Mode-specific Inhibition of Sodium-Calcium Exchange during Protein Phosphatase Blockade*

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The effects of the protein phosphatase inhibitors calyculin A and okadaic acid on Na+/Ca2+ exchange activity were examined in transfected Chinese hamster ovary cells expressing the bovine cardiac Na+/Ca2+ exchanger. Incubating the cells for 5–10 min with 100 nM calyculin A reduced exchange-mediated 45Ca2+ uptake or Ba2+ influx by 50–75%. Half-maximal inhibition of 45Ca2+ uptake was observed at 15 nM calyculin A. The nonselective protein kinase inhibitors K252a and staurosporine provided partial protection against the effects of calyculin A. Okadaic acid, another protein phosphatase inhibitor, nearly completely blocked exchange-mediated Ba2+ influx. Chinese hamster ovary cells expressing a mutant exchanger in which 420 out of 520 amino acid residues were deleted from the central hydrophilic domain of the exchanger remained sensitive to the inhibitory effects of calyculin A and okadaic acid. Surprisingly, Na1+-dependent Ca2+ efflux appeared to be only modestly inhibited, if at all, by calyculin A or okadaic acid. We conclude that protein hyperphosphorylation during protein phosphatase blockade selectively inhibits the Ca2+ influx mode of Na+/Ca2+ exchange, probably by an indirect mechanism that does not involve phosphorylation of the exchanger itself.

The Na+/Ca2+ exchange system is the primary Ca2+ efflux mechanism in cardiac myocytes and plays a crucial role in regulating the cellular Ca2+ content and the force of myocardial contraction (see reviews in Refs. 1–3). The activity of the Na+/Ca2+ exchanger is regulated by ATP- and Ca2+-dependent mechanisms. The Ca2+-dependent process is thought to involve the binding of Ca2+ to regulatory sites within the central hydrophilic domain of the exchanger (4–6), but the mechanism underlying the ATP-dependent regulatory process has been less clearly defined. Investigations of exchange currents in excised sarcolemmal patches indicate that the effects of ATP on this experimental system are mediated by synthesis of phosphatidylinositol 4,5-bisphosphate (7, 8), rather than protein kinases A and C activation have been reported (11, 12). Studies with rat astrocytes, neurons, and the Xenopus expression system suggest that protein kinase A stimulates the activity of certain exchanger isoforms but not others (13). In contrast to the stimulation of exchange activity described in these studies, inhibition of exchange activity was reported upon activation of protein kinases A and C in bovine chromaffin cells (14) and human mesangial cells (15).

We had previously reported that ATP depletion partially inhibits Na+/Ca2+ exchange activity in transfected Chinese hamster ovary (CHO)1 cells expressing the bovine cardiac Na+/Ca2+ exchanger (16). A phosphorylation mechanism did not appear to be involved, because inhibitors or activators of various protein kinases did not affect Na+/Ca2+ exchange activity in these cells, and phosphorylation of the immunoprecipitated exchange protein was not detectable. As part of these studies, we reported that okadaic acid and calyculin A, potent inhibitors of protein phosphatases 1 and 2A, had no effect on exchange-mediated 45Ca2+ fluxes when the inhibitors were added directly to the assay medium. In the present report, we reinvestigated the effects of these agents to determine whether exposing the cells to the inhibitors for longer incubation periods would reveal an acceleration of exchange activity through a phosphorylation-dependent process. Instead, we found that calyculin A and okadaic acid strongly inhibited the Ca2+ influx (reverse) mode of Na+/Ca2+ exchange. Curiously, these treatments had little or no effect on the Ca2+ efflux (forward) mode of exchange. The mechanism involved is uncertain, but it does not appear to entail phosphorylation of the exchanger itself.

EXPERIMENTAL PROCEDURES

Cells—CHO cells expressing Na+/Ca2+ exchange activity (CK1.4 cells) were prepared by transfecting the cells (CCL 61, American Type Culture Collection) with the expression vector pCDNA1/Neo (Invitrogen Corp., Carlsbad, CA) containing a cDNA insert coding for the bovine cardiac Na+/Ca2+ exchanger (17, 18). The cells were grown in Iscove’s modified Dulbecco’s medium containing 10% fetal calf serum and antibiotics as described (18). CK138 cells were transfected with a deletion mutant of the exchanger, 3(241–680), missing 440 out of the 520 amino acids of the central hydrophilic domain of the exchanger, as described (16). The kinetic and regulatory behavior of this mutant in excised patches (19) and in the transfected CHO cells (16, 20) has been described previously.

Materials and Solutions—Na-PSS contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 10 mM glucose, and 20 mM Mops, adjusted to pH 7.4

1 The abbreviations used are: CHO, Chinese hamster ovary; NMDG, N-methyl-d-glucamine; PP, protein phosphatase; PSS, physiological salts solution; Mops, 3-(N-morpholino)propanesulfonic acid.

* This work was supported by National Institutes of Health Grant HL 49632. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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(37 °C) with Tris, K-PSS, and NMDG-PSS had the same composition as Na-PSS except that NaCl was replaced with KCl (total concentration, 140 mM) or NMDG. Na- and K-PSS were mixed to yield the Na⁺ and K⁺ concentrations specified as 20/120, 28/112, or 40/100 Na/K-PSS. Fura-2-AM was purchased from Molecular Probes, Inc. (Eugene, OR). Calyculin A and okadaic acid were from Alexis Biochemicals (San Diego, CA). All other biochemicals were purchased from Sigma.

**45Ca²⁺ Uptake and Efflux Assay**—These assays were conducted essentially as previously described (16). Briefly, the culture medium was removed from cells grown in 24-well fibronectin-coated plastic dishes and replaced with 1 ml of nominally Ca⁺⁺-free Na-PSS containing 0.4 mM ouabain. For calyculin-treated cells, calyculin A (100 nM) was added to the preincubation medium after 20 min. After 30 min of incubation at 37°C, the medium in each well was replaced with 0.2 ml of 40/100 Na/K-PSS containing 1 mM 4CaCl₂ (37°C). After the desired interval, the cells were washed four times with termination fluid consisting of 100 mM MgCl₂ + 10 mM LaCl₃ + 5 mM Mops/Tris, pH 7.4. The radioactivity was solubilized in 0.1 N HNO₃, counted in a scintillation counter, and normalized to the amount of protein in the wells. For the 4Ca⁺⁺ efflux assay, ouabain-treated cells were loaded with 4Ca⁺⁺ by reverse Na⁺/Ca⁺⁺ exchange in 40/100 Na/K-PSS as described above with or without 100 mM calyculin A present. After the 10-min loading period, the medium was replaced with 1 ml of either Na-PSS or NMDG-PSS, and the reaction was terminated after various intervals as described above.

**Fura-2-based Assays of Ba²⁺ Influx and Ca²⁺ Efflux**—The Ba²⁺ influx assay for Na⁺/Ca⁺⁺ exchange activity is fully described in Ref. 21. Cells were grown to confluence in 75-cm² culture flasks, washed three times with Na-PSS, and incubated for 1 min at 37 °C with Na-PSS containing 5 mM EDTA to detach cells from the flask. The suspended cells were centrifuged at 700 × g for 1 min, resuspended in Na-PSS + 1 mM CaCl₂ centrifuged again, and resuspended in 4–5 ml of Na-PSS + 1 mM CaCl₂ containing 1% bovine serum albumin. The cells were divided into 300-μl aliquots and incubated for 30 min with 3 mM fura-2-AM and 0.25 mM sulfinpyrazone (to retard transport of fura-2 out of the cells). The fura-2 and sulfinpyrazone were added as 1000-fold concentrated stock solutions in dimethyl sulfoxide.

After a 30-min loading period, the cells were centrifuged for several seconds in an Eppendorf mini-centrifuge, washed, and preincubated for 5 min in 100 μl of Na-PSS + 1 mM CaCl₂ or with other additions and conditions as indicated in individual experiments. These cells were then added directly to fluorescence cuvettes containing 3 ml of 20/120 Na/K-PSS + 0.3 mM EGTA. Gramicidin (2 μg/ml) was added to the cuvettes to equilibrate transmembrane Na⁺ and K⁺ gradients. After monitoring fluorescence using cells that had not been loaded with fura-2, data were presented as the ratio of fluorescence for excitation at 350 and 390 nm using a Photon Technology International RF-M trace). A related broad spectrum protein kinase inhibitor, staurosporine (1 μM), also protected against inhibition by calyculin A (data not shown). Other protein kinase inhibitors were tested but were found not to protect against the effects of calyculin. These included 1 μM KN-62 (calmodulin-dependent protein kinase), 200 μM genistein (tyrosine kinases), 0.5 μM calphostin C (protein kinase C), 10 μM H-89 (protein kinase A), and another relatively nonselective inhibitor, 50 μM H-7. When calyculin-treated cells were subsequently incubated in a calyculin-free medium for an additional 10 min, the rates of 4Ca⁺⁺ uptake continued to decline. Including K252a in the calyculin-free medium enhanced recovery, but the rates of 4Ca⁺⁺ uptake remained less than 40% of untreated controls (data not shown).

The Na⁺/Ca⁺⁺ exchange protein contains 11 transmembrane segments with a cytosolically disposed hydrophilic domain of 520 amino acids residing between the 5th and 6th transmembrane segments (1–3). The hydrophilic domain is thought to be essential for regulation of activity, because its elimination by procedures (15) or 50 nM calyculin A plus 1 μM K252a (CA + K252a, n = 2) was included during the last 10 min of the preincubation period. B, CK138 cells were treated as described in A for the CK1.4 cells (n = 4 for each trace).

**RESULTS**

**Calyculin A Inhibits Ca²⁺ Influx by Na⁺/Ca⁺⁺ Exchange**—To measure Na⁺/Ca⁺⁺ exchange activity, CK1.4 cells were pretreated with ouabain for 30 min in Na-PSS to elevate the cytosolic Na⁺ concentration and then placed in a low [Na⁺] medium (40/100 Na/K-PSS) containing 1 mM 4CaCl₂. As shown in Fig. 1A, 4Ca⁺⁺ uptake was diminished by 55–75% when the cells were treated with 50 mM calyculin A for the final 10 min of the preincubation period. A 5–10-min exposure to calyculin A was necessary to elicit this inhibitory effect. As previously reported (16), calyculin A did not inhibit 4Ca⁺⁺ uptake when added to the assay medium without preincubation, and it had no effect on 4Ca⁺⁺ uptake in cardiac sarcolemmal vesicles (data not shown). Thus, calyculin A does not directly act on the Na⁺/Ca⁺⁺ exchanger but presumably modulates its activity through a phosphorylation-dependent mechanism.

The nonselective protein kinase inhibitor K252a (1 μM) offered partial protection against the inhibitory effects of calyculin A when included in the preincubation medium (Fig. 1). In the absence of calyculin, K252a had no effect on 4Ca⁺⁺ uptake (data not shown). A related broad spectrum protein kinase inhibitor, staurosporine (1 μM), also protected against inhibition by calyculin A (data not shown). Other protein kinase inhibitors were tested but were found not to protect against the effects of calyculin. These included 1 μM KN-62 (calmodulin-dependent protein kinase), 200 μM genistein (tyrosine kinases), 0.5 μM calphostin C (protein kinase C), 10 μM H-89 (protein kinase A), and another relatively nonselective inhibitor, 50 μM H-7. When calyculin-treated cells were subsequently incubated in a calyculin-free medium for an additional 10 min, the rates of 4Ca⁺⁺ uptake continued to decline. Including K252a in the calyculin-free medium enhanced recovery, but the rates of 4Ca⁺⁺ uptake remained less than 40% of untreated controls (data not shown).

The Na⁺/Ca⁺⁺ exchange protein contains 11 transmembrane segments with a cytosolically disposed hydrophilic domain of 520 amino acids residing between the 5th and 6th transmembrane segments (1–3). The hydrophilic domain is thought to be essential for regulation of activity, because its elimination by chymotrypsin treatment or site-directed mutagenesis abrogates normal regulatory behavior (19). CHO cells expressing a mutant exchanger in which 440 residues from this domain have been deleted (CK138 cells) were treated with calyculin A and assayed for 4Ca⁺⁺ uptake by reverse Na⁺/Ca⁺⁺ exchange. As shown in Fig. 1B, calyculin A inhibited 4Ca⁺⁺ uptake by 40–65% in these cells. As with the CK1.4 cells, inhibition by calyculin A was partially alleviated by K252a. We conclude that the effects of calyculin A are not mediated by phosphorylation of residues within the deleted segment of this mutant. Although phosphorylation of one or more of the residual serine or threonine residues remains a possible mechanism for these

**Fig. 1.** Inhibition of exchange-mediated 4Ca⁺⁺ uptake by calyculin A. A, CK1.4 cells were pretreated with ouabain (30 min) and then assayed for 4Ca⁺⁺ uptake as described under “Experimental Procedures” (control, n = 10). For the other traces, 50 nM calyculin A (CA, n = 5) or 50 nM calyculin A plus 1 μM K252a (CA + K252a, n = 2) was included during the last 10 min of the preincubation period. B, CK138 cells were treated as described in A for the CK1.4 cells (n = 4 for each trace).
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![Graph](image)

**Fig. 2.** Concentration dependence of inhibition of \(^{45}\)Ca\(^{2+}\) uptake by calyculin A. CK1.4 cells were pretreated with ouabain for 30 min. The indicated concentrations of calyculin A were added to the medium for the final 10 min of the preincubation period. The cells were then assayed for \(^{45}\)Ca\(^{2+}\) uptake (15 s) in 40/100 Na/K-PSS as described under “Experimental Procedures” (n = 5–6). The results are expressed as the percentage of \(^{45}\)Ca\(^{2+}\) uptake for calyculin-free controls.

effects, this seems implausible due to the absence of normal regulatory behavior in this mutant.

The concentration dependence for inhibition of the initial rate of \(^{45}\)Ca\(^{2+}\) uptake by calyculin A is shown in Fig. 2. Maximal inhibition (64 ± 4%) was observed at 100 nM, and the half-maximally effective concentration was approximately 15 nM. Longer incubation periods with 100 nM calyculin did not increase the degree of inhibition.

**Exchange-mediated Ba\(^{2+}\) Uptake**—\(^{45}\)Ca\(^{2+}\) entering the cells by reverse Na\(^+\)/Ca\(^{2+}\) exchange is accumulated by intracellular organelles, especially mitochondria. Thus, the inhibition of \(^{45}\)Ca\(^{2+}\) uptake observed with calyculin A might have involved a reduction in organelar Ca\(^{2+}\) sequestration rather than an inhibition of Na\(^+\)/Ca\(^{2+}\) exchange activity. To examine this possibility, we measured the exchange-mediated influx of Ba\(^{2+}\) in fura-2-loaded cells treated with 2 \(\mu\)g/ml gramicidin as described in Ref. 21. The presence of gramicidin, a channel-forming monovalent cation ionophore, eliminates transmembrane Na\(^+\) and K\(^+\) gradients and provides a continuous supply of cytosolic Na\(^+\) for the activation of exchange activity (21). As shown in Fig. 3, treatment of CK1.4 cells or CK138 cells for 5 min with 100 nM calyculin A markedly inhibited the initial rate of Ba\(^{2+}\) influx in both cell types. Because Ba\(^{2+}\) is not accumulated by the endoplasmic reticulum in these cells and does not appear to be extensively accumulated by mitochondria (21), we conclude that the effects of calyculin A reflect the inhibition of exchange activity itself rather than an impairment of organelar Ca\(^{2+}\) sequestration.

The residual rate of Ba\(^{2+}\) influx in the calyculin-treated CK138 cells was greater (p < 0.025) than for the calyculin-treated CK1.4 cells (Fig. 3C). Moreover, the degree of inhibition by calyculin A for the CK138 cells (44 ± 4.6%) was significantly less than for the CK1.4 cells (74 ± 2.4%; p < 10\(^{-5}\)). Thus, the effects of calyculin A are less pronounced for the mutant than for the wild-type exchanger. A similar pattern was evident in the \(^{45}\)Ca\(^{2+}\) uptake studies (Fig. 1). We conclude that the hydrophilic domain of the exchanger mediates a portion of the inhibitory effects of protein phosphatase blockade but that substantial inhibition was still observed even in the absence of most of the hydrophilic domain.

When CK1.4 cells were treated with okadaic acid, a protein phosphatase inhibitor with greater potency toward PP-2A than PP-1, we found that exchange-mediated Ba\(^{2+}\) influx was nearly completely blocked (87 ± 2.3% inhibition of initial rate) (Fig. 4, A and C). Incubations of 60 min with okadaic acid were required to observe this inhibition, consistent with a slow entry of this inhibitor into cells (24, 25). Under identical conditions, okadaic acid methyl ester, an inactive analog of okadaic acid, had no effect on Ba\(^{2+}\) influx (Fig. 4D). The okadaic acid treatment inhibited the initial rate of Ba\(^{2+}\) influx in CK138 cells by 65 ± 3.4% (Fig. 4B). Although the percentage of inhibition was less than for the CK1.4 cells, the rates of Ba\(^{2+}\) uptake after okadaic acid treatment for both cell types were similar to those seen for passive Ba\(^{2+}\) leakage into the cell in the absence of exchange activity (21). Thus, the values given must be seen as lower limits for the percentage of inhibition of exchange activity by okadaic acid. Tautomycin (1 \(\mu\)M), a third protein phosphatase inhibitor with reduced affinity for PP-2A compared with PP-1, had no effect on Ba\(^{2+}\) influx after either a 5- or a 60-min preincubation (Fig. 4D). The results suggest that the effects of calyculin A and okadaic acid are mediated by blockade of protein phosphatase 2A (see under “Discussion”).

Other experiments demonstrated that K252a, as in the experiments with calyculin A, partially protected against inhibition of Ba\(^{2+}\) influx by okadaic acid (data not shown). We were unable to mimic the effects of the protein phosphatase inhibitors on Ba\(^{2+}\) influx by incubating the cells with phorbol 12-myristate 13-acetate (10 \(\mu\)M, 5 min), dibutyryl cAMP (1 mM, 5 or 60 min) or 8-bromo-cGMP (1 mM, 5 or 60 min).

To determine whether the inhibition of Ba\(^{2+}\) entry could be overcome by increasing the cytosolic Na\(^+\) concentration, we carried out the experiment shown in Fig. 5. CK1.4 cells were preincubated for 5 min with 2 \(\mu\)g/ml gramicidin in either Na-PSS or 28/112 Na/K-PSS, with or without 100 nM calyculin A. Under these preincubation conditions, the cytosol was loaded with either a high concentration (140 mM) or a low concentration (28 mM) of Na\(^+\) due to the presence of the monovalent cation ionophore gramicidin; the K\(_{m}\) for activation of exchange activity by cytosolic Na\(^+\) is 18–28 mM (19, 26). The cells were then diluted into K-PSS, and 1 mM Ba\(^{2+}\) was added after 30 s. As expected, the cells with high cytosolic Na\(^+\) (Fig. 5A) showed a greater rate and extent of Ba\(^{2+}\) uptake than cells with low cytosolic Na\(^+\) (Fig. 5B). Calyculin A treatment inhibited the initial rates of Ba\(^{2+}\) influx by 57 ± 3.6 \textit{versus} 68 ± 3.3% at 140 and 28 mM Na\(^+\), respectively (Fig. 5C). The difference in the two values (p < 0.05) is quite small and suggests that calyculin A did not markedly affect the affinity of the exchanger for Na\(^+\). In other experiments (data not shown), Ba\(^{2+}\) influx was inhibited to the same extent by calyculin A at Ba\(^{2+}\) concentrations of 1 mM and 5 mM; the K\(_{m}\) for external Ba\(^{2+}\) uptake by Na\(^+\)/Ca\(^{2+}\) exchange in these cells is 3 mM (21). The results indicate that calyculin A inhibition is characterized by a reduction in the V\(_{max}\) for exchange activity, rather than changes in the affinity of the exchanger for the transported ions.

**Protein Phosphatase Inhibitors and Ca\(^{2+}\) Efflux**—We expected the protein phosphatase inhibitors to reduce the Ca\(^{2+}\) efflux mode of exchange activity to a similar extent as for the Ca\(^{2+}\) influx mode. Surprisingly, this was not the case. We adopted three different protocols to assess Ca\(^{2+}\) efflux mediated by Na\(^+\)/Ca\(^{2+}\) exchange. In the first approach, ouabain-treated CK1.4 cells were loaded with \(^{45}\)Ca\(^{2+}\) by a 10 min incubation in 40/100 Na/K-PSS containing 1 mM \(^{45}\)CaCl\(_2\), with or without 100 nM calyculin A. The medium was then replaced with either NMDG-PSS or Na-PSS, and the rate of loss of \(^{45}\)Ca\(^{2+}\) from the cells was monitored. As shown in Fig. 6, \(^{45}\)Ca\(^{2+}\) efflux was more rapid in Na-PSS than in NMDG-PSS, presumably reflecting the acceleration of Ca\(^{2+}\) efflux by Na\(^+\)/Ca\(^{2+}\) exchange. Remarkably, calyculin A had no effect on Na\(^+\)/Ca\(^{2+}\) exchange. In other experiments (data not shown), \(^{45}\)Ca\(^{2+}\) efflux was inhibited to the same extent by calyculin A at Ba\(^{2+}\) concentrations of 1 mM and 5 mM; the K\(_{m}\) for external Ba\(^{2+}\) uptake by Na\(^+\)/Ca\(^{2+}\) exchange in these cells is 3 mM (21). The results indicate that calyculin A inhibition is characterized by a reduction in the V\(_{max}\) for exchange activity, rather than changes in the affinity of the exchanger for the transported ions.
dependent $^{45}\text{Ca}^{2+}$ efflux. In other experiments, the cells were treated with calyculin A in Na-PSS for 10 min before beginning the 10 min $^{45}\text{Ca}^{2+}$ loading period in 40/100 Na/K-PSS. Although the initial level of $^{45}\text{Ca}^{2+}$ uptake was lower under these conditions than in Fig. 6, the results were essentially the same; i.e. the presence of Na$^+$ stimulated $^{45}\text{Ca}^{2+}$ efflux, indicating that exchange activity had not been blocked by the calyculin A treatment (data not shown).
Another approach to measuring Ca\(^{2+}\) efflux was to assess the effect of extracellular Na\(^+\) on the [Ca\(^{2+}\) transient elicited by the Ca\(^{2+}\) ionophore ionomycin, which releases Ca\(^{2+}\) from intracellular stores. For the data shown in Fig. 7, A and B, ionomycin (2 \(\mu\)M) was added to suspensions of CK1.4 cells that had been preincubated for 5 min with or without 100 nM calyculin A. The suspension medium was either Na-PSS + 0.3 mM EGTA or K-PSS + 0.3 mM EGTA, as indicated in the figures. As shown for untreated cells in Fig. 7A, the [Ca\(^{2+}\)] transient was reduced in amplitude and shorter in duration in Na-PSS compared with K-PSS, a result that reflects the activity of the Na\(^+\)/Ca\(^{2+}\) exchanger in mediating Ca\(^{2+}\) efflux (22, 23). Half-times for the decline in [Ca\(^{2+}\)], from the peak value were 8.5 and 16 s in Na- and K-PSS, respectively, as determined from first-order plots of the data (see legend to Fig. 7 for details.)

With calyculin-treated cells (Fig. 7B), the half-times were 13 and 26 s in Na- and K-PSS, respectively, indicating that calyculin A prolonged the [Ca\(^{2+}\)] transient in both Na- and K-PSS. The reasons for this behavior are not known. As discussed in detail elsewhere (23), the time course of the Ca\(^{2+}\) transient reflects a complex interplay between the Ca\(^{2+}\) content of intracellular stores, rates of Ca\(^{2+}\) efflux from the cell, and the time dependence of Ca\(^{2+}\) release from internal stores, which occurs over several tens of seconds in these cells. Calyculin treatment could have affected any of these contributing processes. We assumed that the rate constant for the decline in [Ca\(^{2+}\)], in Na-PSS was the sum of the Na-dependent component (due to Na\(^+\)/Ca\(^{2+}\) exchange) and the Na-independent component, as measured in K-PSS. Subtracting the rate constants in K-PSS from those obtained in Na-PSS (cf. legend to Fig. 7), the Na\(^-\) dependent component was found to be reduced by 37% in the calyculin-treated cells. Although this value suggests that exchange activity may have been inhibited by the calyculin A treatment, the results with okadaic acid do not support such a conclusion, as discussed below.

The ionomycin-induced [Ca\(^{2+}\)] transients for untreated cells and cells incubated for 60 min with 1 \(\mu\)M okadaic acid are shown in Fig. 7, C and D. The half-times for the decline in [Ca\(^{2+}\)], in untreated cells (Fig. 7C) were 14 and 18 s in Na- and K-PSS, respectively. With the batch of cells used for this experiment, the effect of Na\(^+\) was somewhat reduced compared with the results described for the cells in Fig. 7A. For cells treated with okadaic acid (Fig. 7D), the corresponding half-times were 26 and 46 s. In this case, the Na\(^-\) dependent component was not reduced but increased by 16% following the okadaic acid treatment, suggesting that there was no impairment of exchange activity.

This analysis must be viewed cautiously because the Na-dependent component of the decline in [Ca\(^{2+}\)], provides only a rough estimate of exchange activity. As discussed elsewhere (23), ionomycin-induced Ca\(^{2+}\) release is not instantaneous in these cells but appears to continue at a declining rate over several tens of seconds. Thus, the duration of the [Ca\(^{2+}\)] transient will be determined to a significant degree by the underlying rate of Ca\(^{2+}\) release. Both calyculin A and okadaic acid induce major cytoskeletal alterations (cf. “Discussion”), which could influence the quantity of stored Ca\(^{2+}\) and/or the rate of Ca\(^{2+}\) release. These factors make it difficult to quantitate exchange activity with certainty in these experiments. Nevertheless, the results clearly indicate that the exchanger remains capable of carrying out Ca\(^{2+}\) efflux following treatment with the phosphatase inhibitors. The results with okadaic acid are especially noteworthy because this agent almost completely abrogated exchange-mediated Ba\(^{2+}\) influx (cf. Fig. 4) but did not appear to markedly impair Ca\(^{2+}\) efflux.

A final index of exchange-mediated Ca\(^{2+}\) efflux is shown in Fig. 8. In this experiment, CK1.4 cells were pretreated for 10 min, with or without 100 nM calyculin A, in the presence of ionomycin to deplete internal stores. The cells were then added to cuvettes containing either Na-PSS or K-PSS (final ionomycin concentration, 1.3 \(\mu\)M), and the rate of Ca\(^{2+}\) entry upon addition of 1 mM CaCl\(_2\) was monitored. In these experiments, Ca\(^{2+}\) entry occurs both through the ionophoretic behavior of ionomycin and through store-dependent Ca\(^{2+}\) entry channels. For the CK1.4 cells in K-PSS, reverse Na\(^+\)/Ca\(^{2+}\) exchange also contributes to Ca\(^{2+}\) entry (22). As shown in Fig. 8, the initial rate of

Fig. 6. \(^{45}\)Ca\(^{2+}\) efflux from CK1.4 cells: effects of calyculin A. Cells were assayed for \(^{45}\)Ca\(^{2+}\) efflux in Na- or NMDG-PSS as described under “Experimental Procedures.” For calyculin-treated cells (filled symbols), 100 nM calyculin A was included in the medium during the 10-min \(^{45}\)Ca\(^{2+}\) uptake period (n = 5–6).

Fig. 7. Ionomycin-induced Ca\(^{2+}\) release in Na- or K-PSS following treatment with either calyculin A or okadaic acid. Fura-2-loaded CK1.4 cells were preincubated in Na-PSS + 1 mM CaCl\(_2\) for 5 min with (B) or without (A) 100 nM calyculin A (n = 6). In separate experiments with a different batch of CK1.4 cells, cells were preincubated for 60 min with (D) or without (C) 1 \(\mu\)M okadaic acid (n = 5). The cells were added to fluorescence cuvettes containing 3 ml of Na- or K-PSS as indicated; ionomycin (2 \(\mu\)M) was added to initiate Ca\(^{2+}\) release from internal stores at 30 s (arrow). See text for further details. First order plots were constructed for the decline in [Ca\(^{2+}\)] from the peak of the transient (not shown), and the slopes of the initial portions of these plots yielded the following rate constants for the data in Na- and K-PSS, respectively: A, 0.0818 and 0.0423 s\(^{-1}\); B, 0.0517 and 0.0268 s\(^{-1}\); C, 0.0491 and 0.0390 s\(^{-1}\); D, 0.0269 and 0.0151 s\(^{-1}\).
Compared with K-PSS, whereas the amplitude of the rise in Ca\(^{2+}\) in untreated cells, probably because calyculin A-treatment inhibits the initial rate of Ca\(^{2+}\) influx by nearly 100% in control cells. Thus, the response of protein phosphatases to inhibitors in intact cells is often slow and incomplete, and this behavior complicates the interpretation of results.

In the present study, calyculin A inhibited “reverse-mode” exchange activity by 60–75% after incubation periods of 5–10 min, and okadaic acid inhibited Ba\(^{2+}\) influx by nearly 100% after incubation periods of 60 min. The relative effectiveness of calyculin A and okadaic acid is similar to that reported by Favre et al. (27) for PP-2A inhibition, and we therefore suggest that the effects of these inhibitors are probably mediated by blockade of PP-2A. We also tested tautomycin (1 \(\mu\)M), which has a reduced affinity for PP-2A (30 nM) compared with PP-1 (0.5 nM) (25), and did not observe inhibition of exchange activity (Fig. 4C). The significance of this finding is uncertain, however, because different cell types vary greatly in their response to tautomycin. In the experiments of Favre et al. (27), for example, exposure to 10 \(\mu\)M tautomycin for more than 2 h was required for 50% inhibition of PP-1 activity. On the other hand, studies in our laboratory with L6 cells revealed significant effects of 1 \(\mu\)M tautomycin within 5–10 min of incubation. 2

The effects of the phosphatase inhibitors probably result from hyperphosphorylation of cellular proteins, because protein kinase inhibitors such as K252a (Fig. 1) and staurosporine partially protected against the effects of calyculin A and okadaic acid. More selective inhibitors of protein kinase A, protein kinase C, or calmodulin-dependent protein kinase did not influence the effects of calyculin A. Moreover, we were unable to mimic the effects of the protein phosphatase inhibitors by incubating the cells with agents known to activate protein kinases A, C, and G. Thus, the protein kinase(s) responsible for the phosphorylation-dependent inhibition of exchange activity remain unidentified.

Direct phosphorylation of the exchanger itself is probably not involved in these effects, although this possibility cannot be completely eliminated. Previously published immunoprecipitation experiments with \(^{38}\text{P}\)-labeled CK1.4 cells failed to disclose detectable amounts of phosphorylated exchanger, even in calyculin-treated cells (16). (Exchanger phosphorylation has been detected in other cell types, however (11, 12).) Moreover, cells expressing a deletion mutant (CK138 cells), which is missing

2 B. M. Hantash, unpublished observations.
mush of the central hydrophilic domain of the exchanger, remained susceptible to the effects of protein phosphatase blockade (Figs. 1, 3, and 4). Many residual serines and threonines remain in this mutant, including a potential casein kinase 2 phosphorylation site at 720-SAGEDDDD. However, it is unlikely that the deletion mutant could mediate a regulatory response to putative phosphorylation, because previous studies have demonstrated that all well characterized modes of exchanger regulation are absent in this mutant (19). Calyculin A and okadaic acid were somewhat less effective in blocking Ba$^{2+}$ influx in the mutant than the wild-type exchanger (Figs. 1 and 3). These results suggest that the hydrophilic domain mediates a portion of the inhibitory effect of these agents on exchange activity. Although it is possible, but unlikely, that this portion of the response is due to exchanger phosphorylation, other aspects of exchanger function mediated by the hydrophilic domain, e.g. possible interactions with cytoskeletal proteins (16), could equally well be involved.

It is remarkable that exchange-mediated Ca$^{2+}$ efflux activity appeared to be resistant to the effects of protein phosphatase blockade (Figs. 6–8). Na$_{	ext{i}}$-dependent 4$^{2+}$Ca$^{2+}$ efflux was unaffected by calyculin A treatment (Fig. 6). Na$^{+}$ also remained effective in attenuating the ionomycin-induced increase in [Ca$^{2+}$], in cells treated with calyculin A or okadaic acid (Fig. 7). Although the results with calyculin A were consistent with partial inhibition of exchange-mediated Ca$^{2+}$ influx, the results with okadaic acid were not. The okadaic acid data are particularly noteworthy because this agent blocked exchange-mediated Ca$^{2+}$ influx nearly completely (Fig. 4, A and C). Finally, the presence of extracellular Na$^{+}$ blunted the rise in [Ca$^{2+}$], produced by the addition of extracellular Ca$^{2+}$ in ionomycin-treated CK1.4 cells, whether or not the cells had been pretreated with calyculin A (Fig. 8). The results of each of these approaches are consistent with the conclusion that the protein phosphatase blockade did not inhibit exchange activity operating in the Ca$^{2+}$ influx mode.

The selective inhibition of the reverse mode of exchange activity did not appear to involve changes in the affinity for the transported ions, or alterations in normal regulatory behavior. Thus, the inhibition by calyculin A was nearly the same at concentrations of cytosolic Na$^{+}$ (Fig. 5) or external Ba$^{2+}$ (data not shown) that were above the $K_m$ value compared to concentrations at or below the $K_m$ value. Moreover, the CK138 cells, which express an unregulated exchanger (19), remain susceptible to inhibition by protein phosphatase blockade, albeit with reduced sensitivity compared with the CK1.4 cells (Figs. 1, 3, and 4). Thus, an explanation for the results in terms of the conventional regulatory or kinetic properties of the exchanger remains elusive. Recently, the novel exchange antagonist KB-R7943 was shown to be considerably more potent in inhibiting inhibition of protein phosphatase activity (32–35). These cytoskeletal changes might influence exchanger location or its interactions with associated proteins, thereby inhibiting its activity. Preliminary microscopic observations using fluorescently labeled probes or antibodies have verified that CK1.4 cells exposed to calyculin A exhibit extensive disruption of intermediate filaments, microtubules and stress fibers (data not shown). The relationship, if any, between these cytoskeletal alterations and exchange activity is unclear. Treatment of CK1.4 cells with nocodazole and/or cytochalasin D, agents that disrupt microtubules and F-actin filaments, respectively, did not inhibit exchange activity in preliminary experiments (data not shown). Disruption of the intermediate filament system could conceivably affect exchange activity, because the cytoskeletal protein ankyrin interacts with both intermediate filaments (36, 37) and the Na$^{+}$/Ca$^{2+}$ exchanger (38). This suggestion is entirely speculative, however. Protein phosphatase inhibitors often cause extensive vacuolization and membrane internalization (39, 40), and this process could lead to loss of exchanger protein from the external membrane surface. However, this possibility is difficult to reconcile with the Ca$^{2+}$ influx results, which suggest little or no impairment of exchanger function. Moreover, wholesale internalization of plasma membrane transporters seems unlikely because calyculin A treatment did not inhibit Na$^{+}$/H$^{+}$ exchange or bumetanide-sensitive $^{86}$Rb$^{+}$ uptake activities (data not shown). At present, therefore, it is uncertain whether the extensive cytoskeletal alterations produced during protein phosphatase blockade are an important factor in the inhibition of exchange activity.

A final consideration involves the effects of the protein phosphatase inhibitors on other Ca$^{2+}$ influx mechanisms. Calyculin A and okadaic acid strongly inhibit store-dependent Ca$^{2+}$ entry in several different cell types (41–45), although there have also been reports that okadaic acid stimulates store-dependent Ca$^{2+}$ entry under some conditions (46, 47). We have found that calyculin A reduces store-dependent entry of Ca$^{2+}$, Ba$^{2+}$, and Mn$^{2+}$ in CHO cells. We have also found that calyculin A and okadaic acid treatments block Ba$^{2+}$ entry through i-type Ca$^{2+}$ channels in the L6 rat skeletal muscle cell line. These findings raise the possibility that protein phosphatase blockade elicits a general inhibition of divalent cation entry, perhaps involving cytoskeletal changes as a common mechanistic element. Studies examining this possibility are currently in progress.

In summary, the results have revealed a profound inhibition of the Ca$^{2+}$ influx mode of Na$^{+}$/Ca$^{2+}$ exchange activity during protein phosphatase blockade. Surprisingly, the Ca$^{2+}$ influx mode of exchange activity appeared to be relatively unaffected. The mechanism involved is unclear, but it probably does not involve phosphorylation of the exchanger itself. The selective inhibition of Ca$^{2+}$ influx could involve an intrinsic difference in the susceptibility of the two modes of exchange activity to protein hyperphosphorylation or to cytoskeletal changes, or possibly a more general mechanism that suppresses divalent cation entry.

REFERENCES

1. Hryshko, L. V., and Philipson, K. D. (1997) Basic. Res. Cardiol. 92, Suppl. 1, 45–51.
2. Reeves, J. P. (1998) J. Bioenerg. Biomembr. 30, 151–160.
3. Blauwstein, M. P., and Lederer, W. J. (1999) Physiol. Rev. 79, 763–854.
4. Matsuoka, S., Nicoll, D. A., Hryshko, L. V., Levitsky, D. O., Weiss, J. N., and Philipson, K. D. (1995) J. Gen. Physiol. 105, 403–420.
5. Levitsky, D. O., Nicoll, D. A., and Philipson, K. D. (1994) J. Biol. Chem. 269, 22847–22852.
6. DiPolo, R. (1979) J. Gen. Physiol. 73, 91–113.
7. Hilgemann, D. W., and Ball, R. (1996) Science 273, 956–959.
8. Hilgemann, D. W. (1997) Annu. Rev. Physiol. 59, 193–220.

M. Condrescu, B. M. Hantash, Y. Fang, and J. P. Reeves, unpubl. observations.
