Barium Influx Mediated by the Cardiac Sodium-Calcium Exchanger in Transfected Chinese Hamster Ovary Cells

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ABSTRACT We examined Ba^{2+} influx using isotopic and fura-2 techniques in transfected Chinese hamster ovary cells expressing the bovine cardiac Na^+/Ca^{2+} exchanger (CK1.4 cells). Ba^{2+} competitively inhibited exchange-mediated $^{45}Ca^{2+}$ uptake with a $K_i \sim 3$ mM. Ba^{2+} uptake was stimulated by pretreating the cells with ouabain and by removing extracellular Na^+, as expected for Na^+/Ba^{2+} exchange activity. The maximal velocity of Ba^{2+} accumulation was estimated to be 50% of that for Ca^{2+}. When the monovalent cation ionophore gramicidin was used to equilibrate internal and external concentrations of Na^+, Ba^{2+} influx was negligible in the absence of Na^+ and increased to a maximum at 20–40 mM Na^+. At higher Na^+ concentrations, Ba^{2+} influx declined, presumably due to the competition between Na^+ and Ba^{2+} for transport sites on the exchanger. Unlike Ca^{2+}, Ba^{2+} did not appear to be taken up by intracellular organelles: Thus, $^{133}Ba^{2+}$ uptake in ouabain-treated cells was not reduced by mitochondrial inhibitors such as Cl-CCP or oligomycin-rotenone. Moreover, intracellular Ca^{2+} stores that had been depleted of Ca^{2+} by pretreatment of the cells with ionomycin (a Ca^{2+} ionophore) remained empty during a subsequent period of Ba^{2+} influx. Ca^{2+} uptake or release by intracellular organelles secondarily regulated exchange activity through alterations in $[Ca^{2+}]_i$. Exchange-mediated Ba^{2+} influx was inhibited when cytosolic $[Ca^{2+}]$ was reduced to 20 nM or less and was accelerated at cytosolic Ca^{2+} concentrations of 25–50 nM. We conclude that (*a*) Ba^{2+} substitutes for Ca^{2+} as a transport substrate for the exchanger, (*b*) cytosolic Ba^{2+} does not appear to be sequestered by intracellular organelles, and (*c*) exchange-mediated Ba^{2+} influx is accelerated by low concentrations of cytosolic Ca^{2+}.

KEY WORDS: fura-2 • endoplasmic reticulum • Ba uptake • Na/Ca exchange • CHO cells

INTRODUCTION

The cardiac Na^+/Ca^{2+} exchanger couples the transmembrane movement of 3 Na^+ ions to that of a single Ca^{2+} ion in the opposite direction. It is the principal mechanism for mediating Ca^{2+} efflux in cardiac myocytes. It plays a critical role in regulating cardiac contractility by competing with the sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA)[1] for cytosolic Ca^{2+}, thereby modulating the amount of releasable Ca^{2+} stored in the sarcoplasmic reticulum (reviewed in Reeves, 1995). Kinetic results are compatible with a consecutive exchange mechanism, in which Ca^{2+} and Na^+ are translocated in separate steps (Khananshvili, 1990; Hilgemann et al. 1991).

Exchange activity is regulated by Ca^{2+}- and/or ATP-dependent processes that affect the exchanger's distribution between an active state and either of two inactive states (Hilgemann et al., 1992*a*, *b*). Entry of the exchanger into the first inactive state (I_1 inactivation) is thought to occur when the exchanger is fully loaded with Na^+ at the cytoplasmic membrane surface; this mode of inactivation is observed experimentally as a time-dependent decrease in "reverse" Na^+/Ca^{2+} exchange (Na^+_i-dependent Ca^{2+} influx) following a step increase in $[Na^+]_i$ (Hilgemann, 1990). The second (I_2) mode of inactivation is promoted by the absence of cytosolic Ca^{2+} and is detected experimentally as an activation of reverse exchange activity by submicromolar concentrations of cytosolic Ca^{2+} (secondary Ca^{2+} activation) (DiPolo, 1979; DiPolo and Beaugé, 1987). Cytosolic ATP counteracts both modes of inactivation, although the precise mechanism(s) involved have not been delineated. Despite the advances in our understanding of the regulatory behavior of the exchanger in subcellular or internally dialyzed cellular preparations, much less information is available on how exchange activity is regulated in intact cells, or how it interacts with other Ca^{2+} homeostatic mechanisms. Part of the difficulty in studying these issues stems from the technical limitations of Ca^{2+} influx measurements. Both $^{45}Ca^{2+}$ fluxes and fura-2 mea-

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[1]*Abbreviations used in this paper:* CHO, Chinese hamster ovary; $InsP_3$, inositol (1,4,5)trisphosphate; PSS, physiological salts solution; SERCA, sarco(endo)plasmic reticulum Ca^{2+} ATPase; Tg, thapsigargin.

surements are greatly affected by the sequestration and release of Ca^{2+} from the endoplasmic reticulum and the mitochondria. A previous report (Chernaya et al., 1996) used Ba^{2+} as a substitute for Ca^{2+} to assess effects of thapsigargin (Tg) on Na^+/Ca^{2+} exchange activity in transfected Chinese hamster ovary cells expressing the bovine cardiac Na^+/Ca^{2+} exchanger. Here we describe detailed studies of Ba^{2+} transport by the Na^+/Ca^{2+} exchanger in these cells. Consistent with results obtained with other cells, cytosolic Ba^{2+} is not significantly accumulated by either the endoplasmic reticulum or the mitochondria. Measurements of Na^+_i-dependent Ba^{2+} influx therefore provide a more direct measure of exchange activity than the corresponding Ca^{2+} flux measurements. Moreover, regulatory activation of Na_i-dependent Ba^{2+} influx by $[Ca^{2+}]_i$ can be readily observed under appropriate conditions. Our results suggest that Ca^{2+}_i-dependent activation of exchange activity involves a complex interplay between $[Ca^{2+}]_i$ and various intracellular Ca^{2+} compartments.

METHODS

Cells

CK1.4 cells were prepared by transfection of *dhfr*⁻ CHO cells (CCL 61; American Type Culture Collection, Rockville, MD) with a mammalian expression vector (pcDNA I/Neo; Invitrogen Corp., San Diego, CA) containing a cDNA insert coding for the bovine cardiac Na^+/Ca^{2+} exchanger (Aceto et al. 1992; Pijuan et al. 1993). Control cells were prepared by transfection of the CHO cells with pcDNA3 (Invitrogen Corp.), a closely related expression vector with no cDNA insert. The cells were grown in Iscove's modified Dulbecco's medium containing 500 μg/ml geneticin (G418; GIBCO, Gaithersburg, MD), either 10% FCS (JRH Biosciences, Lenexa, KS) or 10% supplemented calf serum (Cool Calf 2; Sigma Chemical Co., St. Louis, MO) and antibiotics as described (Pijuan et al., 1993). Unless otherwise specified, all biochemicals were obtained from Sigma Chemical Co.

Solutions

Na-PSS (physiological salts solution) contains 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, and 20 mM MOPS, pH adjusted to 7.4 (37°C) with Tris. NMDG-, Li-, and K-PSS have the same composition as Na-PSS, except that Na^+ is replaced with $NMDG^+$, Li^+, and K^+, respectively. The termination medium for the $^{45}Ca^{2+}$ transport assay consists of 100 mM $MgCl_2$, 10 mM $LaCl_3$, and 5 mM MOPS, pH 7.4 (Tris).

$^{45}Ca^{2+}$ and $^{133}Ba^{2+}$ Uptake

Cells were grown to confluence in 24-well plastic dishes and preincubated for 30 min at 37°C with 1 ml/well of nominally Ca^{2+}-free Na-PSS with or without 0.4 mM ouabain as indicated. The preincubation medium was then aspirated and replaced with 200 μl of assay medium (Na- or NMDG-PSS, as indicated) containing 100 μM $^{45}Ca^{2+}$ or $^{133}Ba^{2+}$. Radioisotopes were obtained from DuPont NEN Research Products (Boston, MA). For cells preincubated with ouabain, the assay solutions also contained 0.4 mM ouabain. After the desired interval, the wells were washed four times with 1 ml of termination medium; the contents of the wells were then extracted with 1 ml of 0.1 N HNO_3 and counted. Protein was determined in separate sample wells by the Lowry method (Lowry, 1951). Data are presented as the mean values ± SEM (error bars shown in figures) for the indicated number (*n*) of experiments.

Fura-2 Assays

Cells were grown to confluence in 75-cm² plastic culture flasks and washed three times with Na-PSS. The cells were released from the flask with 5 ml of Na-PSS + 5 mM EDTA, centrifuged, and resuspended in 5 ml of Na-PSS + 1 mM $CaCl_2$, centrifuged again, and resuspended in 4–5 ml of Na-PSS + 1 mM $CaCl_2$ + 1% BSA; the BSA aids in solubilizing the added fura-2-AM (Thomas and Delaville, 1991). The cells were distributed in individual 0.3-ml aliquots to plastic tubes and allowed to incubate for 30 min at 37°C to recover from isolation. Individual tubes of cells were loaded at 10-min intervals for 30 min (37°C) with 3 μM fura-2-AM (Molecular Probes, Eugene, OR) and 0.25 mM sulfinpyrazone to retard transport of fura-2 out of the cell (DiVirgilio et al., 1988; Pijuan et al., 1993). The fura-2 and sulfinpyrazone were added as 1,000-fold concentrated stock solutions in dimethyl sulfoxide. Where indicated, ouabain (0.4 mM) was also added as a 1,000-fold concentrated solution in dimethyl sulfoxide. After the 30-min loading period, the cells were rapidly centrifuged in an Ependorf Mini-centrifuge, washed, and preincubated in 0.1 ml of the desired medium for 1 min as specified in individual experiments. The cells were then added directly to a cuvette containing 3 ml of either Na-PSS or Li-PSS and fura-2 fluorescence was monitored at 510-nm emission with alternate excitation at 350/390 nm (Schilling et al., 1989), using a Photon Technology International (South Brunswick, NJ) RF-M 2001 fluorometer. All fluorescence values were corrected for autofluorescence using cells that had not been loaded with fura-2. Data are presented as the ratio of fluorescence values at the 350/390 excitation wavelengths and represent the mean values ± SEM (error bars shown in figures) for the indicated number (*n*) of experiments. Calibrations were conducted with digitonin-permeabilized cells according to the procedure of Grynkiewicz et al. (1985) and yielded values of R_{max} = 7.5, R_{min} = 1.4 and Sf/Sb = 2.75 under our experimental conditions. K_D values for Ba^{2+} and fura-2 of 0.8 μM (Schilling et al., 1989), 1.4 μM (Kwan and Putney, 1990), and 2.4 μM (McCormack and Osbaldeston, 1990) have been reported; the average K_D (1.5 μM) yields $[Ba^{2+}]$ values of 0.5, 1.5, 3.1, and 6.0 μM at 350/390 ratios of 2, 3, 4, and 5, respectively. Fura-2 also responds to Ca^{2+} at the excitation wavelengths used for the Ba^{2+} measurements. Assuming K_D = 224 nM for the Ca^{2+}/fura-2 complex (Grynkiewicz et al., 1985), the following 350/390 excitation ratios correspond to the indicated values for $[Ca^{2+}]_i$: 2.0 (67 nM), 2.5 (135 nM), 3.0 (219 nM), 4.0 (457 nM), and 5.0 (887 nM).

RESULTS

Isotope Flux Studies

The data in Fig. 1 show the effects of various concentrations of Ba^{2+} on the rates of $^{45}Ca^{2+}$ uptake by ouabain-treated CK1.4 cells in a Na^+-free medium. Ba^{2+} inhibited $^{45}Ca^{2+}$ uptake at both 0.1 and 1.0 mM $[Ca^{2+}]$, but was a more potent inhibitor at the lower Ca^{2+} concentration. Curiously, low concentrations of Ba^{2+} (0.1 mM) slightly stimulated $^{45}Ca^{2+}$ uptake; the explanation for this is unclear, and this phenomenon was not investigated further. At Ba^{2+} concentrations >1 mM, the in-

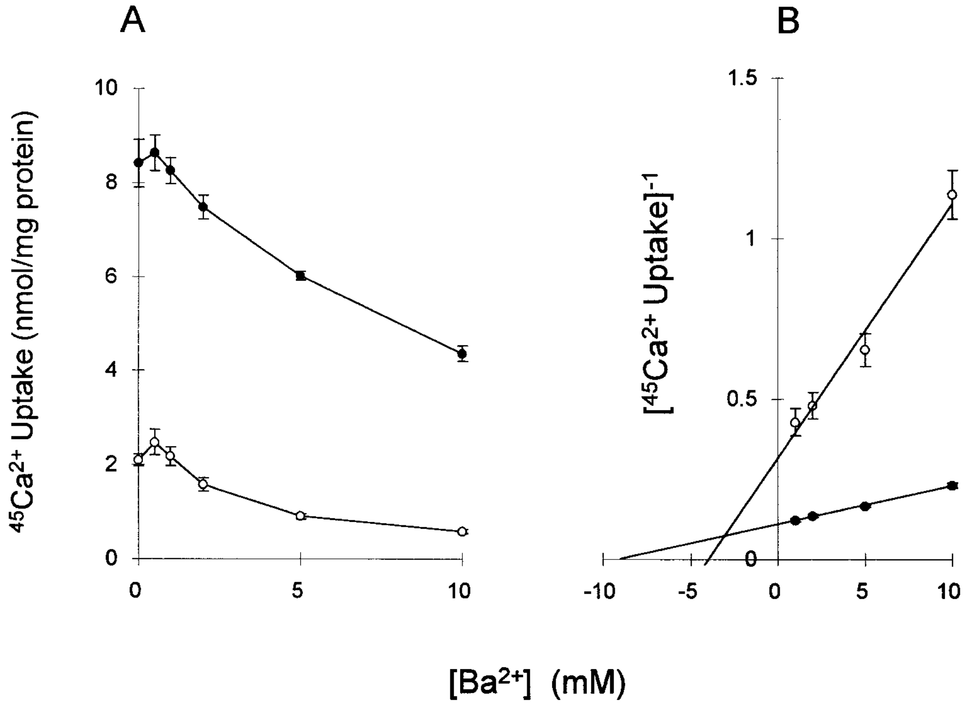

FIGURE 1. Ba^{2+} inhibits exchange-mediated $^{45}Ca^{2+}$ uptake by CK1.4 cells. (*Left*) Cells were pretreated with 0.4 mM ouabain and assayed for $^{45}Ca^{2+}$ uptake (15 s) in NMDG-PSS containing the indicated concentrations of $BaCl_2$. The concentrations of $^{45}Ca^{2+}$ used are 1 mM (*filled circles*) and 0.1 mM (*open circles*). (*Right*) The data from the *left panel* for 1–10 min are presented as a Dixon plot. The point of intersection of the two lines yields an apparent K_i for Ba^{2+} of 3 mM (n = 5).

hibition was competitive (K_i = 3.1 mM) as indicated by the Dixon plot in the right panel of Fig. 1; including the full range of Ba^{2+} concentrations in the Dixon plot shifted the apparent K_i to 4.5 mM (data not shown). The results imply that Ba^{2+} interacts with the Ca^{2+} transport site on the Na^+/Ca^{2+} exchanger.

To determine whether Ba^{2+} is transported by the exchanger, we examined $^{133}Ba^{2+}$ uptake. As shown in Fig. 2 *A* (*open symbols*), $^{133}Ba^{2+}$ uptake in an Na-free medium was stimulated by prior treatment of the cells with ouabain to elevate intracellular Na^+. In the presence of physiological concentrations of extracellular Na^+, $^{133}Ba^{2+}$ uptake by ouabain-treated cells was strongly inhibited (Fig. 2 *B*, *open squares*). For vector transfected control cells (Fig. 2, *filled symbols*), which do not exhibit Na^+/Ca^{2+} exchange activity (Pijuan et al., 1993; Chernaya et

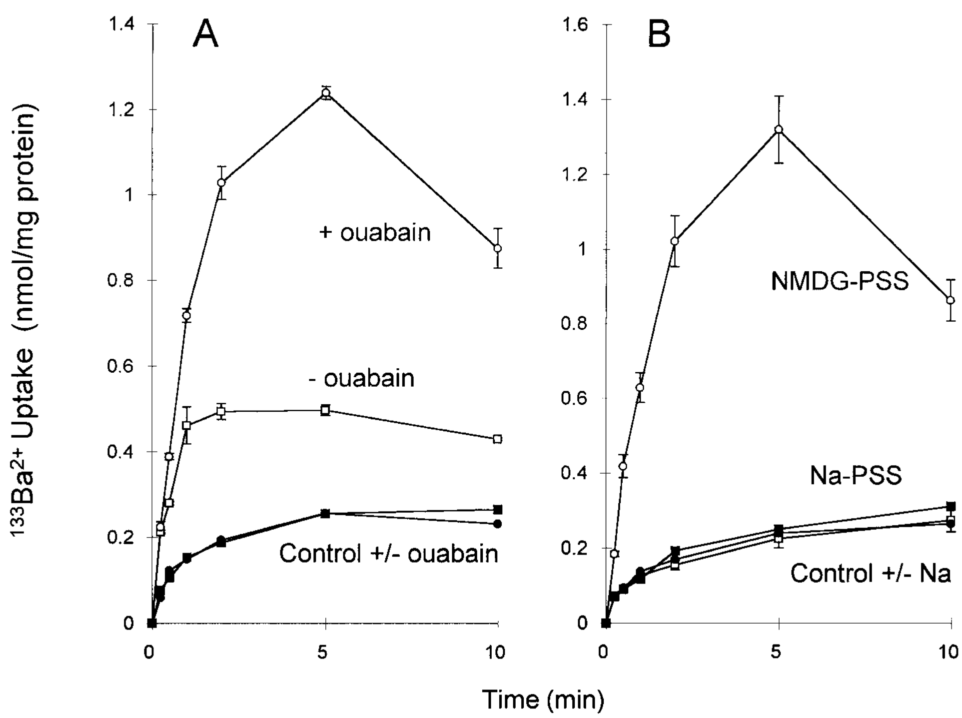

FIGURE 2. $^{133}Ba^{2+}$ uptake by CK1.4 cells and vector-transfected control cells. (*A*) Cells were preincubated with or without 0.4 mM ouabain, as indicated, and assayed for $^{133}Ba^{2+}$ uptake in NMDG-PSS. (*B*) Cells were preincubated with ouabain and assayed for $^{133}Ba^{2+}$ uptake in NMDG-PSS or Na-PSS, as indicated. *Open symbols*, CK1.4 cells (n = 3); *closed symbols*, control cells (n = 2).

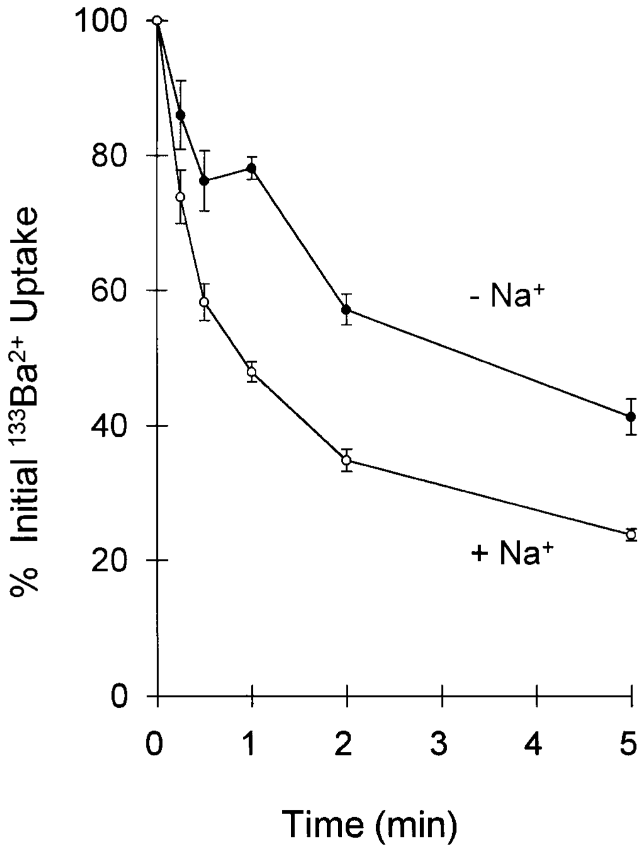

FIGURE 3. $^{133}Ba^{2+}$ efflux from CK1.4 cells. Ouabain-treated cells were allowed to accumulate $^{133}Ba^{2+}$ for a period of 5 min and then placed in Na-PSS (*open circles*) or NMDG-PSS (*closed circles*) for the indicated intervals ($n = 4$).

al., 1996), $^{133}Ba^{2+}$ uptake was <20% of the maximal levels shown by CK1.4 cells and was unaffected by ouabain treatment or Na-free conditions. The results with $^{133}Ba^{2+}$ are qualitatively similar to those obtained with $^{45}Ca^{2+}$; the maximal levels of $^{45}Ca^{2+}$ uptake, however, are generally three to five times higher than for $^{133}Ba^{2+}$ (see, for example, Fig. 6). Based on the initial rates of exchange-mediated $^{133}Ba^{2+}$ uptake in Fig. 2, and assuming a K_m of 3 mM, we calculate a V_{max} for Ba^{2+} uptake of 3.7 nmol/mg protein/15 s, or ~50% of that reported previously for $^{45}Ca^{2+}$ uptake (Condrescu et al., 1995). As shown in Fig. 3, extracellular Na^+ stimulated $^{133}Ba^{2+}$ efflux from preloaded CK1.4 cells, suggesting that the exchanger also transports Ba^{2+} out of the cells. The efflux data with $^{133}Ba^{2+}$ are again qualitatively similar to those obtained using $^{45}Ca^{2+}$.

Fura-2 Measurement of Ba^{2+} Influx

An alternate method of assaying Ba^{2+} movements is to use the Ca^{2+}-indicating dye fura 2 (Schilling et al., 1989). As shown in Fig. 4 *A*, the addition of Ba^{2+} to fura 2–loaded CK1.4 cells produced an increase in the 350/390 excitation ratio which was enhanced by Na^+_o-removal (Li$^+$-substitution). Pretreating the cells with ouabain to elevate $[Na^+]_i$ (Fig. 4 *B*) increased the 350/390 excitation ratio under Na^+-free conditions, consistent with the expected increase in Na^+_i-dependent Ba^{2+} influx via the exchanger. Similar results were obtained when *N*-methyl-D-glucamine was used as the Na^+ substitute instead of Li^+ (data not shown). When vector-transfected control cells were used instead of CK1.4 cells, the increase in the 350/390 ratio was not affected by Na^+-removal or by ouabain-treatment and was approximately equivalent to that seen in the presence of Na^+ for the CK1.4 cells (data not shown).

Adding 10 mM EGTA after a period of Ba^{2+} accumulation in the Na-free medium resulted in little or no decline in the 350/390 ratio (Fig. 4) suggesting that cytosolic Ba^{2+} is transported poorly or not at all by the ATP-dependent Ca^{2+} pumps. In comparable experiments conducted with Ca^{2+} instead of Ba^{2+}, EGTA addition results in a rapid decline in $[Ca^{2+}]_i$ (see Condrescu et al., 1995; Chernaya et al., 1996). The results with Ba^{2+} in the fura-2 experiments differ from the results of the $^{133}Ba^{2+}$ flux studies (Fig. 3), which indicated that $^{133}Ba^{2+}$ was lost from the CK1.4 cells, even in the absence of extracellular Na^+ (cf., DISCUSSION).

Dependence of Ba^{2+} Influx on $[Na^+]_i$

The effects of ouabain treatment indicate that Ba^{2+} entry is accelerated when $[Na^+]_i$ is increased. The influence of $[Na^+]_i$ is examined more directly in the experiments shown in Fig. 5. Fura-2–loaded CK1.4 cells were placed in cuvettes containing K-PSS with various concentrations of Na^+ (mM concentrations given in Fig. 5 next to individual traces) and treated with 1 μM gramicidin to bring about rapid equilibration of monovalent cations across the plasma membrane. Thus, in the presence of gramicidin, Na^+ concentrations should be equal on both sides of the cell membrane. In the absence of Na^+, Ba^{2+} influx was negligible. (The initial, abrupt rise in the fura-2 ratio upon addition of Ba^{2+} is due to the presence of small amounts of extracellular fura-2.) Increasing concentrations of Na^+ produced progressively higher rates of Ba^{2+} influx with maximal rates at 20–40 mM Na^+. With higher Na^+ concentrations, Ba^{2+} influx declined to a level that was only slightly higher, at 140 mM Na^+, than that observed in the absence of Na^+. The slopes of the fura-2 traces are presented as a function of $[Na^+]$ in the inset to the right panel of Fig. 5. The increasing rates of Ba^{2+} influx within the range of 0–20 mM $[Na^+]$ most likely reflect the stimulatory effects of cytosolic Na^+ in activating exchange activity. The decline in Ba^{2+} influx rates at higher Na^+ concentrations is probably due to competition between external Na^+ and Ba^{2+} for transport sites on the exchange carrier. When control transfected cells were used in similar experiments, Ba^{2+} influx was

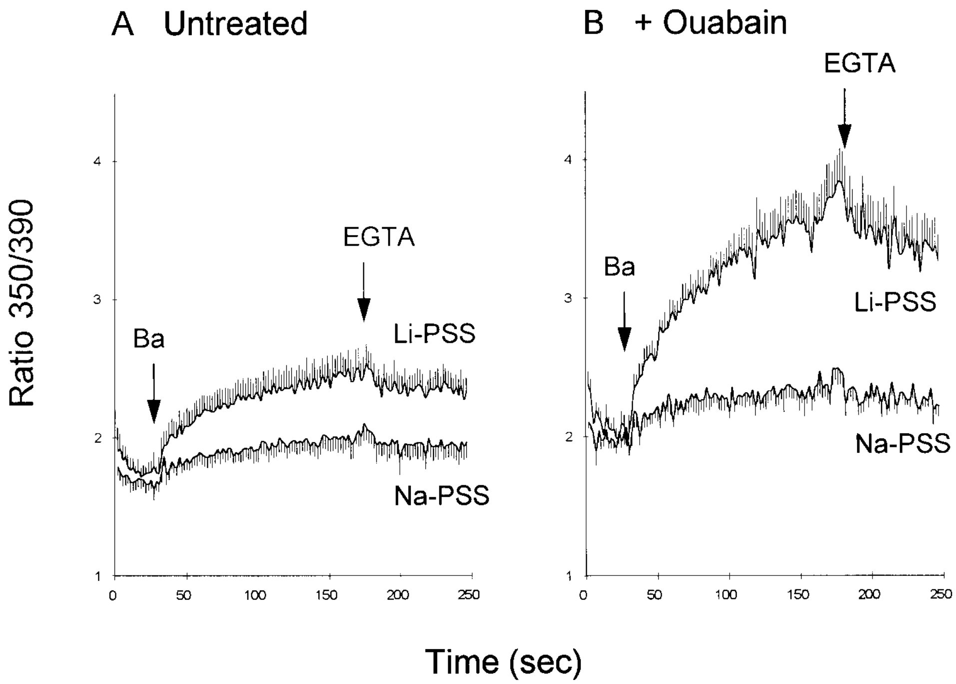

FIGURE 4. Ba^{2+} influx in CK1.4 cells detected with fura-2. Cells were loaded with fura-2 without (*A*) or with (*B*) 0.4 mM ouabain, washed, and preincubated for 1 min in Na-PSS + 1 mM $CaCl_2$ and then diluted 30-fold into cuvettes containing either Li-PSS or Na-PSS, as indicated; 0.3 mM EGTA was included in both media. $BaCl_2$ (1 mM) and EGTA (10 mM) were added as indicated by the arrows ($n = 5$).

low (comparable to that seen in the absence of Na^+ for the CK1.4 cells), and variations in $[Na^+]$ had no effect. We conclude that the Na^+/Ca^{2+} exchanger provides the major route of Ba^{2+} entry in the CK1.4 cells.

Ba^{2+} Uptake and Organellar Sequestration

Previous reports have suggested that Ba^{2+} is not significantly sequestered by the endoplasmic reticulum (Kwan and Putney, 1990; Rasgado-Flores et al., 1986). Ba^{2+} uptake by mitochondria has been reported, but the results vary markedly with different cell types (cf., DISCUSSION). We conducted the following experiments to determine whether Ba^{2+} was sequestered by intracellular organelles in CK1.4 cells and to assess whether the presence of cytosolic Ba^{2+} interfered with other Ca^{2+} transport processes.

To examine mitochondrial accumulation of Ba^{2+}, we measured the effects of mitochondrial inhibitors on $^{45}Ca^{2+}$ and $^{133}Ba^{2+}$ uptake. As shown in Fig. 6, the uncoupler Cl-CCP (2 μM) and the combination of oligomycin (2.5 μg/ml) + rotenone (2 μM) inhibited ex-

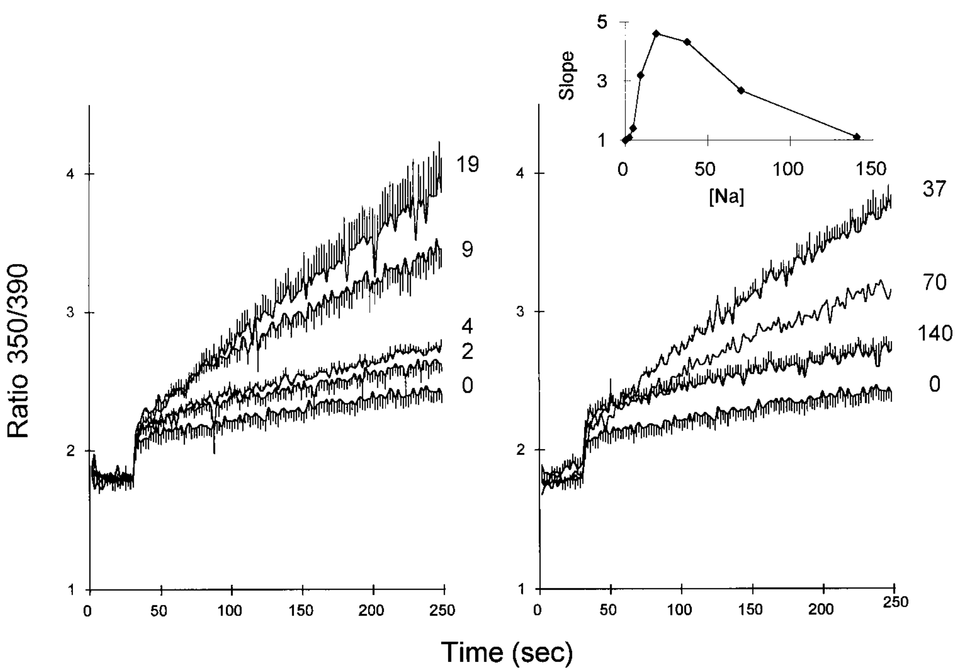

FIGURE 5. Ba^{2+} influx in gramicidin-treated CK1.4 cells. Cells were loaded with fura-2, washed, and preincubated in K-PSS + 1 mM $CaCl_2$, centrifuged, and resuspended in mixtures of K-PSS + Na-PSS that yielded the final Na^+ concentrations (in mM) indicated next to each trace; each solution also contained 0.3 mM EGTA. Gramicidin (1 μM) was added immediately after the cells and 1 mM $BaCl_2$ was added at 30 s. (n = 3–5; for 70 mM Na^+, $n = 2$). *Inset*: Dependence of the rate of Ba^{2+} influx on $[Na^+]$. The slopes of the traces at each $[Na^+]$, normalized to that for $[Na^+] = 0$, are plotted on the ordinate scale.

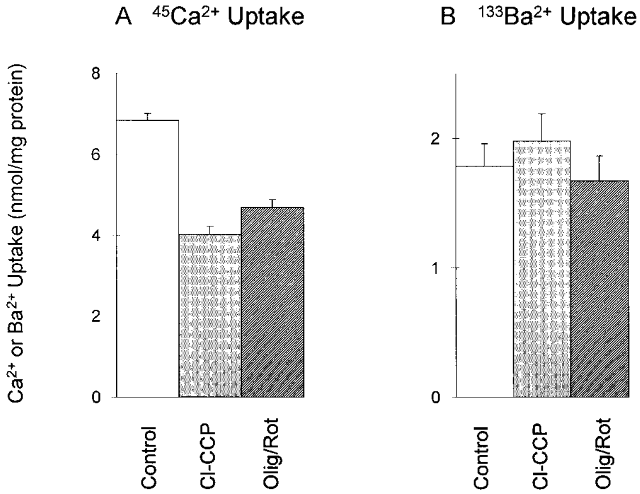

FIGURE 6. Effect of mitochondrial antagonists on $^{45}Ca^{2+}$ and $^{133}Ba^{2+}$ uptake by CK1.4 cells. Cells were treated with ouabain and assayed for in NMDG-PSS for $^{45}Ca^{2+}$ (0.1 mM) or $^{133}Ba^{2+}$ (0.1 mM) uptake (5 min); where indicated 10 μM Cl-CCP or 2.5 μg/ml oligomycin + 2 μM rotenone were present in the assay medium (n = 6, $^{133}Ba^{2+}$; n = 4, $^{45}Ca^{2+}$).

change-mediated $^{45}Ca^{2+}$ uptake in ouabain-treated CK1.4 cells by 42 ± 2 and 32 ± 2%, respectively, but had no effect on $^{133}Ba^{2+}$ uptake (−11 ± 3 and 6 ± 4% inhibition, respectively). The results suggest that any mitochondrial accumulation of Ba^{2+} was small compared to that observed with Ca^{2+}, consistent with the reported selectivity of the mitochondrial uniporter toward divalent cations ($Ca^{2+} >> Ba^{2+}$; Saris and Åkerman, 1980). To address the question of whether cytosolic Ba^{2+} could be sequestered by the endoplasmic reticulum, we depleted intracellular Ca^{2+} stores using the Ca^{2+} ionophore ionomycin and then asked whether the stores would refill with Ba^{2+} during a subsequent period of Ba^{2+} influx. We assessed the degree of filling of intracellular stores by measuring the response of fura-2–loaded cells to the addition of extracellular ATP. ATP binds to P_{2U} receptors in Chinese hamster ovary cells and elicits the production of inositol (1,4,5)-*tris*phosphate ($InsP_3$), leading to release of sequestered Ca^{2+} from $InsP_3$-sensitive stores (Iredale and Hill, 1993; Pijuan et al., 1993). This is illustrated in the inset to Fig. 7, where ATP elicits a pronounced $[Ca^{2+}]_i$ transient when added to CK1.4 cells (control trace). In contrast to this behavior, when an aliquot of cells was pretreated for 1 min with 10 μM ionomycin before adding the cells to the cuvette, no Ca^{2+} transient was observed (Fig. 7, *inset*). This indicates that the ionomycin had released essentially all the sequestered Ca^{2+} from the stores during the 1-min preincubation. It is important to note that in all traces shown in Fig. 7, 0.3% BSA was present in the cuvette to scavenge residual ionomycin (cf., Chernaya et al., 1996); this was done to ensure that the presence of the ionophore would not interfere with possible Ba^{2+} accumulation in the stores. In comparable experiments carried out with Ca^{2+}, the $InsP_3$-sensitive stores refilled rapidly in the presence of extracellular Ca^{2+} (Chernaya et al., 1996).

In the main panel of Fig. 7, ouabain-treated CK1.4 cells with intact Ca^{2+} stores were allowed to accumulate Ba^{2+} for 2.5 min in an Na^+-free medium; then 10 mM EGTA was added to block any further Ba^{2+} influx, and

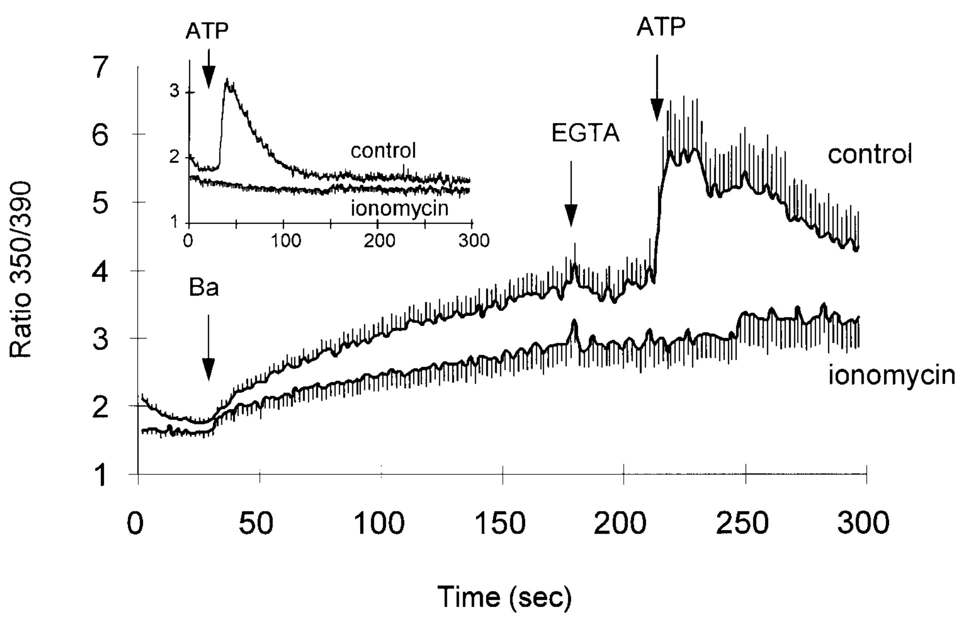

FIGURE 7. Test for Ba^{2+} sequestration by $InsP_3$-sensitive stores. (*control*) Cells were preloaded with fura-2 in the presence of 0.4 mM ouabain, washed, and incubated for 1 min in Na-PSS + 1 mM $CaCl_2$ and then diluted 30-fold into cuvettes containing Li-PSS + 0.3 mM EGTA + 0.3% BSA. $BaCl_2$ (1 mM), EGTA (10 mM) and ATP (0.3 mM) were added as indicated. (*ionomycin*) Cells were loaded with fura-2 in the presence of ouabain, washed, and incubated for 1 min in Na-PSS + 0.3 mM EGTA containing 10 μM ionomycin; the cells were then centrifuged and resuspended in Li-PSS + 0.3 mM EGTA + 0.3% BSA. $BaCl_2$, EGTA, and ATP were added as in the control trace. (*Inset*) Experimental conditions are as described for the control and ionomycin-treated cells above, except that ATP (0.3 mM) was added as indicated (n = 6).

ATP was subsequently added (after a 30-s delay) to measure the amount of Ca^{2+} present in the stores. As shown, ATP evoked a robust $[Ca^{2+}]_i$ transient under these conditions (control trace). These results indicate that the $InsP_3$-sensitive stores had retained their Ca^{2+} load during the period of Ba^{2+} accumulation and responded normally to ATP addition. Note that after the peak of the $[Ca^{2+}]_i$ transient, the fura-2 signal declined toward the level seen before ATP addition; continued incubation resulted in stabilization of the fura-2 signal at the level observed before ATP addition, consistent with the absence of Ba^{2+} removal from the cytosol (data not shown). Similar results were seen when 2 μM ionomycin was used to elicit Ca^{2+} release instead of ATP (data not shown). Thus, the presence of cytosolic Ba^{2+} did not block either the ATP-evoked Ca^{2+} release pathway or Ca^{2+} removal from the cytosol. The latter process may occur through either the SERCA or the plasma membrane Ca^{2+} pumps, or both. In the case where Ca^{2+} release was elicited by ionomycin, only the plasma membrane Ca^{2+} pump would be expected to contribute to Ca^{2+} removal from the cytosol since intracellular organelles would be unable to accumulate Ca^{2+} in the presence of the ionophore. These results therefore indicate that Ca^{2+} extrusion by the plasma membrane Ca^{2+} ATPase is not blocked by the presence of cytosolic Ba^{2+}.

When ionomycin-pretreated cells (with depleted Ca^{2+} stores) were subjected to the protocol described above, ATP did not elicit an increase in the 350/390 ratio (Fig. 7, *main panel*). The absence of an increase in $[Ba^{2+}]_i$ indicates that the $InsP_3$-sensitive stores remained empty during the period of Ba^{2+} uptake. Note that Ba^{2+} is rapidly conducted by $InsP_3$-gated Ca^{2+} channels (Bezprozvanny and Ehrlich, 1994) and would therefore have been released from the stores if present. The results indicate that Ba^{2+} is not sequestered by $InsP_3$-sensitive stores in these cells, probably due to its inability to serve as a transport substrate for the SERCA Ca^{2+} pump.

An unexpected feature of these results was that the ionomycin-treated cells showed a sharply reduced rate of Ba^{2+} influx compared to control cells that had not been treated with ionomycin. As described in more detail below, this appears to be a secondary consequence of the reduced $[Ca^{2+}]_i$ in the cells with depleted Ca^{2+} stores: following removal of the ionomycin with BSA, the stores would be expected to re-sequester residual cytosolic Ca^{2+} and lower $[Ca^{2+}]_i$. The average value of $[Ca^{2+}]_i$ during the 15 s before the addition of Ba^{2+} in this experiment was 23 ± 7 nM for the ionomycin/BSA-treated cells vs. 41 ± 5 nM for the control cells ($P < 0.001$, paired t test; $n = 6$).

Store Depletion and Ba^{2+} Influx

To examine the issues raised above in greater detail, we studied the effects of ionomycin on exchange-mediated Ba^{2+} influx in cells treated with thapsigargin (Tg), a selective blocker of SERCA Ca^{2+} pumps (Lytton et al., 1991). CK1.4 cells were pretreated with ionomycin, Tg, or both agents and then washed with 1% BSA to scavenge residual ionomycin. The cells were then placed in Li-PSS + 0.3 mM EGTA and fura-2 fluorescence was monitored after the addition of 1 mM $BaCl_2$. In each case, the cells had also been loaded with cytosolic Na^+ by including 0.4 mM ouabain in the fura-2 loading medium (see legend to Fig. 8 for further details).

As shown in Fig. 8, Ba^{2+} influx was greatest in the Tg-treated cells and least in the cells pretreated with ionomycin; in the latter case, Ba^{2+} influx was essentially identical to that seen in Na-PSS (data not shown), implying that the exchanger was inactive under these conditions. Remarkably, Ba^{2+} influx in cells that had been treated with both ionomycin and Tg was greatly reduced compared to cells treated with Tg alone. Control cells that had been subjected to the same preincubation protocol, but in the absence of either ionomycin or Tg, also showed reduced Ba^{2+} influx; this is due to the mechanical manipulations involved in the protocols, which lead to release of ATP, depletion of the Ca^{2+} stores, and a decline in $[Ca^{2+}]_i$ (unpublished observations; cf. below). The rate of Ba^{2+} influx shows a reasonably good correlation with the values of $[Ca^{2+}]_i$ observed during the 15 s interval before the addition of Ba^{2+}. The $[Ca^{2+}]_i$ values corresponding to Tg, control, Tg + ionomycin or ionomycin treatments were 34 ± 3, 26 ± 1, 20 ± 3, and 17 ± 1 nM respectively ($n = 3$ in each case).

Our interpretation of these results (cf. DISCUSSION) is that exchange activity is regulated secondarily through the influence of intracellular organelles on the cytosolic Ca^{2+} concentration. Release of Ca^{2+} from internal stores elevates $[Ca^{2+}]_i$ and accelerates exchange activity (cf. Chernaya et al., 1996), whereas the exchanger is deactivated when these organelles resequester residual cytosolic Ca^{2+} and lower $[Ca^{2+}]_i$. The effects of organellar Ca^{2+} release and sequestration are further documented in the accompanying manuscript (Vázquez et al., 1997), which describes time-dependent changes in exchange activity associated with ATP-induced Ca^{2+} release from $InsP_3$-sensitive Ca^{2+} stores. Two surprising aspects of results in Figs. 7 and 8 deserve special emphasis: (*a*) exchange activity becomes activated at quite low values of $[Ca^{2+}]_i$ (25–50 nM) and (*b*) exchange activity can be deactivated even when the SERCA pumps are blocked by Tg, implying that Tg-resistant Ca^{2+} pumps also participate in regulation of cytosolic Ca^{2+}.

DISCUSSION

The results presented here show that Ba^{2+} provides an advantageous alternative to Ca^{2+} for measurements of exchange activity in intact cells. Ba^{2+} competitively in-

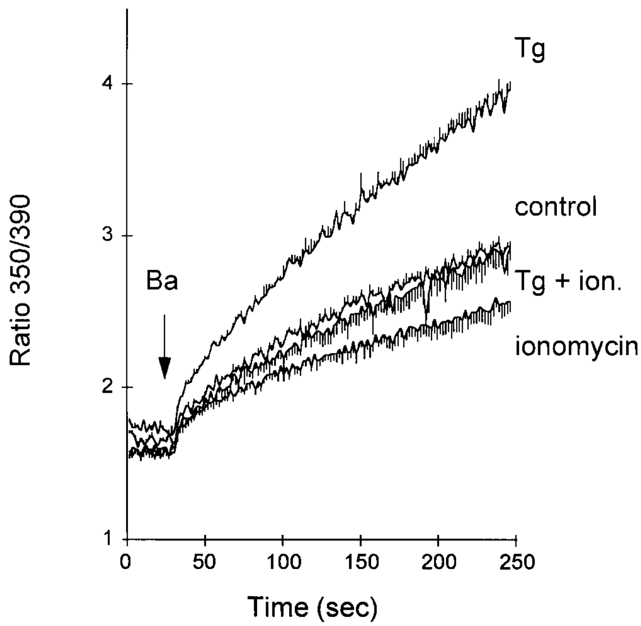

FIGURE 8. Ba^{2+} influx in CK1.4 cells pretreated with Tg, ionomycin or both. Aliquots (0.3 ml) of CK1.4 cells were loaded with fura-2 in the presence of 0.4 mM ouabain, washed, and preincubated for 1 min in 0.1 ml of Na-PSS + 0.3 mM EGTA containing either 200 nM Tg, 10 μM ionomycin, both Tg and ionomycin, or neither agent (*control*). The cells were then centrifuged, resuspended in 0.3 ml of Na-PSS + 0.3 mM EGTA + 1% BSA and incubated for an additional 1 min. After an additional centrifugation, the cells were resuspended in Li-PSS + 0.3 mM EGTA in the cuvette; $BaCl_2$ (1 mM) was added as indicated ($n = 3$).

hibited $^{45}Ca^{2+}$ uptake by Na^+/Ca^{2+} exchange and therefore presumably binds to the Ca^{2+} transport sites on the exchanger (Fig. 1). $^{133}Ba^{2+}$ uptake was stimulated by ouabain-treatment and was completely suppressed by 140 mM extracellular Na^+ (Fig. 2); similar behavior was observed using fura-2–loaded cells to assess Ba^{2+} influx (Fig. 4). The effects of Na^+ in the gramicidin-treated cells (Fig. 5) indicated that Ba^{2+} influx was blocked in the absence of cytosolic Na^+ and was accelerated by increasing concentrations of cytosolic Na^+. We conclude that the exchanger provides the major pathway for Ba^{2+} entry in these cells. The data with gramicidin-treated cells also suggests that exchange activity is quite sensitive to Na^+_i; a small, but significant increase in Ba^{2+} entry was observed at concentrations as low as 2.3 mM Na^+ (Fig. 5).

Previously published studies addressed the question of whether Ba^{2+} is transported by the Na^+/Ca^{2+} exchanger. Trosper and Philipson (1983) reported that $^{45}Ca^{2+}$ efflux from preloaded cardiac sarcolemmal vesicles was accelerated in the presence of Ba^{2+} and suggested that this was due to Ca^{2+}/Ba^{2+} exchange. Tibbits and Philipson (1985) measured exchange-mediated $^{133}Ba^{2+}$ uptake by cardiac sarcolemmal vesicles; maximal rates of uptake were 20-fold lower than for Sr^{2+} or Ca^{2+}. Measurements of outward exchange currents in cardiac myocytes indicated that Ba^{2+} was transported only poorly, if at all, by the exchanger (Kimura et al., 1987; Shimoni and Giles, 1987). However, more recent experiments with excised membrane patches from cardiac myocytes and/or frog oocytes expressing the cardiac exchanger indicate that significant outward exchange currents can be observed with Ba^{2+} as the transported substrate (personal communications from Dr. Donald Hilgemann, University of Texas Southwestern Medical Center, Dallas, TX and Dr. Larry Hryshko, St. Boniface General Hospital Research Center, Winnipeg, Canada). In contrast to reports that Ba^{2+} is transported only poorly by the exchanger, our estimated maximal influx rates with $^{133}Ba^{2+}$ are ~50% of those observed with $^{45}Ca^{2+}$. It is not clear why there is such a disparity among the different reports on the relative rates of Ca^{2+} and Ba^{2+} as exchange substrates. We have recently observed that exchange-mediated Ba^{2+} influx in CHO cells is much more temperature sensitive than Ca^{2+} influx (unpublished observations); while this in itself does not explain the differing results among the various experimental reports, it does suggest that Ba^{2+} translocation by the exchanger might involve mechanistic constraints that do not apply to Ca^{2+}. Thus, the varying results obtained among different investigators might reflect subtle differences in experimental conditions, species differences, or variations in membrane composition that exert disproportionate effects on exchange-mediated Ba^{2+} movements.

Approximately 1–2 nmol/mg protein of $^{133}Ba^{2+}$ were accumulated by ouabain-treated cells under Na^+-free conditions (Figs. 2 and 6). With a cellular water content of 6 μl/mg protein, this is equivalent to 200–300 μM total intracellular Ba^{2+}. In fura-2 experiments, the 350/390 ratios during Ba^{2+} influx approached 4.0, which is equivalent to a cytosolic concentration of approximately 3 μM. Thus, much of the intracellular Ba^{2+} is buffered, although it is unclear which cellular constituents are involved in this process. With $^{45}Ca^{2+}$, the cells accumulated 7 nmol/mg protein under the conditions of Fig. 6; with different conditions (1 mM $^{45}Ca^{2+}$, 40 mM Na^+ + 100 mM K^+ in the assay media), $^{45}Ca^{2+}$ accumulations of up to 40 nmol/mg protein have been observed (data not shown). $[Ca^{2+}]_i$ rarely exceeded 1 μM during exchange-mediated Ca^{2+} influx, even under conditions favoring extensive Ca^{2+} accumulation. Thus, the ratio of total cellular cation to the free cytosolic concentration was much higher for Ca^{2+} than for Ba^{2+}, a result consistent with a greater degree of organellar sequestration of Ca^{2+} (cf. below).

An unexpected disparity was observed between the efflux data obtained with $^{133}Ba^{2+}$ and fura-2. In the $^{133}Ba^{2+}$ studies, preaccumulated Ba^{2+} was lost from the cells with a half-time of 3–4 min under Na^+-free conditions

(Fig. 3). With fura-2–loaded cells, however, little or no decline in cytosolic [Ba^{2+}] could be detected when 10 mM EGTA was added (under Na^+-free conditions) following a period of Ba^{2+} influx (Fig. 4). This ratio of EGTA to Ba^{2+}, under the conditions of our experiments, yields a free [Ba^{2+}] of 1.6 μM (MAXC program; Bers et al., 1994) and completely blocks Ba^{2+} influx. Moreover, despite the data in Fig. 3 indicating that extracellular Na^+ stimulates $^{133}Ba^{2+}$ efflux, we were unable to demonstrate an Na^+_o-dependent decline in cytosolic [Ba^{2+}] in fura-2–loaded cells using several different protocols to preload cells with Ba^{2+} (data not shown). This behavior remains unexplained at the present time.

A major advantage of Ba^{2+} over Ca^{2+} in measuring exchange activity is that Ba^{2+} was not sequestered by the endoplasmic reticulum in these cells (Fig. 7). This conclusion confirms results reported previously using other cell types (Rasgado-Flores et al., 1987; Kwan and Putney, 1990). Moreover, Ba^{2+} did not appear to be significantly accumulated by mitochondria, as judged by the absence of an effect of mitochondrial inhibitors on $^{133}Ba^{2+}$ uptake (Fig. 6). Previous studies of Ba^{2+} uptake by mitochondria yielded conflicting results. Uptake of divalent cations by isolated mitochondria showed a selectivity sequence $Ca^{2+} > Sr^{2+} >> Mn^{2+} > Ba^{2+}$ (Saris and Åkerman, 1980; Vanio et al., 1970). In rat liver mitochondria, Ba^{2+} uptake was further inhibited by K^+ and Mg^{2+} (Vanio et al., 1970), suggesting that the cytosolic concentrations of these ions would greatly reduce the capacity of mitochondria to accumulate Ba^{2+}. Studies with permeabilized synaptosomes also showed little if any Ba^{2+} accumulation by mitochondria (Rasgado-Flores et al., 1987). On the other hand, Ba^{2+} accumulation was readily detectable in fura-2–loaded rat heart mitochondria (McCormack and Osbaldeston, 1990). Mitochondria in pancreatic B cells (Howell and Tyhurst, 1976) and rabbit vascular smooth muscle (Somlyo et al., 1974) showed dense Ba^{2+} deposits when the cells were exposed to high external concentrations of Ba^{2+}. While our results do not rule out mitochondrial Ba^{2+} accumulation in the CHO cells, they suggest that this process is likely to be negligible compared to Ca^{2+} sequestration. Thus, measurements of Ba^{2+} uptake by either isotopic flux measurements or fura-2 should be a more direct indicator of exchange activity than the corresponding measures of Ca^{2+} uptake.

A second advantage of using Ba^{2+} for studies of exchanger regulation is that activation of exchange activity by $[Ca^{2+}]_i$ can be readily observed (cf. below). This is experimentally difficult to demonstrate with Ca^{2+} influx measurements because Ca^{2+} entering the cells would itself accelerate exchange activity through positive feedback involving secondary Ca^{2+} activation. This implies that cytosolic Ba^{2+} is considerably less effective than Ca^{2+} for activating exchange activity at the Ca^{2+} regulatory sites. Recent measurements of exchange currents in excised patches from frog oocytes expressing the exchanger (NCX1) have verified this conclusion (personal communication, Dr. L. Hryshko).

The effects of Tg and ionomycin shown in Figs. 7 and 8 indicate that exchange activity depends secondarily on the Ca^{2+} sequestering activities of intracellular organelles through their influence on $[Ca^{2+}]_i$. Two aspects of these results deserve special mention. First, the exchanger becomes activated at surprisingly low cytosolic Ca^{2+} concentrations. The experiments with fura-2 (Figs. 4 *B*, 5, 7, and 8) indicate that high levels of exchange-mediated Ba^{2+} influx are observed when cytosolic Ca^{2+} levels (during the period just before the addition of Ba^{2+}) were between 35 and 70 nM; on the other hand, exchange activity was greatly reduced when $[Ca^{2+}]_i$ declined below 20 nM (Figs. 7 and 8). Note that these experiments were carried out in the absence of extracellular Ca^{2+}, and so the lower values of $[Ca^{2+}]_i$ were substantially below "resting" values under physiological conditions (50–75 nM; Pijuan et al., 1993; Vázquez et al., 1997). Because of the relatively simple calibration procedure used, the values cited for $[Ca^{2+}]_i$ may be slightly inaccurate, but any errors are unlikely to be large enough to affect the general conclusions drawn below.

The $[Ca^{2+}]_i$ values that activate exchange activity in our experiments were clearly much lower than values obtained with excised sarcolemmal patches, where concentrations of 300–600 nM are required for half-maximal activation of outward exchange currents (Hilgemann et al., 1992*b*). On the other hand, our results agree closely with those of Miura and Kimura (1989) and of Noda et al.(1988), who observed half-maximal activation of outward exchange currents in guinea pig myocytes at $[Ca^{2+}]_i$ = 22 and 47 nM, respectively. The reasons for the differences between the behavior of excised patches and intact cells are a matter for speculation: They might involve the loss of regulatory cellular components in the patches, or local interactions with intracellular Ca^{2+} storage organelles that elevate $[Ca^{2+}]_i$ in the vicinity of the exchanger above the value in the bulk cytosol. In any event, our results imply that in cells under resting physiological conditions, where $[Ca^{2+}]_i$ is typically 50–75 nM, the exchanger is at least partially activated.

The second aspect of our results that merits detailed consideration is the complex interaction between exchange activity and intracellular organelles. The data in Fig. 7 and 8 indicate that when intracellular Ca^{2+} stores were depleted with ionomycin, subsequent removal of the ionophore allowed resequestration of cytosolic Ca^{2+} by the stores, resulting in a reduction of $[Ca^{2+}]_i$ below levels needed to activate the exchanger.

Deactivation of exchange activity by organellar Ca^{2+} sequestration was not observed in preliminary experiments with cells expressing an exchanger deletion mutant that is not regulated by $[Ca^{2+}]_i$. The unexpected finding that exchange activity was reduced in ionomycin-treated cells where the SERCA Ca^{2+} pump had been blocked with Tg (Fig. 8) suggests that Tg-resistant Ca^{2+} pumps contributed to reducing $[Ca^{2+}]_i$; at present, the identity of these pumps is unknown. It should be noted that these experiments were conducted in the absence of extracellular Ca^{2+}; if Ca^{2+} had been present externally, Ca^{2+} entry through store-dependent influx pathways (Putney, 1990) would have increased $[Ca^{2+}]_i$ and activated exchange activity. These considerations raise the possibility that the exchanger participates in a capacitative feedback mechanism for regulating the filling state of intracellular stores. Thus, when intracellular stores are filled to capacity with Ca^{2+}, their ability to sequester additional Ca^{2+} is reduced, leading to a rise in $[Ca^{2+}]_i$ and activation of exchange activity. Activation of the exchanger under physiological conditions would stimulate Ca^{2+} efflux, thereby reducing net Ca^{2+} entry into the cell and limiting any further increase in store size. Conversely, when the stores contain a reduced Ca^{2+} load, sequestration of Ca^{2+} from the cytosol would reduce $[Ca^{2+}]_i$ and attenuate exchange activity, thereby allowing additional filling of the stores. Capacitative feedback between intracellular stores and exchange activity, in conjunction with capacitative Ca^{2+} entry mechanisms (Putney, 1990), could be an important mechanism for controlling the Ca^{2+} content of $InsP_3$-sensitive stores in neutrophils, pancreatic β cells and vascular smooth muscle cells.

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