The effects of extracellular Na⁺ on store-dependent Ca²⁺ influx were compared for transfected Chinese hamster ovary cells expressing the bovine cardiac Na⁺-Ca²⁺ exchanger (CK1.4 cells) and vector-transfected control cells. Store-dependent Ca²⁺ influx was elicited by depletion of intracellular Ca²⁺ stores with ionomycin, thapsigargin, or extracellular ATP, a purinergic agonist. In each case, the rise in [Ca²⁺], upon the addition of extracellular Ca²⁺ was reduced in CK1.4 cells compared with control cells at physiological [Na⁺]. When Li⁺ or NMDG was substituted for Na⁺, the CK1.4 cells showed a greater rise in [Ca²⁺], than control cells over the subsequent 3 min after the addition of Ca²⁺. Under Na⁺-free conditions, SK&F 96365 (50 μM), a blocker of store-operated Ca²⁺ channels, nearly abolished the thapsigargin-induced rise in [Ca²⁺], in the control cells but only partially inhibited this response in the CK1.4 cells. We conclude that in the CK1.4 cells, Ca²⁺ entry through store-operated channels was counteracted by Na⁺, dependent Ca²⁺ efflux at physiological Na⁺, whereas Ca²⁺ entry was enhanced through Na⁺, dependent Ca²⁺ influx in the Na⁺-free medium. We examined the effects of thapsigargin on Ba²⁺ entry in the CK1.4 cells because Ba²⁺ is transported by the Na⁺-Ca²⁺ exchanger, but it enters these cells only poorly through store-operated channels, and it is not sequestered by intracellular organelles. Thapsigargin treatment stimulated Ba²⁺ influx in a Na⁺-free medium, consistent with an acceleration of Ba²⁺ entry through the Na⁺-Ca²⁺ exchanger. We conclude that organelar Ca²⁺ release induces a regulatory activation of Na⁺-Ca²⁺ exchange activity.

Agents that promote the production of 1,4,5-inositol trisphosphate (InsP₃)² give rise to a biphasic increase in cytosolic Ca²⁺. The initial phase is primarily due to release of Ca²⁺ from intracellular stores, whereas the more prolonged plateau phase involves an accelerated influx of extracellular Ca²⁺. The Ca²⁺ influx pathway involves low conductance Ca²⁺ channels and is activated, through a poorly understood mechanism, by the loss of Ca²⁺ from the InsP₃-sensitive stores (1–4). This process is designated as capacitative Ca²⁺ entry (3) or store-dependent Ca²⁺ influx (SDCI) (4). When cells are exposed to a Ca²⁺-mobilizing agent in the absence of extracellular Ca²⁺, the SDCI pathway remains activated (even after removal of the Ca²⁺-mobilizing agent) until Ca²⁺ is restored and the InsP₃-sensitive stores refill with Ca²⁺. Inhibitors of sarco(endo)plasmic reticulum Ca²⁺ ATPase such as thapsigargin (Tg) prevent Ca²⁺ reaccumulation by the InsP₃-sensitive stores, resulting in prolonged activation of SDCI (5).

The Na⁺-Ca²⁺ exchanger is a carrier-mediated transport process that couples the transmembrane movement of 3 Na⁺ ions to the movement of a single Ca²⁺ ion in the opposite direction. In cardiac cells, it is widely accepted that the exchanger transports a portion of the Ca²⁺ released from the sarcoplasmic reticulum out of the cells by Na⁺-dependent Ca²⁺ efflux and in this way regulates the amount of stored Ca²⁺ available for release during subsequent beats (reviewed in Ref. 6). However, in many other types of cells the downstream response to Ca²⁺-mobilizing agents depends more on the sustained influx of Ca²⁺ following agonist addition than on the magnitude of the Ca²⁺ transient itself. For noncardiac cells, there is a wealth of evidence supporting the generalized activity of Na⁺-Ca²⁺ exchange in mediating Ca²⁺ efflux and in modulating the Ca²⁺ content of intracellular stores (7). However, the precise physiological role of the exchanger and its interactions with other Ca²⁺ homeostatic mechanisms is not as clearly defined in noncardiac cells as in myocardial cells.

A major difficulty in investigations of exchanger function in intact cells is the absence of a selective inhibitor for exchange activity. In this report, we utilize transfected CHO cells to bypass this limitation. CHO cells do not normally express Na⁺-Ca²⁺ exchange activity, but after transfection with an expression vector coding for the bovine cardiac Na⁺-Ca²⁺ exchanger, high levels of activity are observed (8, 9). Comparing the effects of Na⁺ on Ca²⁺ mobilization between transfected and vector-transfected control cells provides a means of identifying functional roles of exchange activity in relation to other cellular mechanisms for intracellular Ca²⁺ handling. The results of this study indicate that Ca²⁺ efflux via the Na⁺-Ca²⁺ exchanger limits the rise in [Ca²⁺] during sustained Ca²⁺ entry, suggesting a potential modulatory function for Na⁺-Ca²⁺ exchange in the Ca²⁺ signaling process. Additional evidence suggests that the exchanger itself undergoes a regulatory activation during Ca²⁺ release from intracellular stores.
**EXPERIMENTAL PROCEDURES**

**Cells**

CK1.4 cells were prepared by transfection of dhfr<sup>−</sup> Chinese hamster ovary cells with a mammalian expression vector (pcDNA-Neo Invitrogen) containing a CDNA insert coding for the bovine cardiac Na<sup>+</sup>-Ca<sup>2+</sup>-exchanger (8). Control cells were prepared by transfection of the CHO cells with the vector alone (i.e. no insert). The cells were grown in Iscove’s modified Dulbecco’s medium containing 10% fetal calf serum and 500 μg/ml geneticin (G418) as described (8). Unless specified otherwise, biochemicals were obtained from Sigma.

**Fura-2 Assays**

Cells grown in 75-cm<sup>2</sup> plastic culture flasks were washed three times with Na-PSS, which consists in (mM) 140 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 5 glucose, and 20 mM HEPES buffered to pH 7.4 at 37°C with Tris. PSS prepared by substituting 140 mM NaCl with 140 mM LiCl is designated as Li-PSS, and nominally Ca<sup>2+</sup>-free PSS refers to PSS in which 1 mM CaCl<sub>2</sub> has been omitted. The cells were released from the flask by the addition of 5 ml of EDTA to Ca<sup>2+</sup>-free Na-PSS, centrifuged, and resuspended and 500 μl of nominally Ca<sup>2+</sup>-free Na-PSS containing 0.3 mM EGTA and 0.25 mM sulfinpyrazone (to retard transport of fura-2 out of the cells; Ref. 8). After loading, the cells were centrifuged and resuspended in nominally Ca<sup>2+</sup>-free Na-PSS containing 0.3 mM EGTA and, as indicated, either 10 μM ionomycin or 200 μM thapsigargin. After 1 min, the cells were centrifuged, resuspended in the desired medium (specified in the individual experiments), and placed in a fluorescence cuvette. 0.25 mM sulfinpyrazone was included in the cuvette solutions. Occasional variations in this protocol are designated in individual experiments. All experiments were conducted at 37°C. Fluorescence was monitored at 510 nm emission with excitation at 340 and 380 nm in a Photon Technology International RF-M 2001 fluorometer. Calibration of the 340/380 ratios was accomplished by adding 10 μM digitonin to the cuvettes in the presence of excess EGTA or Ca<sup>2+</sup> for determination of R<sub>340</sub> and R<sub>380</sub>, using the formula of Grynkiewicz et al. (10) to calculate the corresponding values of [Ca<sup>2+</sup>]<sup>−</sup>. No differences in calibration between CK1.4 and control cells were observed. All fluorescence values were corrected for autofluorescence determined for each set of experiments using unloaded cells. For experiments involving Ba<sup>2+</sup> entry, the excitation wavelengths were 350 and 390 nm (11); calibrations were not conducted for the Ba<sup>2+</sup> experiments. The results are presented for multiple experiments as mean values ± S.E. (error bars shown in figures). Significance testing was carried out using Student’s t test (two-tailed) for unpaired samples.

**<sup>46</sup>Ca Uptake**

Cells were grown in 24-well plastic dishes. The medium was replaced with 1 ml of nominally Ca<sup>2+</sup>-free Na-PSS, and the cells were preincubated for 30 min at 37°C. Midway through the preincubation period, Tg (50 μg/ml) and 20% dimethyl sulfoxide was added to the desired wells. The preincubation medium was replaced with the 200 μl of assay medium (either Na-PSS or NMDG-PSS, pH 8.0, containing 1 mM <sup>46</sup>CaCl<sub>2</sub>) and after the desired intervals, the wells were washed 4 times with 1 ml of termination medium (100 mM MgCl<sub>2</sub> + 10 mM La<sub>2</sub>Cl<sub>6</sub> + 5 mM Mops/Tris, pH 7.4). The contents of the wells were extracted with 0.1 N HNO<sub>3</sub> and counted.

**Protocols**

**Refilling of Ca<sup>2+</sup> Stores—Ca<sup>2+</sup> stores were depleted by treating 0.1 ml of fura-2-loaded cells for 1 min with 10 μM ionomycin in Ca<sup>2+</sup>-free Na-PSS containing 0.3 mM EGTA (37°C). The cells were centrifuged and resuspended in nominally Ca<sup>2+</sup>-free PSS (with substitutions for Na<sup>+</sup> as indicated) containing 0.3% fatty acid free bovine serum albumin to scavenge residual ionomycin. After 30 s, 1 mM CaCl<sub>2</sub> was added to the medium, and [Ca<sup>2+</sup>]<sup>−</sup> was monitored. After the desired interval, 3 mM EGTA and, 30 or 30 s later, 0.3 mM ATP were added to bring about Ca<sup>2+</sup> release from the InsP<sub>3</sub>-sensitive stores (8). The peak of the Ca<sup>2+</sup> transient was taken as a measure of the amount of Ca<sup>2+</sup> in the InsP<sub>3</sub>-sensitive pool.

**Tg Treatment**—Cell suspensions (0.1 ml) were loaded with fura-2 and treated with 200 nM Tg in Ca<sup>2+</sup>-free Na-PSS containing 0.3 mM EGTA for 1 min at 37°C. The cells were centrifuged and suspended in nominally Ca<sup>2+</sup>-free PSS containing 0.3 mM EGTA (with or without Na<sup>+</sup>-substitutes as indicated) in the cuvette. CaCl<sub>2</sub> (1 mM) was added as indicated, and [Ca<sup>2+</sup>]<sup>−</sup>, was monitored.

**RESULTS**

**Refilling of Intracellular Ca<sup>2+</sup> Stores—**Intracellular Ca<sup>2+</sup> stores in fura-2-loaded cells were depleted by treatment with ionomycin in a Ca<sup>2+</sup>-free medium. The cells were then re-exposed to Ca<sup>2+</sup> and the state of filling of the InsP<sub>3</sub>-sensitive Ca<sup>2+</sup> stores was assessed after the desired intervals by the addition of 3 mM EGTA followed by 0.3 mM ATP. ATP elicits the formation of InsP<sub>3</sub> in these cells (8, 12) through its interaction with a P<sub>2</sub> purinergic receptor (12). As shown in Fig. 1 (top trace, left panel) when 0.3 mM ATP was added to CK1.4 cells after pretreatment with ionomycin, no [Ca<sup>2+</sup>]<sup>−</sup> transient was observed, indicating that the InsP<sub>3</sub>-sensitive stores had been completely depleted; similar results were obtained with control cells (data not shown). In each of the other traces in Fig. 1, CaCl<sub>2</sub> (1 mM) was added at 30 s (arrows), and then 3 mM EGTA was added after various intervals, followed by 0.3 mM ATP 30 s later. For the control cells (right panel, Fig. 1), [Ca<sup>2+</sup>]<sup>−</sup> increased markedly upon the addition of 1 mM CaCl<sub>2</sub>, and the Ca<sup>2+</sup> stores refilled rapidly with Ca<sup>2+</sup>, attaining a maximal level within 2.5 min. For the CK1.4 cells (left panel, Fig. 1), the increase in [Ca<sup>2+</sup>]<sup>−</sup>, upon the addition of 1 mM CaCl<sub>2</sub>, was smaller than for the control cells, and the rate of store refilling was slower, requiring 5 min of exposure to Ca<sup>2+</sup> to achieve a maximal level.

For the CK1.4 cells, the rate of store refilling was markedly increased in a Na<sup>+</sup>-free medium (Li substitution). As shown in Fig. 2 (left panels), the increase in [Ca<sup>2+</sup>]<sup>−</sup>, upon the addition of CaCl<sub>2</sub> in Li-PSS was larger than in Na-PSS, and the [Ca<sup>2+</sup>]<sup>−</sup> transients elicited by ATP were markedly increased; in contrast, there was no significant difference between the sodium-and lithium-based media for the control cells (right panels, Fig. 2). The peaks of the Ca<sup>2+</sup> transients in the CK1.4 cells after 30 and 150 s of exposure to Ca<sup>2+</sup> were 48 ± 2 nm (n = 5) and 140 ± 13 nm (n = 3) in Na-PSS as compared with 140 ± 22 nm (n = 3) and 427 ± 83 nm (n = 5) in Li-PSS, respectively. The differences between the sodium- and lithium-based media were highly significant (p < 0.01). The comparable values for the control cells were 112 ± 31 nm (n = 5) and 327 ± 46 nm (n = 8) in Na-PSS versus 109 ± 25 nm (n = 3) and 312 ± 64 nm (n = 5) in Li-PSS. The difference between the CK1.4 cells and the
Ca²⁺-loaded cells were treated with ionomycin and placed in nominally Ca²⁺-free Na-PSS (light traces; error bars down) or Li-PSS (light traces; error bars up). CaCl₂ (1 mM) was added at 30 s (arrows), and after either 30 s (A and C) or 150 s (B and D), 3 mM EGTA was added (♀) followed by 0.3 mM ATP 10 s later (♂). The results are the mean values for three to five determinations in two (control) and three (CK1.4) independent experiments.

Control cells in Na-PSS (150 s) was significant (p < 0.01); other comparisons showed no significant differences. The larger size of the [Ca²⁺] transient for the CK1.4 cells in the Na⁺-free medium was primarily a reflection of the increased amount of Ca²⁺ in the InsP₃-sensitive stores. As shown below (see Fig. 8), the ATP-evoked [Ca²⁺] transient in cells containing equivalent amounts of stored Ca²⁺ was smaller in Na-PSS than in Li-PSS, but the effect of Na⁺ was small compared with that shown in Fig. 2. The results in Figs. 1 and 2 indicate that extracellular Na⁺ reduces the rate of store refilling in CHO cells expressing the Na⁺-Ca²⁺ exchanger.

*Tg-induced Ca²⁺ Entry in CK1.4 Cells—Another method of depleting intracellular Ca²⁺ stores is by treating the cells with Tg, an irreversible inhibitor of sarco(endoplasmic reticulum Ca²⁺-ATPases (13, 14). In cells pretreated with 200 nM Tg, ATP does not induce a [Ca²⁺] transient either before or after the addition of extracellular Ca²⁺ (data not shown). This indicates that the InsP₃-sensitive Ca²⁺ stores were fully depleted by the Tg treatment and that refilling of the stores does not occur, as expected from the blockade of sarco(endoplasmic reticulum Ca²⁺-ATPase activity. As in other cell types, Tg treatment increases the influx of Mn²⁺, a Ca²⁺ surrogate, as measured by the decline in fluorescence due to quenching of fura-2 by intracellular Mn²⁺. As shown in Fig. 3, Mn²⁺ entry was stimulated by Tg to the same extent in vector-transfected and CK1.4 cells, indicating that intracellular store depletion is equally effective in activating store-operated Ca²⁺ channels in the two types of cells. No difference was observed in the rate of Mn²⁺ entry between Na- and Li-PSS for either type of cell (data not shown). Mn²⁺ is not transported by the Na⁺-Ca²⁺ exchanger in these cells (data not shown).

The right panel of Fig. 4 shows that the addition of 1 mM CaCl₂ to Tg-treated control cells produced a marked increase in [Ca²⁺], which was essentially identical in Na-PSS or Li-PSS. For CK1.4 cells, however (left panel, Fig. 4), the rise in [Ca²⁺] was greatly reduced in Na-PSS (trace c) compared with Li-PSS (trace a). Traces b and d depict the rise in [Ca²⁺] in Li-PSS and Na-PSS, respectively, for CK1.4 cells that had not been treated with Tg. For these cells, the rise in [Ca²⁺] in Li-PSS (trace b) was significantly greater than in Na-PSS (trace c) but was still much less than that shown by Tg-treated cells in Li-PSS (trace a). Similar results were obtained when NMDG was used as the Na⁺ substitute instead of Li⁺ (data not shown). The average values of [Ca²⁺] observed between 90 and 120 s after the addition of CaCl₂ for the data shown in Fig. 4 are summarized in Table I. Compared with the vector-transfected control cells, Tg-treated CK1.4 cells exhibited a reduced [Ca²⁺] in Na-PSS but an elevated [Ca²⁺] in Li-PSS (p < 0.01 in each case). The initial portions of the traces from the CK1.4 cells and control cells in Na-PSS are directly compared in the inset of Fig. 4. The increases in [Ca²⁺] between the two types of cells are significant (p < 0.05 or less) for all times after the asterisk shown in the inset.

A similar pattern of results was obtained in ⁴⁵Ca²⁺ flux experiments. The data in Fig. 5 depict ⁴⁵Ca²⁺ uptake by vector-transfected control and CK1.4 cells in Na-PSS and in Na-free PSS (NMDG substitution). ⁴⁵Ca²⁺ accumulation under these conditions is markedly enhanced at alkaline pH, and so these experiments were conducted at an external pH of 8.0. As shown...
Effects of thapsigargin on [Ca\textsuperscript{2+}] in CK1.4 and control cells

Data for the [Ca\textsuperscript{2+}] determinations were taken from the experiments shown in Fig. 4. The average [Ca\textsuperscript{2+}] values 90–120 s after the addition of 1 mM CaCl\textsubscript{2} were determined for each experiment, and these values were averaged over the number of experiments shown.

| Cells     | Medium   | Tg | [Ca\textsuperscript{2+}] | n  |
|-----------|----------|----|--------------------------|----|
| CK1.4     | Na-PSS   | –  | 92 ± 8                   | 3  |
| CK1.4     | Li-PSS   | –  | 162 ± 6                  | 5  |
| CK1.4     | Na-PSS   | +  | 134 ± 15                 | 5  |
| CK1.4     | Li-PSS   | +  | 496 ± 68                 | 5  |
| Control   | Na-PSS   | +  | 218 ± 11                 | 9  |
| Control   | Li-PSS   | +  | 225 ± 23                 | 5  |

In the right panel of Fig. 5, Tg treatment (filled symbols) stimulated \(45\text{Ca}^{2+}\) uptake in the control cells in both Na-PSS and in NMDG-PSS. For the CK1.4 cells, however (left panel, Fig. 5), Tg had practically no effect on \(45\text{Ca}^{2+}\) uptake in Na-PSS, although in NMDG-PSS it stimulated \(45\text{Ca}^{2+}\) uptake as well as in the control cells. The results in NMDG-PSS were similar to those obtained with Li-PSS (data not shown). Thus, the conclusions of the \(45\text{Ca}^{2+}\) experiments are consistent with those obtained in the fura-2 measurements: Tg-induced \(\text{Ca}^{2+}\) entry was markedly reduced in the CK1.4 cells by the presence of extracellular Na\textsuperscript{+}.

In many types of cells, \(\text{Ca}^{2+}\) entry via the SDCI pathway is blocked by SK&F 96365 (15, 16). As shown in the right panel of Fig. 6, 50 \(\mu\text{M}\) SK&F 96365 sharply reduced the rise in [Ca\textsuperscript{2+}], in vector-transfected control cells that had been treated with Tg prior to adding 1 mM CaCl\textsubscript{2}; SK&F was equally effective in Na- or Li-PSS. In contrast, SK&F only partially inhibited the rise in [Ca\textsuperscript{2+}] for Tg-treated CK1.4 cells in Li-PSS (left panel). Thus, in the CK1.4 cells a substantial fraction of Ca\textsuperscript{2+} enters the cell via a pathway that is insensitive to inhibition by SK&F 96365, because this pathway is absent in the control cells, it probably reflects \(\text{Ca}^{2+}\) influx via reverse Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange. Incubation of the Tg-treated cells under Na-free conditions for 5 min prior to adding CaCl\textsubscript{2} reduced the increase in [Ca\textsuperscript{2+}], and increased its sensitivity to SK&F 96365, consistent with a reduced [Na\textsuperscript{+}], and decreased contribution of Ca\textsuperscript{2+} influx via the exchanger (data not shown). Note also that the decline in [Ca\textsuperscript{2+}], after addition of EGTA was slowed by the presence of SK&F 96365 (left panel, Fig. 6), consistent with previous reports of an inhibitory effect of this agent on \(\text{Ca}^{2+}\) efflux (16, 17).

\(\text{Ca}^{2+}\) Efflux from CK1.4 Cells—The results presented above suggest that Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} efflux via the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger is responsible for the attenuation of Tg-induced Ca\textsuperscript{2+} entry in the presence of Na\textsuperscript{+}. To assess the magnitude of Na\textsuperscript{+}-dependent Ca\textsuperscript{2+} efflux from CK1.4 cells, we adopted the following protocol. After loading with fura-2, \([\text{Ca}^{2+}]_i\), was elevated by preinhibiting a concentrated suspension of the cells for 1 min with Li-PSS containing 200 \(\mu\text{M}\) Tg and 1 mM CaCl\textsubscript{2}.

The cell suspension was then diluted into a cuvette containing 0.3 mM EGTA in either Li- or Na-PSS, and the rate of decline in \([\text{Ca}^{2+}]_i\) was monitored. As shown in Fig. 7, the rate of decline of \([\text{Ca}^{2+}]_i\), was more rapid in Na-PSS than in Li-PSS, consistent with a contribution of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange to Ca\textsuperscript{2+} efflux. We cannot be certain that the decline in \([\text{Ca}^{2+}]_i\), under these conditions is entirely due to Ca\textsuperscript{2+} efflux from the cell because Ca\textsuperscript{2+} sequestration by Tg-resistant compartments could also play a role. However, when control cells were used instead of the CK1.4 cells in similar experiments, there was no difference between Na- or Li-PSS, and the rate of decline in \([\text{Ca}^{2+}]_i\), was similar to that observed in Li-PSS for the CK1.4 cells (data not shown). Thus, the Na\textsuperscript{+}-dependent component of the decline in \([\text{Ca}^{2+}]_i\), probably represents Ca\textsuperscript{2+} efflux via the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger.

The data in the inset of Fig. 7 depict the decline in \([\text{Ca}^{2+}]_i\), during the first 40 s of the experiment, expressed as a first order plot. Nonlinear first order plots were observed in both Na- and Li-PSS. At equivalent concentrations of cytosolic Ca\textsuperscript{2+}, the rate of decline in \([\text{Ca}^{2+}]_i\), was approximately 2.5-fold greater in Na-PSS than in Li-PSS throughout the concentration range examined.

Effects of ATP on \([\text{Ca}^{2+}]_i\), in the Presence of Extracellular Ca\textsuperscript{2+}—It is important to know if the exchanger modulates Ca\textsuperscript{2+} movements elicited by physiological agonists such as ATP. Therefore, we examined the effects of Na\textsuperscript{+} on the shape of the \([\text{Ca}^{2+}]_i\), transient elicited by ATP in CK1.4 cells in the absence of extracellular Ca\textsuperscript{2+}. As shown in Fig. 8 (left panel), the peak of the \([\text{Ca}^{2+}]_i\), transient was smaller in Na-PSS than in Li-PSS, and the transient decayed to prestimulation levels more quickly. A similar pattern was observed when a \([\text{Ca}^{2+}]_i\), transient was evoked with 2 \(\mu\text{M}\) ionomycin instead of ATP (right panel, Fig. 8). With ionomycin, organelar Ca\textsuperscript{2+} sequestration would be blocked, and the decay of the \([\text{Ca}^{2+}]_i\), transient should reflect \(\text{Ca}^{2+}\) efflux from the cell; the acceleration of \(\text{Ca}^{2+}\) efflux in Na-PSS compared with Li-PSS therefore provides an index of Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange activity. For control cells, no dif-
The difference between Na- and Li-PSS was observed in comparable experiments (cf. Fig. 9 for data with ATP). The results confirm the importance of Na+-dependent Ca²⁺ influx in regulating [Ca²⁺] in the CK1.4 cells.

The effects of ATP in the presence of extracellular Ca²⁺ are compared for vector-transfected control cells and the CK1.4 cells in Fig. 9. The cuvette solutions in these experiments initially contained 0.3 mM EGTA and PSS with 140 mM NaCl, 140 mM LiCl, or 40 mM NaCl + 100 mM KCl as the principal salts. The effects of adding 0.3 mM ATP plus 1 mM CaCl₂ (upper traces, Fig. 9), ATP alone (center traces, Fig. 9), or CaCl₂ alone (lower traces, Fig. 9) were examined for each cell type. For the control cells (right panels, Fig. 9), the addition of ATP alone produced a transient rise in [Ca²⁺], whereas the addition of ATP + 1 mM CaCl₂ elicited a sustained increase in [Ca²⁺], which was higher than that evoked by the addition of Ca²⁺ alone. There were no major differences in the behavior of the control cells among the various media, although there was a tendency for the sustained phase of Ca²⁺ entry to be reduced in the 40/100 sodium/potassium medium (trace b, upper right panel, Fig. 9); the latter effect is probably due to the reduced driving force for Ca²⁺ entry in cells depolarized by the high potassium concentration.

For the CK1.4 cells (left panel, Fig. 9), dramatic differences were observed among the different media when ATP and Ca²⁺ were added together. In 140 mM Na⁺ (trace c; upper left panel, Fig. 9), the initial [Ca²⁺] transient was followed by a sustained phase that was slightly but significantly reduced (p < 0.05) compared with that observed in the control cells. The levels of [Ca²⁺] attained in the Na-free or 40 mM Na⁺ media (traces a and b; upper left panel, Fig. 9) were much higher than those produced by the addition of Ca²⁺ alone (lower left panel, Fig. 9) and were markedly elevated compared with the sustained [Ca²⁺] levels in the vector-transfected control cells, particularly in the case of Li-PSS. Ca²⁺ entry was similarly enhanced when NMDG was used as the Na⁺ substitute (data not shown). As in the case of Tg-treated cells, the sustained increases in [Ca²⁺] in the low [Na⁺] media were only partially inhibited by SK&F 96365 (data not shown), suggesting that a portion of the Ca²⁺ entry under these conditions was conducted by the Na⁺-Ca²⁺ exchange system. Intracellular Ca²⁺ stores refilled rapidly after ATP addition (assessed by ionomycin-induced Ca²⁺ release; data not shown) indicating that Ca²⁺ sequestration was not blocked under these experimental conditions. The pronounced increase in [Ca²⁺] in the low [Na⁺] media suggests that exchange activity might have been accelerated during the release of Ca²⁺ from InsP₃-sensitive stores. This possibility is
Barium influx in Tg-treated CK1.4 Cells—Ba$^{2+}$ is transported by the cardiac-type Na$^{-}$-Ca$^{2+}$ exchanger (18) and reportedly enters several types of cells via the SDCI pathway (11, 20–22), although in either pathway it is less effectively transported than Ca$^{2+}$. Importantly, however, Ba$^{2+}$ is not sequestered by intracellular organelles such as the endoplasmic reticulum or the mitochondria (20, 23). Experiments to be reported elsewhere confirm the exchanger’s ability to transport Ba$^{2+}$ and the absence of organellar Ba$^{2+}$ sequestration in the CK1.4 cells. Because Ba$^{2+}$ is not accumulated by intracellular organelles, the fura-2 signal should provide a relatively direct assessment of Ba$^{2+}$ influx.

The data in Fig. 10 (right panel) show the effects of adding 1 mM BaCl$_2$ to control cells with or without prior treatment with Tg. Ba$^{2+}$ entry produces an initial abrupt rise in the 350/390 ratio in these experiments, which is probably due to small amounts of extracellular fura-2. The subsequent gradual rise in the 350/390 ratio reflects Ba$^{2+}$ entry into the cells and is only slightly enhanced in Tg-treated cells (trace a, Fig. 10). Compared with untreated cells (trace b, Fig. 10), no difference was observed in Li- or Na-PSS and the results with both media have been combined in each trace. Ba$^{2+}$ influx therefore occurs only weakly through the SDCI pathway in these cells. Note that after the addition of EGTA at 180 s, only a small decline in the fura-2 signal was observed, indicating that Ba$^{2+}$ is not readily transported out of the cell under these conditions.

In CK1.4 cells (left panel, Fig. 10), Ba$^{2+}$ entry in Li-PSS is substantially increased by Tg (trace a, Fig. 10) compared with untreated cells (trace b, Fig. 10); the fura-2 ratios attained in trace a (Fig. 10) are significantly higher than observed in the vector-transfected control cells. SK&F 96365 (50 μM) had no effect on Ba$^{2+}$ entry in the CK1.4 cells (data not shown), consistent with the absence of significant barium entry via store-operated channels. In Na-PSS, Ba$^{2+}$ influx (trace c, Fig. 10) was only slightly less than in the non-treated cells in Li-PSS (trace b, Fig. 10); no difference was observed between Tg-treated and untreated cells in Na-PSS, and it is conceivable that increased Na$^{+}$ influx might be responsible for the subsequent acceleration of Ba$^{2+}$ influx by the Na$^{+}$-Ca$^{2+}$ exchanger. Therefore, we determined whether Tg could accelerate Ba$^{2+}$ entry when added to the cells in a Na$^{+}$-free medium. CK1.4 cells were loaded with fura-2 and placed in a cuvette containing either Ca$^{2+}$-free Na-PSS or Li-PSS and 0.3 mM EGTA. After 30 s, either 200 mM Tg or vehicle (dimethyl sulfoxide, 6 μl) was added to the cuvette, and 1 mM BaCl$_2$ was added 2 min later. As shown in Fig. 11, in the absence of Tg treatment, there was little or no difference between Na- or Li-PSS for barium entry in the CK1.4 cells (A) or the control cells (C). For both types of cells (Fig. 11, B and D), the addition of Tg resulted in a slowly developing transient rise in the 350/390 fluorescence ratio, which undoubtedly reflects the gradual release of Ca$^{2+}$ from internal stores. The choice of 350/390 excitation wavelengths optimizes the Ba$^{2+}$ signal but still allows detection of increases in Ca$^{2+}$.

For the Tg-treated control cells (Fig. 11D), no difference was observed in Ba$^{2+}$ entry between the Na-PSS and the Li-PSS; the ratios were not significantly higher than those observed in the absence of Tg, again indicating that Ba$^{2+}$ does not enter these cells efficiently by the SDCI pathway. However, the Tg-treated CK1.4 cells (Fig. 11B) exhibited an accelerated Ba$^{2+}$ entry in Li-PSS (trace a) compared with Na-PSS (trace b). We conclude that the Tg-induced acceleration of Ba$^{2+}$ entry via Na$^{+}$-Ca$^{2+}$ exchange is independent of a possible effect of Tg on Na$^{+}$ entry. When the CK1.4 cells were treated with 200 mM ionomycin (which does not transport Ba$^{2+}$; Ref. 24) in other-

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2 M. Condescu, G. Chernaya, J. G. Patel, and John P. Reeves, manuscript in preparation.
wise identical experiments, similar results were obtained, i.e., ionomycin stimulated Ba\(^{2+}\) uptake in Li-PSS but not in Na-PSS (data not shown).

**DISCUSSION**

The expression of the cardiac Na\(^+-\)Ca\(^{2+}\) exchanger in a cell type that does not normally exhibit this activity confers several new attributes to cellular Ca\(^{2+}\) homeostasis. In the CK1.4 cells, physiological concentrations of extracellular Na\(^+\) retard store refilling and attenuate Tg-induced Ca\(^{2+}\) entry compared with control cells. Upon the addition of Ca\(^{2+}\) to Tg-treated cells, the initial rise in [Ca\(^{2+}\)]\(_i\) is identical in CK1.4 cells and in control cells until [Ca\(^{2+}\)]\(_i\) reaches 100 nM, when the increase slackens markedly in the CK1.4 cells (inset, Fig. 4). Na\(^{+}\)-dependent Ca\(^{2+}\) efflux provides the simplest explanation for these results. We suggest that a portion of the Ca\(^{2+}\) entering the cell through store-operated Ca\(^{2+}\) channels is transported back out of the cell by the exchanger, thereby reducing net Ca\(^{2+}\) entry and attenuating the rise in [Ca\(^{2+}\)]\(_i\), during SDCI. In this view, the exchanger generates circulatory movements of Ca\(^{2+}\) across the plasma membrane during Ca\(^{2+}\) channel activity; this circulation could play an important role in Ca\(^{2+}\) signaling processes, as suggested by Alkon and Rasmussen (27).

The effects of Na\(^{+}\) in stimulating Ca\(^{2+}\) efflux (Fig. 7) and reducing the [Ca\(^{2+}\)]\(_i\) transients elicited by ATP or ionomycin (Fig. 8) lend support to this interpretation. However, these data were obtained in Ca\(^{2+}\)-free media and do not necessarily reflect the exchanger’s activity in the presence of physiological [Ca\(^{2+}\)]\(_o\). On one hand, the efficiency of the exchanger as a Ca\(^{2+}\) pump could be substantially reduced by Ca\(^{2+}\) entry via Na\(^{+}\)-, Ca\(^{2+}\)-, or Ca\(^{2+}\)-Ca\(^{2+}\) exchanges. On the other hand, local gradients due to Ca\(^{2+}\) channel activity might elevate [Ca\(^{2+}\)]\(_i\), beneath the plasma membrane compared with that of the bulk cytosol. The exchanger’s rapid turnover (>2,000 s\(^{-1}\); Refs. 25 and 26) and high K\(_{m}\) for Ca\(^{2+}\) (4 \(\mu\)M; ref. 26) would ensure a high level of efficiency in transporting Ca\(^{2+}\) from a region with locally elevated [Ca\(^{2+}\)]\(_i\).

In the absence of Na\(^{+}\), store refilling and Tg-induced Ca\(^{2+}\) influx are accelerated in CK1.4 cells compared with control cells (Figs. 1, 2, 4, and 5 and Table I). Under these conditions, Ca\(^{2+}\) efflux via the exchanger is blocked and Ca\(^{2+}\) entry and attenuating the rise in [Ca\(^{2+}\)]\(_i\) during SDCI. In this view, the exchanger generates circulatory movements of Ca\(^{2+}\) across the plasma membrane during Ca\(^{2+}\) channel activity; this circulation could play an important role in Ca\(^{2+}\) signaling processes, as suggested by Alkon and Rasmussen (27).

The data in Fig. 9 (ATP) and Fig. 11 (Tg) are consistent with secondary Ca\(^{2+}\) activation as the mechanism accelerating exchange activity, because in both cases the increased Ca\(^{2+}\) or Ba\(^{2+}\) influx was associated with an elevation in [Ca\(^{2+}\)]\(_i\). However, under conditions where the cells were pretreated with Tg, there does not appear to be an elevation of [Ca\(^{2+}\)]\(_i\), compared with untreated cells prior to adding extracellular Ca\(^{2+}\) (Figs. 4 and 6) or Ba\(^{2+}\) (Fig. 10). Thus, it appears that accelerated exchange activity does not necessarily correlate with increased cytosolic Ca\(^{2+}\). Experiments currently under way suggest that Ca\(^{2+}\)-dependent changes in exchange activity involve complex interactions between cytosolic Ca\(^{2+}\) and the Ca\(^{2+}\) content of internal stores. Additional studies will be required to resolve these issues.

Regardless of the precise mechanism(s) involved, our findings indicate that Ca\(^{2+}\) release from intracellular stores is coupled to regulatory activation of Na\(^+-\)Ca\(^{2+}\) exchange activity. The exchanger is potentially a focal point for a variety of regulatory influences, as suggested by reports that the sensitivity of the exchanger to secondary activation by Ca\(^{2+}\) can itself be modulated by an ATP-dependent mechanism (19).

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Activation of exchange activity during Ca\(^{2+}\) release could therefore provide an adjustable negative feedback mechanism for controlling the amount of Ca\(^{2+}\) that is resequestered by the sarcoplasmic reticulum and, as demonstrated in this report, for limiting net Ca\(^{2+}\) entry into the cell during Ca\(^{2+}\) channel activity. Na\(^{+}\)-Ca\(^{2+}\) exchange activity thus provides a potentially rich source of regulatory control for cellular Ca\(^{2+}\) traffic.

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