Acceleration of Sodium-Calcium Exchange Activity during ATP-induced Calcium Release in Transfected Chinese Hamster Ovary Cells

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ABSTRACT The P2U purinergic agonist ATP (0.3 mM) elicited an increase in \([Ca^{2+}]_i\), due to \(Ca^{2+}\), release from intracellular stores in transfected Chinese hamster ovary cells that express the bovine cardiac \(Na^+/Ca^{2+}\) exchanger (CK1.4 cells). The following observations indicate that ATP-evoked \(Ca^{2+}\) release was accompanied by a \(Ca^{2+}\)-dependent regulatory activation of \(Na^+/Ca^{2+}\) exchange activity: Addition of extracellular \(Ca^{2+}\) (0.7 mM) 0–1 min after ATP evoked a dramatic rise in \([Ca^{2+}]_i\), in \(Na^+\)-free media (\(Li^+\) substitution) compared to \(Na^+\)-containing media; no differences between \(Na^+\)- and \(Li^+\)-based media were observed with vector-transfected cells. In the presence of physiological concentrations of extracellular \(Na^+\) and \(Ca^{2+}\), the ATP-evoked rise in \([Ca^{2+}]_i\), declined more rapidly in CK1.4 cells compared to control cells, but then attained a long-lived plateau of elevated \([Ca^{2+}]_i\), which eventually came to exceed the declining \([Ca^{2+}]_i\), values in control cells. ATP elicited a transient acceleration of exchange-mediated \(Ba^{2+}\) influx, consistent with regulatory activation of the \(Na^+/Ca^{2+}\) exchanger. The acceleration of \(Ba^{2+}\) influx was not observed in vector-transfected control cells, or in CK1.4 cells in the absence of intracellular \(Na^+\) or when the \(Ca^{2+}\) content of the intracellular stores had been reduced by prior treatment with ionomycin. The protein kinase C activator phorbol 12-myristate 13-acetate attenuated the exchange-mediated rise in \([Ca^{2+}]_i\), under \(Na^+\)-free conditions, but did not inhibit the ATP-evoked stimulation of \(Ba^{2+}\) influx. The effects of PMA are therefore not due to inhibition of exchange activity, but probably reflect the influence of protein kinase C on other \(Ca^{2+}\) homeostatic mechanisms. We conclude that exchange activity is accelerated during ATP-evoked \(Ca^{2+}\) release from intracellular stores through regulatory activation by increased \([Ca^{2+}]_i\). In the absence of extracellular \(Ca^{2+}\), the stimulation of exchange activity is short-lived and follows the time course of the \([Ca^{2+}]_i\), transient; in the presence of extracellular \(Ca^{2+}\), we suggest that the exchanger remains activated for a longer period of time, thereby stabilizing and prolonging the plateau phase of store-dependent \(Ca^{2+}\) entry.

KEY WORDS: fura-2 • endoplasmic reticulum • \(Ba\) uptake • protein kinase C • \(Na/Ca\) exchange

INTRODUCTION

In Chinese hamster ovary (CHO) cells, the \(P2U\) purinergic agonist ATP elicits the production of 1,4,5-inositol trisphosphate (InsP3) and the release of \(Ca^{2+}\) from InsP3-sensitive stores (Iredale and Hill, 1993; Pijuan et al., 1993). \(Ca^{2+}\) influx is accelerated after \(Ca^{2+}\) release from internal stores, and this leads to an extended period of gradually declining \([Ca^{2+}]_i\), after the peak of the transient as intracellular stores refill and \(Ca^{2+}\) influx subsides. The \(Ca^{2+}\) influx pathway in many cells involves low conductance \(Ca^{2+}\) channels (store-operated channels) that are activated by organellar \(Ca^{2+}\) release (Hoth and Penner, 1993). The mechanism of activation is not well understood but may involve a low molecular weight diffusible second messenger (Parekh et al., 1993; Randriamampita and Tsien, 1993). The \(Ca^{2+}\) influx pathway has been termed capacitative \(Ca^{2+}\) entry (Putney, 1990) or store-dependent \(Ca^{2+}\) influx (Clementi et al., 1992).

Recent work in our laboratory has been directed toward understanding the regulation of the cardiac \(Na^+/Ca^{2+}\) exchange system and its interactions with other \(Ca^{2+}\) homeostatic mechanisms. The experimental approach we have used involves examining the effects of \(Na^+\) on \(Ca^{2+}\) mobilization in transfected CHO cells permanently expressing the bovine cardiac \(Na^+/Ca^{2+}\) exchanger (CK1.4 cells; Pijuan et al., 1993). Since non-transfected (or vector-transfected) CHO cells do not normally express \(Na^+/Ca^{2+}\) exchange activity, comparing the behavior of CK1.4 cells with vector-transfected cells provides a means of identifying functional roles of exchange activity in relation to other \(Ca^{2+}\) homeostatic processes (Chernaya et al., 1996).

In the accompanying manuscript (Condrescu et al., 1997), we described the advantages of using \(Ba^{2+}\) as a surrogate for \(Ca^{2+}\) in studies of exchanger regulation. We concluded that \(Ba^{2+}\) is transported by the ex-
changer but is not significantly accumulated by intracellular organelles and that the exchanger becomes deactivated as [Ca$^{2+}$], is lowered by the Ca$^{2+}$ sequestering activities of intracellular organelles. We had previously presented indirect evidence, based on fura-2 measurements of cytosolic Ca$^{2+}$ ([Ca$^{2+}$]), that exchange activity was accelerated during ATP-evoked Ca$^{2+}$ release in CK1.4 cells (Chernaya et al., 1996); however, in that study the various contributions of exchange activity, Ca$^{2+}$ channel activity and changes in organellar Ca$^{2+}$ sequestration to the [Ca$^{2+}$], response could not be clearly defined. In the present report, we show that ATP elicits a transient increase in exchange-mediated Ba$^{2+}$ influx and that this acceleration of exchange activity is dependent upon an increase in [Ca$^{2+}$]. Other results provide additional information on the ATP-evoked Ca$^{2+}$ movements and suggest that activation of the exchanger stabilizes and prolongs the plateau phase of store-dependent Ca$^{2+}$ entry.

**METHODS**

The cells, materials, solutions, and procedures used are essentially identical to those described in the accompanying report (Condrescu et al., 1997). Calibration of the fura-2 signal in representative cell preparations for Ca$^{2+}$ at 340 and 380 nm excitation as described (Condrescu et al., 1997) yielded S/S$_{0} = 4.8$, R$_{min} = 0.9$, R$_{max} = 12$. Assuming $K_{p} = 224$ nM for the Ca$^{2+}$/fura-2 complex (Grynkiewicz et al., 1985), the following ratios correspond to the indicated values for [Ca$^{2+}$]: 1.5 (60 nM), 2.0 (120 nM), 3.0 (250 nM), 4.0 (420 nM), and 5.0 (630 nM). Calibrations for Ba$^{2+}$ and for Ca$^{2+}$ at the 350/390 excitation wavelengths used for the Ba$^{2+}$ influx studies were reported in Condrescu et al. (1997).

**RESULTS**

**Effect of Na$^{+}$ on ATP-induced Ca$^{2+}$ Influx**

The expression of the Na$^{+}$/Ca$^{2+}$ exchanger in the transfected CHO cells profoundly affects their response to extracellular ATP. The data in Fig. 1 depict the changes in [Ca$^{2+}$], when ATP and Ca$^{2+}$ are added to CK1.4 cells in either Na–physiological salts solution (PSS) or Li-PSS; traces are shown for the simultaneous addition of ATP and Ca$^{2+}$ (Fig. 1 A), the addition of Ca$^{2+}$ 1, 2 or 3 min after ATP (Fig. 1 B–D), or the addition of Ca$^{2+}$ alone (E). As shown in Fig. 1, B, C, and D, the presence of Na$^{+}$ accelerates the decline in the [Ca$^{2+}$], transient elicited by ATP under Ca$^{2+}$-free conditions, as reported previously (Chernaya et al., 1996). Upon addition of Ca$^{2+}$, the rise in [Ca$^{2+}$], was higher in Li-PSS than in Na-PSS, but the relative increase in Li-PSS was most pronounced when Ca$^{2+}$ was added either simultaneously with ATP (cf. Chernaya et al., 1996) or within 1 min after ATP. When Ca$^{2+}$ was added 2 or 3 min after ATP, the changes in [Ca$^{2+}$], in both media were similar to that observed upon the addition of Ca$^{2+}$ alone.

![Figure 1. Effect of ATP on [Ca$^{2+}$], in CK1.4 cells.](image)

In contrast to this behavior, control-transfected cells showed no increase in [Ca$^{2+}$], in Li-PSS compared to Na-PSS; indeed, the changes in [Ca$^{2+}$], tended to be somewhat reduced in Li-PSS (Fig. 2). Note that in Li-PSS, the increase in [Ca$^{2+}$], upon adding Ca$^{2+}$ 1 min after ATP was much greater in CK1.4 cells than in control cells (compare B in Figs. 1 and 2); the average [Ca$^{2+}$], values in Li-PSS 30–60 s after the addition of Ca$^{2+}$ were 221 ± 8 nM (n = 5) for CK1.4 cells and 71 ± 11 nM (n = 3) for control cells.

The responses of control and CK1.4 cells to ATP addition under physiological conditions, i.e., in Na-PSS containing 1 mM CaCl$_{2}$, are directly compared in Fig. 3. The peak increase in [Ca$^{2+}$], upon ATP addition was higher for control cells than for CK1.4 cells. After the peak of the [Ca$^{2+}$], transient in the control cells, [Ca$^{2+}$], gradually declined toward the initial “resting” value (70–75 nM) over the time course of the experiment. With CK1.4 cells, the changes in [Ca$^{2+}$], elicited by ATP followed a markedly different time course: [Ca$^{2+}$], fell quickly after the peak of the transient and...
attained a stable plateau (100 ± 1 nM at 150–200 s; n = 6), which remained elevated compared to the initial \([\text{Ca}^{2+}]_i\) (74 ± 2 nM). The more rapid decline in \([\text{Ca}^{2+}]_i\) in the CK1.4 cells was clearly evident when the results were normalized for differences in the peak height of the transient (Fig. 3, inset). Although reduced in magnitude compared to control cells, the plateau of elevated \([\text{Ca}^{2+}]_i\) in the CK1.4 cells was maintained for a longer period of time and indeed “crossed over” the declining \([\text{Ca}^{2+}]_i\) trace for the control cells at ~170 s in the figure.

**ATP-induced Ba\(^{2+}\) Influx in CK1.4 Cells**

In the experiments depicted in Fig. 4, we examined the effects of extracellular ATP on \(\text{Ba}^{2+}\) influx. In general, ATP accelerated \(\text{Ba}^{2+}\) influx in Li-PSS compared to Na-PSS, and the time dependence of this acceleration was consistent with a transient activation of exchange activity which roughly coincided with the time course of the \([\text{Ca}^{2+}]_i\) transient. The fura-2 traces in these experiments are highly complex because they reflect the combined influence of changes in \([\text{Ca}^{2+}]_i\) and \([\text{Ba}^{2+}]_i\). For example, in the experiment where ATP and \(\text{Ba}^{2+}\) are added simultaneously (Fig. 4A), the contribution of \([\text{Ca}^{2+}]_i\) to the fura-2 signal would initially be expected to predominate because of the release of \(\text{Ca}^{2+}\) from

**Figure 2.** Effect of ATP on \([\text{Ca}^{2+}]_i\) in control cells. Conditions are identical to those described in Fig. 1 except that vector-transfected control CHO cells were used instead of CK1.4 cells. ATP and \(\text{CaCl}_2\) were added either simultaneously (A), or \(\text{CaCl}_2\) was added 60, 120, or 180 s after ATP in B, C, and D, respectively. Fura-2 fluorescence was monitored with alternate excitation at 340 and 380 nm. For all experiments, \(n = 1–2\) (no error bars) except for B, where \(n = 3–4\).

**Figure 3.** Comparison of control and CK1.4 cells: effect of ATP on \([\text{Ca}^{2+}]_i\) in presence of extracellular Na\(^{+}\) and Ca\(^{2+}\). CK1.4 or control transfected cells were loaded with fura-2, preincubated for 1 min in Na-PSS + 1 mM \(\text{CaCl}_2\) and then diluted 30-fold into cuvettes containing Na-PSS + 1 mM \(\text{CaCl}_2\). Fura-2 fluorescence was monitored (340/380 excitation) and ATP (0.3 mM) was added as shown (\(n = 5–6\)).

**Figure 4.** ATP and \(\text{Ba}^{2+}\) influx in CK1.4 cells. CK1.4 cells were loaded with fura-2, preincubated for 1 min in Na-PSS + 1 mM \(\text{CaCl}_2\) and diluted 30-fold into cuvettes containing either Li- or Na-PSS plus 0.3 mM EGTA. Fura-2 fluorescence was monitored with alternate excitation at 350 and 390 nm. ATP (0.3 mM) was added at 30 s in each panel and \(\text{BaCl}_2\) (1 mM) was added at increasing intervals following ATP, as indicated; in A, ATP and \(\text{BaCl}_2\) were added simultaneously (\(n = 4–6\) [A, B]; 3–4 [D, E] and 6–8 [C]).
InsP₃-sensitive stores. The concentration of Ca²⁺ would then decline as Ca²⁺ is removed from the cytosol due to efflux or organellar sequestration. In Na-PSS, this is reflected in a decline in the fura-2 ratio, but in Li-PSS, the fura-2 ratio remains elevated because Ba²⁺ rapidly enters the cell via exchange activity. Two observations lend support to this interpretation: (a) The presence of extracellular or cytosolic Ba²⁺ does not interfere with the decline in [Ca²⁺], after ATP addition (Condrescu et al., 1996). (b) When excess EGTA was added to the cells after Ba²⁺ influx under these conditions, the fura-2 signal did not decline (data not shown), as expected for cytosolic Ba²⁺ (Condrescu et al., 1997); in contrast, a rapid decline would be expected upon EGTA addition if the elevated fura-2 ratio were due to cytosolic Ca²⁺ (Chernaya et al., 1996).

When Ba²⁺ was added 0.5, 1, 2, or 3 min after ATP, the difference between the fura-2 signals in Li-PSS and Na-PSS diminished (Fig. 4 B–E) as the delay between ATP and Ba²⁺ addition increased. We conclude that exchange activity decreased progressively after ATP addition, consistent with the results obtained with Ca²⁺ (Fig. 1) and that the acceleration of exchange activity corresponded closely with the period of elevated [Ca²⁺]. In the absence of ATP, no difference in Ba²⁺ influx between Li-PSS and Na-PSS (Fig. 4 F) could be detected when Ba²⁺ was added at 90 s (compare with Fig. 4 C); this indicates that the exchanger is inactive at this time in the absence of ATP addition. When control-transfected cells were used instead of CK1.4 cells, we observed little or no stimulation of Ba²⁺ influx by ATP and could detect no differences between Li- and Na-PSS (Fig. 5). This result strongly supports our interpretation that Ba²⁺ influx in Li-PSS in the CK1.4 cells is due to exchange activity.

As an additional test of this conclusion, we examined the effects of removing cytosolic Na⁺, a treatment that should block Ba²⁺ influx via the exchange activity. To remove cytosolic Na⁺, the gramicidin-treatment protocol described in the accompanying manuscript (Condrescu et al., 1997) was used (Fig. 6). In this experiment, fura-2-loaded cells were added to a cuvette containing 20/120 Na/K-PSS and gramicidin (1 µM) was added to permeabilize the membrane to monovalent cations. Results for CK1.4 cells are shown in Fig. 6 A. In the absence of ATP addition, the rate of Ba²⁺ influx (trace b) was essentially identical to that shown in Fig. 5 in the accompanying manuscript (Condrescu et al., 1997) for gramicidin-treated cells under very similar conditions ([Na⁺] = 19 mM). However, when ATP was added 60 s before Ba²⁺, the rate of Ba²⁺ influx was dramatically accelerated (trace a) compared to that seen in the absence of ATP addition. Ba²⁺ influx was completely abolished when ATP was added to cells in the absence of Na⁺ (trace c), although the transient due to Ca²⁺ release from InsP₃-sensitive stores was normal. When the same experiment was carried out with vector-transfected control cells (Fig. 6 B), Ba²⁺ influx was essentially the same under all experimental conditions, and was identical to that shown in the absence of Na⁺ (trace e, Fig. 6 A) for the CK1.4 cells.

In the experiments with gramicidin-treated cells, considerable exchange-mediated Ba²⁺ influx was observed even in the absence of ATP addition (trace b; Fig. 6). This appears to reflect stimulation of the exchanger by the increase in [Ca²⁺], brought about by gramicidin through its uncoupling action on mitochondria. A similar increase in [Ca²⁺], was observed with the uncoupler carbonyl cyanide m-chlorophenylhydrazone (2 µM) or with the combination of oligomycin (2.5 µg/ml) plus rotenone (2 µM), and these
agents also accelerated exchange activity (data not shown).

\[[Ca^{2+}]_i\] and Exchange Activity

The effect of ATP on exchange-mediated Ba^{2+} influx is dependent upon the release of Ca^{2+} from internal stores. Thus, as shown in Fig. 7, when intracellular Ca^{2+} stores were depleted by pretreatment with ionomycin and the ionomycin was subsequently removed with BSA (Fig. 7 A), ATP elicited no increase in \[[Ca^{2+}]_i\], and the addition of Ba^{2+} 30 s later revealed no differences in Ba^{2+} influx between Li- and Na-PSS. The data in the

Fig. 7 B are for cells in which the ionomycin treatment was omitted and show the expected increase in Ba^{2+} influx in Li- vs. Na-PSS. Thus, a rise in cytosolic Ca^{2+} appears to be necessary for the activation of exchange activity evoked by extracellular ATP.

Protein Kinase C and Exchange-mediated Ca^{2+} Influx

The protein kinase C activator phorbol 12-myristate 13-acetate (PMA) attenuated the effects of ATP on exchange-mediated Ca^{2+} entry in the CK1.4 cells. The data in Fig. 8 show that Ca^{2+} influx in Li-PSS was re-

\[\text{Figure 6. ATP and Ba}^{2+}\text{ influx in gramicidin-treated cells. CK1.4 (A) or vector-transfected control (B) cells were loaded with fura-2, preincubated for 1 min in Na-PSS + 1 mM CaCl}_2\text{ and diluted 30-fold into cuvettes containing 20/120 Na/K-PSS + 0.3 mM EGTA (traces a and c). For trace c, the cells were preincubated in K-PSS + 1 mM CaCl}_2\text{ and then transferred to cuvettes containing K-PSS + 0.3 mM EGTA. Gramicidin (1\mu M) was added immediately after adding the cells to the cuvette. ATP (0.3 mM final concentration) was added at 30 s for traces a and c and was omitted for trace b; 1 mM BaCl}_2\text{ was added for all traces at 90 s (n = 5).}\]

\[\text{Figure 7. Acceleration of Ba}^{2+}\text{ influx by ATP requires Ca}^{2+}\text{ release. CK1.4 cells were preincubated for 1 min in Na-PSS + 0.3 mM EGTA + 10 \mu M ionomycin (A), or Na-PSS + 1 mM CaCl}_2\text{ (B). The cells in A were centrifuged and then incubated for an additional 1 min in Na-PSS + 0.3 mM EGTA + 1% BSA to scavenge residual ionomycin; after a final centrifugation, the cells were resuspended in either Na- or Li-PSS + 0.3 mM EGTA and ATP (0.3 mM) and BaCl}_2\text{ (1 mM) were added as indicated. The data in B were obtained using the same batch of cells as in A. In this case, however, the ionomycin pretreatment and BSA wash were omitted and the cells were added directly to the cuvette containing Na- or Li-PSS + 0.3 mM EGTA and additions were made as in A. The data in B are reproduced in B of Fig. 4 (n = 4–6).}\]
Calcium Release and Na/Ca Exchange

Reduced in CK1.4 cells treated with 100 nM PMA (trace 2 in each panel); the effect was observed when ATP and Ca$^{2+}$ were added simultaneously (Fig. 8 A) but appeared to be more pronounced in experiments where Ca$^{2+}$ is added 1 min after ATP (Fig. 8 B). To determine whether the effect of PMA is mediated by protein kinase C, cells were treated overnight with 100 nM PMA, a procedure which down-regulates most isoforms of protein kinase C in many different cell types (Jaken et al., 1981). As shown in Fig. 9, overnight treatment with PMA completely blocked the acute effects of PMA on Ca$^{2+}$ influx in response to ATP, consistent with the conclusion that PMA was acting through protein kinase C in the earlier experiments (Fig. 8). Down-regulation of protein kinase C did not appear to enhance exchange-mediated Ca$^{2+}$ influx in comparison to untreated cells (compare traces in Fig. 9 with those in the lower panel of Fig. 8).

The data in Fig. 10 show the effects of PMA on measured exchange-mediated Ba$^{2+}$ influx after ATP addition. Ba$^{2+}$ influx in Li-PSS was the same whether or not PMA was present, suggesting that the effects of PMA on Ca$^{2+}$ influx (Fig. 8) were not brought about by an alteration in exchange activity itself.
ATP interacts with P2U receptors in CHO cells, promoting Ca\(^{2+}\) release from internal stores and an increased influx of extracellular Ca\(^{2+}\) (Iredale and Hill, 1993; Pijuan et al., 1993). The expression of the cardiac Na\(^{+}\)/Ca\(^{2+}\) exchanger in the CK1.4 cells altered the cellular response to added ATP in comparison to control cells. In Ca\(^{2+}\)-free media, the [Ca\(^{2+}\)]\(_i\) transient evoked by ATP declined more rapidly in the presence of extracellular Na\(^{+}\) than in its absence (Fig. 1; see also Chernaya et al., 1996), whereas Na\(^{+}\) had no effect on the [Ca\(^{2+}\)]\(_i\) transient in control cells (Fig. 2). The effects of Na\(^{+}\) in the CK1.4 cells undoubtedly reflect accelerated Ca\(^{2+}\) efflux due to exchange activity. Previously reported experiments (Chernaya et al., 1996) indicate that Na\(^{+}\) accelerates Ca\(^{2+}\) influx two- to threefold in the CK1.4 cells.

In the absence of Na\(^{+}\), influx of Ca\(^{2+}\) or Ba\(^{2+}\) occurs by "reverse mode" exchange, i.e., Na\(^{+}\)-dependent Ca\(^{2+}\) or Ba\(^{2+}\) influx. ATP accelerated Ca\(^{2+}\) influx in an Na\(^{+}\)-free medium, but the relative contributions of store-operated Ca channels and reverse Na\(^{+}\)/Ca\(^{2+}\) exchange to this process could not be estimated. Since Ba\(^{2+}\) does not pass through store-operated channels in these cells (Chernaya et al., 1996), the acceleration of Ba\(^{2+}\) influx after ATP-induced Ca\(^{2+}\) release (Fig. 4) clearly demonstrated that exchange activity had been activated; no acceleration of Ba\(^{2+}\) influx was observed under identical conditions for vector transfected control cells (Fig. 5).

In cells treated with gramicidin to equalize Na\(^{+}\) concentrations across the plasma membrane, Ba\(^{2+}\) influx for the CK1.4 cells was negligible in the absence of Na\(^{+}\) and was virtually absent in control-transfected cells, regardless of the Na\(^{+}\) concentration (Fig. 6). These results provide compelling evidence that enhanced Ba\(^{2+}\) influx evoked by ATP reflects a regulatory activation of the Na\(^{+}\)/Ca\(^{2+}\) exchanger.

The effects of ATP on exchange activity are most simply explained by secondary activation of the exchanger through increased [Ca\(^{2+}\)]. Although additional mechanisms cannot be ruled out, this interpretation is supported by the following observations: (a) The period of accelerated exchange activity appeared to follow the time course of the [Ca\(^{2+}\)] transient. Thus, the difference in Ba\(^{2+}\) influx between Li- and Na-PSS diminished as the interval between the ATP and Ba\(^{2+}\) additions increased (Fig. 4 A–C), although a small difference was still evident after [Ca\(^{2+}\)] had returned to its resting level (Fig. 4 D and E). (b) In cells pretreated with ionomycin to deplete intracellular Ca\(^{2+}\) stores, the exchanger was essentially inactive after removal of the ionophore with BSA (cf. Condrescu et al., 1997). Addition of ATP to ionomycin-treated cells did not elicit a [Ca\(^{2+}\)] transient, as expected because of the depleted Ca\(^{2+}\) stores, and did not accelerate Ba\(^{2+}\) influx (Fig. 7). (c) Experiments to be presented elsewhere indicate that chelation of [Ca\(^{2+}\)], with cytosolic EGTA (introduced by hydrolysis of the EGTA-acetoxymethyl ester) markedly reduced both the Ca\(^{2+}\) transient and the acceleration of Ba\(^{2+}\) influx by ATP. (In these experiments, EGTA has such a low affinity for Ba\(^{2+}\) that it does not affect the fura-2 signal for Ba\(^{2+}\).) (d) In preliminary results with cells expressing exchanger mutants that are not regulated by [Ca\(^{2+}\)], the rate of Ba\(^{2+}\) influx was independent of [Ca\(^{2+}\)], and was not affected by ATP. Overall, the results are consistent with the conclusions of the accompanying manuscript (Condrescu et al., 1997) and suggest that the exchanger is deactivated as intracellular organelles resequester cytosolic Ca\(^{2+}\). As in the accompanying manuscript, small increases in [Ca\(^{2+}\)], (before Ba\(^{2+}\) addition) are associated with quite large increases in exchange activity (compare traces a and b in Fig. 6, for example).

When both extracellular Na\(^{+}\) and Ca\(^{2+}\) were present, i.e., under physiological conditions, the [Ca\(^{2+}\)] transient elicited by ATP declined more rapidly in CK1.4 cells than in control cells (Fig. 3 inset) but then achieved a stable plateau of elevated [Ca\(^{2+}\)], which outlasted the corresponding phase of elevated [Ca\(^{2+}\)], in the control cells (Fig. 3). Although the precise mechanism underlying this phenomenon remains to be determined, the following speculative scenario provides a reasonable interpretation of the results: (a) In comparison to the control cells, Ca\(^{2+}\) influx via the exchanger in the CK1.4 cells accelerates the decline in [Ca\(^{2+}\)] after the peak of the transient; this leads to a greater depletion of the InsP\(_3\)-sensitive stores in the CK1.4 cells and also retards refilling of the stores (Chernaya et al., 1996). (b) As a result of the greater store depletion, the period during which store-operated Ca\(^{2+}\) channels remain active is prolonged in the CK1.4 cells. (c) Ca\(^{2+}\) influx through store-operated channels elevates [Ca\(^{2+}\)] beneath the plasma membrane and maintains the exchanger in an activated state. Thus, we suggest that the exchanger and store-dependent Ca\(^{2+}\) entry mechanisms mutually reinforce each other’s activity so as to prolong the sustained phase of Ca\(^{2+}\) entry during continuous receptor stimulation. Although highly speculative, this interpretation suggests a potential mechanism by which the Na\(^{+}\)/Ca\(^{2+}\) exchanger could modulate and sustain Ca\(^{2+}\) signalling processes.

Activation of protein kinase C with PMA attenuated the rise in [Ca\(^{2+}\)], associated with Ca\(^{2+}\) influx via the exchanger after ATP administration (Fig. 8). This effect was not exerted directly on exchange activity, however, since PMA failed to affect exchange-mediated Ba\(^{2+}\) influx after ATP addition (Fig. 10); other experiments showed that PMA also had no effect on Ba\(^{2+}\) influx by ouabain-loaded cells under Na\(^{+}\)-free conditions (data not shown). These results are consistent with previous
results from our laboratory (Condrescu et al., 1995), but contrast sharply with a recent report by Iwamoto et al. (1996) who observed that PMA stimulated phosphorylation of the exchanger, and modestly accelerated exchange-mediated 45Ca2+ fluxes, in cardiac myocytes and transfected CCL39 cells. At present, the difference between the two sets of results is unexplained. We conclude that the effects of PMA in the CK1.4 cells reflect a secondary influence of protein kinase C activity through Ca2+ homeostatic mechanisms other than Na+/Ca2+ exchange. Recent results with other cell types provide evidence for at least two such mechanisms: protein kinase C activity inhibits influx of Ca2+ through store-operated Ca2+ channels (Parekh and Penner, 1995) and accelerates the activity of the plasma membrane Ca2+ ATPase (see Balasubramanyam and Gardner [1995] and references cited therein).

Our results suggest that Na+/Ca2+ exchange activity in these cells is regulated in a sensitive and dynamic fashion through Ca2+ uptake and release by intracellular stores. The reduction in exchange activity by intracellular stores as they sequester cytosolic Ca2+ and the corresponding activation of the exchanger as the stores fill to capacity would act to maintain both [Ca2+]i and the Ca2+ content of InsP3-sensitive stores at relatively constant levels (Condrescu et al, 1997). Factors which modulate the driving force for Na+/Ca2+ exchange and/or the affinity of the exchanger’s regulatory sites for Ca2+ would therefore be expected to exert corresponding influences on the Ca2+ content of the stores. For cells which exhibit both Na+/Ca2+ exchange and InsP3-sensitive Ca2+ stores (e.g., vascular smooth muscle cells, chromaffin cells, neutrophils, pancreatic β cells) these feedback relations are likely to be important components of the Ca2+ signalling process.

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