+}, but not Mg^{2+}, produces an outward Na^+ current in cardiac myocytes (Kimura, Miyamae & Noma, 1987), the exchanger in heart also does not transport Mg^{2+} at an appreciable rate. Mg^{2+} inhibits exchange activity in cardiac sarcolemmal vesicles (Trosper & Philipson, 1983), although it appears to be more potent in aortic myocytes (Smith et al., 1987).

External Potassium Increases the Selectivity of the Exchanger for External Calcium

The most extraordinary feature of the Mg^{2+} inhibition is its modulation by external K^+. High K^+ decreases the potency of Mg^{2+} by seven to eightfold in LLC-MK_2 cells and aortic myocytes (Table 2; Smith et al., 1987). Schnetkamp (1980) found that Mg^{2+} competitively inhibited $^{45}Ca^{2+}$-Ca^{2+} exchange in rod outer segments from vertebrate photoreceptor cells and that K^+ or Rb^+ strikingly decreased the inhibition. The exchanger in rod outer segments exchanges 4 Na^+ per 1 Ca^{2+} + 1 K^+ (Cervetto et al., 1989; Schnetkamp et al., 1989). The exchanger in LLC-MK_2 cells does not appear to require or transport K^+. First, there was substantial $^{45}Ca^{2+}$ influx that depended on loading the cells with Na^+ in the absence of external K^+ (Fig. 1, Table 1; Smith et al., 1987). Second, addition of 5 mM KCl to the $^{45}Ca^{2+}$ uptake solution had no effect on uptake in the presence of Na^+ or NMG (*data not shown*). Third, there was no stimulation of K^+ influx (measured with $^{86}Rb^+$) under the conditions that markedly increased $^{45}Ca^{2+}$ influx via the exchanger (Table 4).

External potassium had no effect on the K_m for external Ca^{2+}, but decreased the inhibitory potency of Mg^{2+} ~eightfold (Table 2). The mechanism by which raising external K^+ decreases the potency of Mg^{2+} is unknown. Previously we (Smith et al., 1987) speculated that the binding of K^+ to the monovalent "B" site (Reeves, 1985) might increase the selectivity of the divalent "A" site for Ca^{2+}. If potassium binding to the "B" site increases the selectivity of the "A" site for Ca^{2+}, it would have to do so by decreasing its affinity for Mg^{2+} without affecting its affinity for Ca^{2+} because potassium had no effect on the apparent K_m for Ca^{2+} (Table 2). Alternatively, potassium may modulate the inhibitory potency of Mg^{2+} indirectly, e.g., by altering membrane potential, as noted previously (Smith et al., 1987). Whatever the mechanism is by which potassium decreases the potency of Mg^{2+}, it applies generally to divalent cations that inhibit the exchanger, because high K^+ decreased the inhibitory effectiveness of cadmium, manganese, cobalt, and barium by 4- to 8.6-fold (Smith et al., 1987).

In conclusion, the present data show that the LLC-MK_2 line of monkey kidney epithelial cells has substantial Na^+-Ca^{2+} exchange activity which shares several kinetic features with the exchanger in aortic myocytes. Because no exchange activity was detected in four other renal epithelial cell lines or primary cultures from the distal collecting tubule (R.-M. Lyu, J.A. Schaeffer and J.B. Smith, *unpublished data*), the gene for the Na^+-Ca^{2+} exchanger may be selectively expressed in only certain epithelial cells of the nephron.

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