

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

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**ABSTRACT** The  $P_{2U}$  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)<sup>1</sup> cells, the  $P_{2U}$  purinergic agonist ATP elicits the production of 1,4,5-inositol trisphosphate ( $InsP_3$ ) and the release of  $Ca^{2+}$  from  $InsP_3$ -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 mo-

lecular 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-

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<sup>1</sup>Abbreviations used in this paper: CHO, Chinese hamster ovary;  $InsP_3$ , inositol (1,4,5)trisphosphate; PSS, physiological salts solution.

changer but is not significantly accumulated by intracellular organelles and that the exchanger becomes deactivated as  $[Ca^{2+}]_i$  is lowered by the  $Ca^{2+}$  sequestering activities of intracellular organelles. We had previously presented indirect evidence, based on fura-2 measurements of cytosolic  $Ca^{2+}$  ( $[Ca^{2+}]_i$ ), 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  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$ . 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  $S_f/S_b = 4.8$ ,  $R_{min} = 0.9$ ,  $R_{max} = 12$ . Assuming  $K_D = 224$  nM for the  $Ca^{2+}$ /fura-2 complex (Grynkiewicz et al., 1985), the following ratios correspond to the indicated values for  $[Ca^{2+}]_i$ : 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  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  transient elicited by ATP under  $Ca^{2+}$ -free conditions, as reported previously (Chernaya et al., 1996). Upon addition of  $Ca^{2+}$ , the rise in  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  in both media were similar to that observed upon the addition of  $Ca^{2+}$  alone.

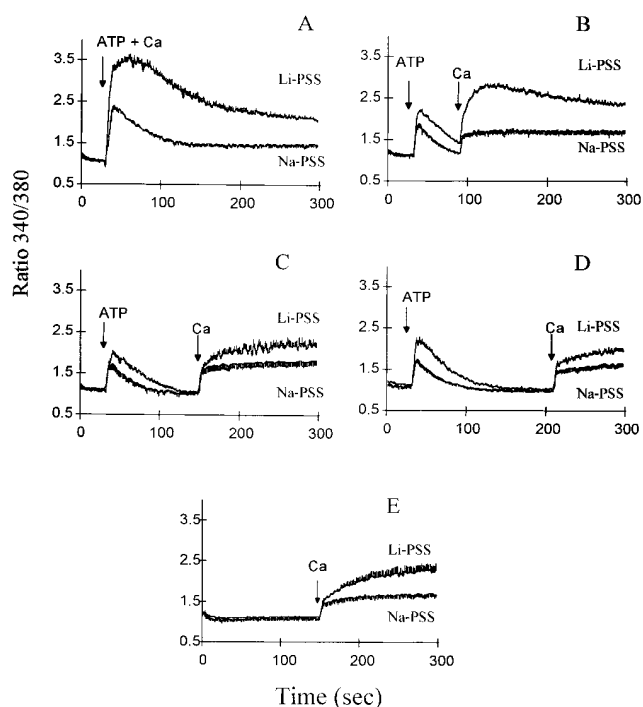


FIGURE 1. Effect of ATP on  $[Ca^{2+}]_i$  in CK1.4 cells. Cells were loaded with fura-2, preincubated for 1 min in Na-PSS + 1 mM  $CaCl_2$  and diluted 30-fold into cuvettes containing Na- or Li-PSS plus 0.3 mM EGTA. ATP (0.3 mM) was added to the cuvette at 30 s in each panel and 1 mM  $CaCl_2$  was added at various intervals after ATP as indicated in the individual panels; in A, ATP and  $CaCl_2$  were added simultaneously and in B, C, and D,  $CaCl_2$  was added 60, 120, and 180 s after ATP. In E, ATP was not added and  $CaCl_2$  was added at 150 s. Fura-2 fluorescence was monitored with alternate excitation at 340 and 380 nm. For all experiments,  $n = 3-4$  except B, where  $n = 5-6$ .

In contrast to this behavior, control-transfected cells showed no increase in  $[Ca^{2+}]_i$  in Li-PSS compared to Na-PSS; indeed, the changes in  $[Ca^{2+}]_i$  tended to be somewhat reduced in Li-PSS (Fig. 2). Note that in Li-PSS, the increase in  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  values in Li-PSS 30-60 s after the addition of  $Ca^{2+}$  were  $221 \pm 8$  nM ( $n = 5$ ) for CK1.4 cells and  $71 \pm 11$  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  $[Ca^{2+}]_i$  upon ATP addition was higher for control cells than for CK1.4 cells. After the peak of the  $[Ca^{2+}]_i$  transient in the control cells,  $[Ca^{2+}]_i$  gradually declined toward the initial "resting" value (70–75 nM) over the time course of the experiment. With CK1.4 cells, the changes in  $[Ca^{2+}]_i$  elicited by ATP followed a markedly different time course:  $[Ca^{2+}]_i$  fell quickly after the peak of the transient and

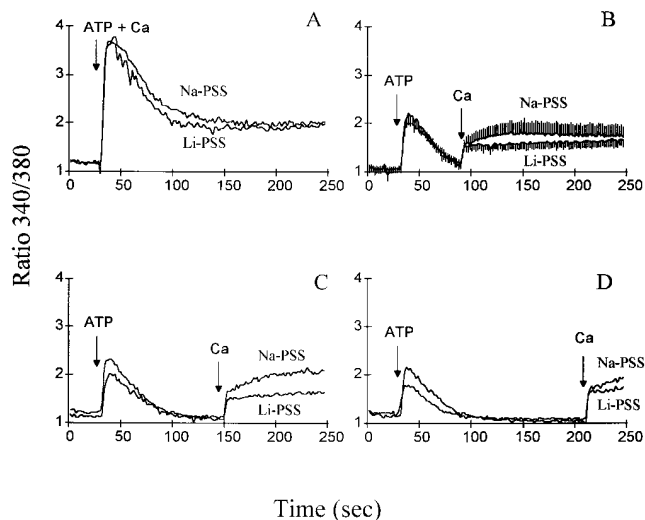


FIGURE 2. Effect of ATP on  $[Ca^{2+}]_i$  in control cells. Conditions are identical to those described in Fig. 1 except that vector-transfected control CHO cells were used instead of CK1.4 cells. ATP and  $CaCl_2$  were added either simultaneously (A), or  $CaCl_2$  was added 60, 120, or 180 s after 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  $n = 3-4$ .

attained a stable plateau ( $100 \pm 1$  nM at 150–200 s;  $n = 6$ ), which remained elevated compared to the initial  $[Ca^{2+}]_i$  ( $74 \pm 2$  nM). The more rapid decline in  $[Ca^{2+}]_i$  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 ele-

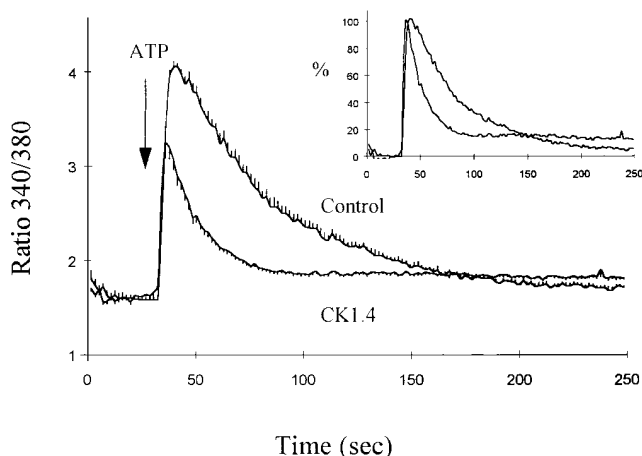


FIGURE 3. Comparison of control and CK1.4 cells: effect of ATP on  $[Ca^{2+}]_i$  in presence of extracellular  $Na^+$  and  $Ca^{2+}$ . CK1.4 or control transfected cells were loaded with fura-2, preincubated for 1 min in Na-PSS + 1 mM  $CaCl_2$  and then diluted 30-fold into cuvettes containing Na-PSS + 1 mM  $CaCl_2$ . Fura-2 fluorescence was monitored (340/380 excitation) and ATP (0.3 mM) was added as shown ( $n = 5-6$ ).

vated  $[Ca^{2+}]_i$  in the CK1.4 cells was maintained for a longer period of time and indeed “crossed over” the declining  $[Ca^{2+}]_i$  trace for the control cells at  $\sim 170$  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  $Ba^{2+}$  influx. In general, ATP accelerated  $Ba^{2+}$  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  $[Ca^{2+}]_i$  transient. The fura-2 traces in these experiments are highly complex because they reflect the combined influence of changes in  $[Ca^{2+}]_i$  and  $[Ba^{2+}]_i$ . For example, in the experiment where ATP and  $Ba^{2+}$  are added simultaneously (Fig. 4 A), the contribution of  $[Ca^{2+}]_i$  to the fura-2 signal would initially be expected to predominate because of the release of  $Ca^{2+}$  from

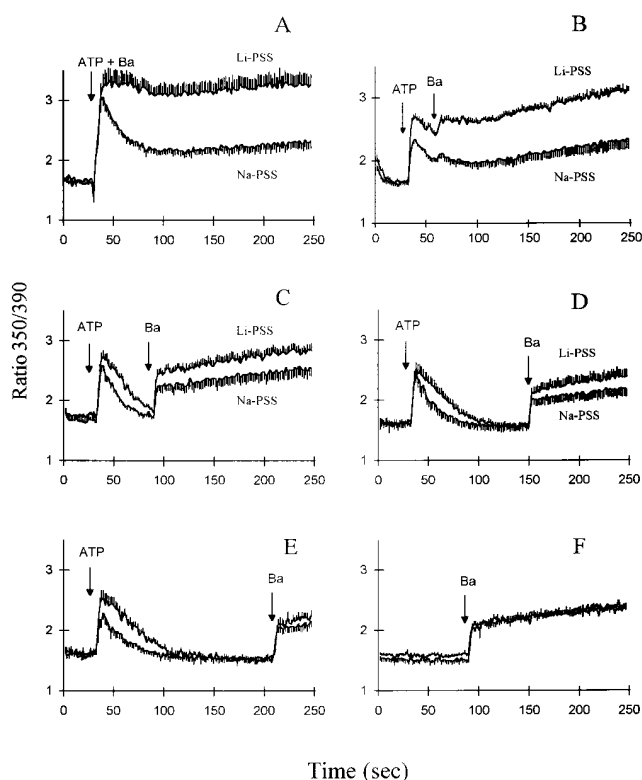


FIGURE 4. ATP and  $Ba^{2+}$  influx in CK1.4 cells. CK1.4 cells were loaded with fura-2, preincubated for 1 min in Na-PSS + 1 mM  $CaCl_2$  and diluted 30-fold into cuvettes containing either Li- or Na-PSS plus 0.3 mM EGTA. Fura-2 fluorescence was monitored with alternate excitation at 350 and 390 nm. ATP (0.3 mM) was added at 30 s in each panel and  $BaCl_2$  (1 mM) was added at increasing intervals following ATP, as indicated; in A, ATP and  $BaCl_2$  were added simultaneously. ( $n = 4-6$  [A, B];  $3-4$  [D, E] and  $6-8$  [C]).

InsP<sub>3</sub>-sensitive stores. The concentration of Ca<sup>2+</sup> would then decline as Ca<sup>2+</sup> 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 Ba<sup>2+</sup> rapidly enters the cell via exchange activity. Two observations lend support to this interpretation: (a) The presence of extracellular or cytosolic Ba<sup>2+</sup> does not interfere with the decline in [Ca<sup>2+</sup>]<sub>i</sub> after ATP addition (Condrescu et al., 1996). (b) When excess EGTA was added to the cells after Ba<sup>2+</sup> influx under these conditions, the fura-2 signal did not decline (data not shown), as expected for cytosolic Ba<sup>2+</sup> (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 Ca<sup>2+</sup> (Chernaya et al., 1996).

When Ba<sup>2+</sup> 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 Ba<sup>2+</sup> addition increased. We conclude that exchange activity decreased progressively after ATP addition, consistent with the results obtained with Ca<sup>2+</sup> (Fig. 1) and that the acceleration of exchange activity corresponded closely with the period of elevated [Ca<sup>2+</sup>]<sub>i</sub>. In the absence of ATP, no difference in Ba<sup>2+</sup> influx between Li-PSS and Na-PSS (Fig. 4 F) could be detected when Ba<sup>2+</sup> 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 Ba<sup>2+</sup> influx by ATP and could detect no differences between Li- and Na-PSS (Fig. 5). This result strongly supports our interpretation that Ba<sup>2+</sup> 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<sup>+</sup>, a treatment that should block Ba<sup>2+</sup> influx via the exchange activity. To remove cytosolic Na<sup>+</sup>, 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 Ba<sup>2+</sup> 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<sup>+</sup>] = 19 mM). However, when ATP was added 60 s before Ba<sup>2+</sup>, the rate of Ba<sup>2+</sup> influx was dramatically accelerated (trace *a*) compared to that seen in the absence of ATP addition. Ba<sup>2+</sup> influx was completely abolished when ATP was added to cells in the absence of Na<sup>+</sup> (trace *c*), although the transient due to

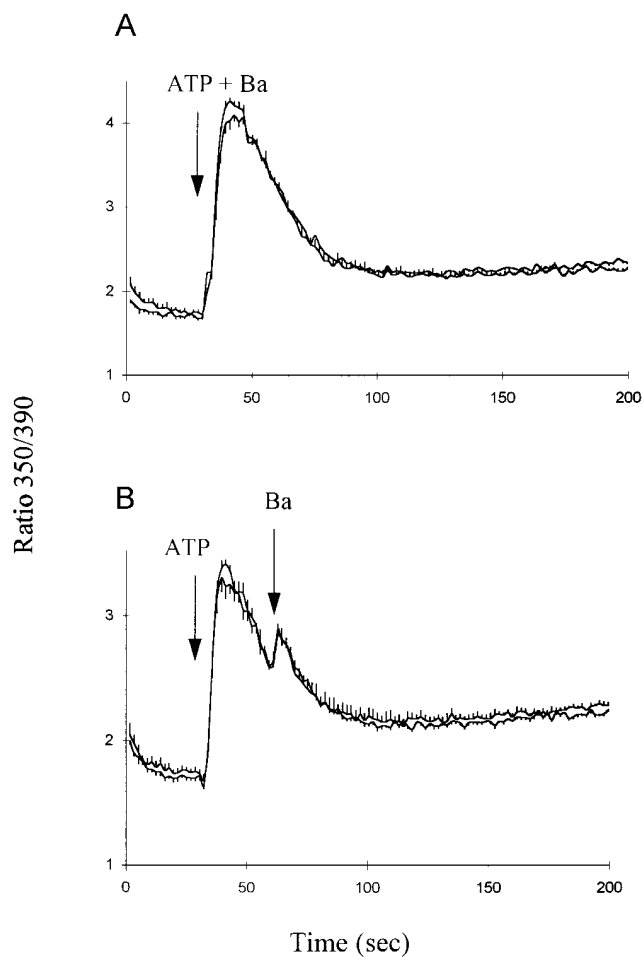


FIGURE 5. ATP and Ba<sup>2+</sup> influx in control cells. Vector transfected control cells were loaded with fura-2, preincubated for 1 min in Na-PSS + 1 mM CaCl<sub>2</sub> and added to cuvettes containing Li- or Na-PSS plus 0.3 mM EGTA. ATP (0.3 mM) and BaCl<sub>2</sub> (1 mM) were added to the cuvettes, either simultaneously (A), or with a 1 min interval between ATP and Ba<sup>2+</sup> additions (B). The traces in Na-PSS and Li-PSS are indistinguishable (*n* = 4).

Ca<sup>2+</sup> release from InsP<sub>3</sub>-sensitive stores was normal. When the same experiment was carried out with vector-transfected control cells (Fig. 6 B), Ba<sup>2+</sup> influx was essentially the same under all experimental conditions, and was identical to that shown in the absence of Na<sup>+</sup> (trace *c*, Fig. 6 A) for the CK1.4 cells.

In the experiments with gramicidin-treated cells, considerable exchange-mediated Ba<sup>2+</sup> 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 [Ca<sup>2+</sup>]<sub>i</sub> brought about by gramicidin through its uncoupling action on mitochondria: A similar increase in [Ca<sup>2+</sup>]<sub>i</sub> 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

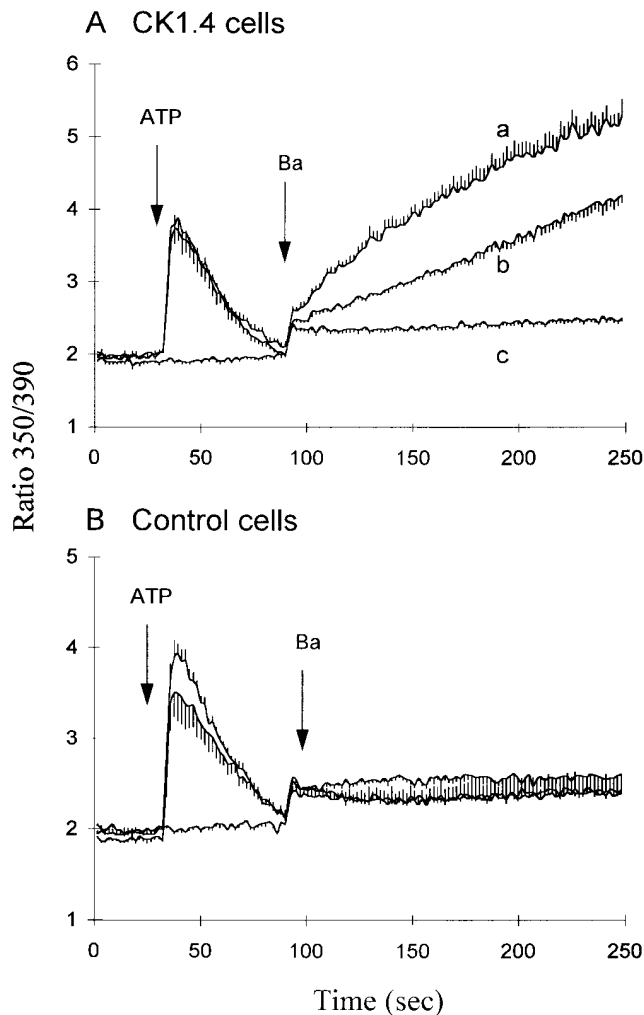


FIGURE 6. ATP and  $Ba^{2+}$  influx in gramicidin-treated cells. CK1.4 (A) or vector-transfected control (B) cells were loaded with fura-2, preincubated for 1 min in Na-PSS + 1 mM  $CaCl_2$  and diluted 30-fold into cuvettes containing 20/120 Na/K-PSS + 0.3 mM EGTA (traces *a* and *c*). For trace *c*, the cells were preincubated in K-PSS + 1 mM  $CaCl_2$  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  $BaCl_2$  was added for all traces at 90 s ( $n = 5$ ).

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

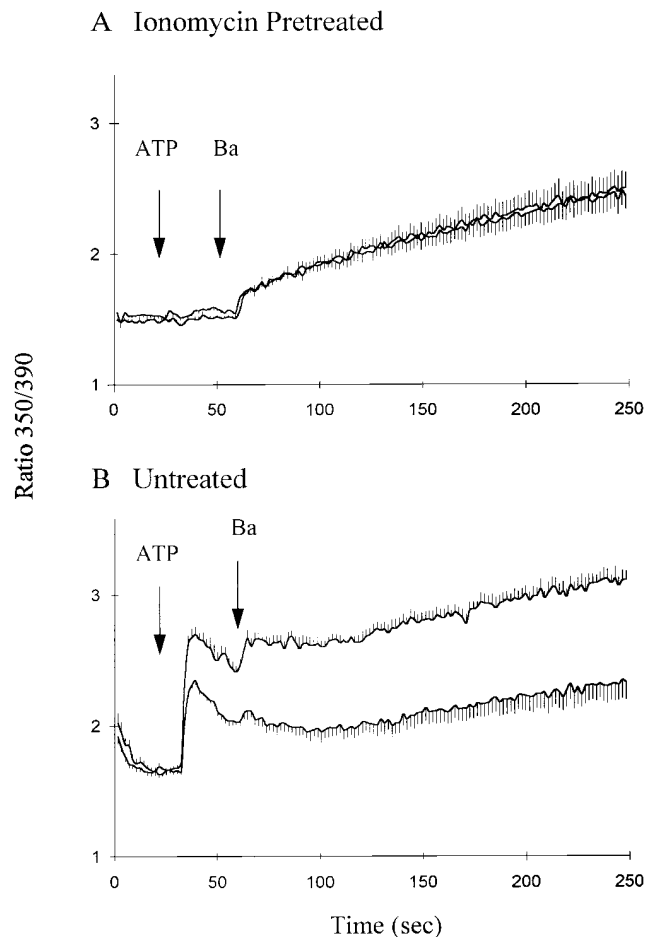


FIGURE 7. Acceleration of  $Ba^{2+}$  influx by ATP requires  $Ca^{2+}$  release. CK1.4 cells were preincubated for 1 min in Na-PSS + 0.3 mM EGTA + 10  $\mu$ M ionomycin (A), or Na-PSS + 1 mM  $CaCl_2$  (B). The cells in A were centrifuged and then incubated for an additional 1 min in Na-PSS + 0.3 mM 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  $BaCl_2$  (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 ( $n = 4-6$ ).

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-

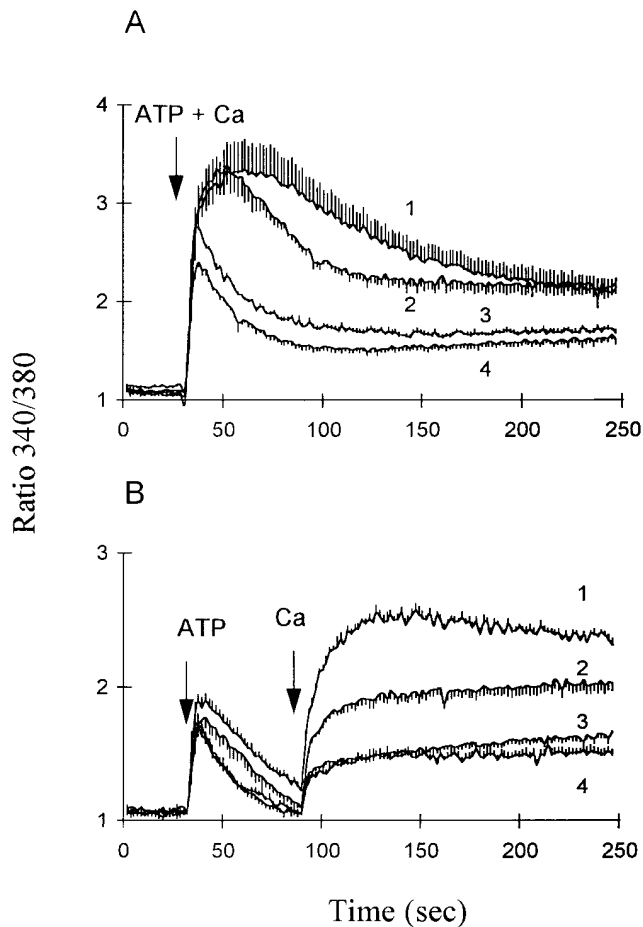


FIGURE 8. PMA attenuates ATP-evoked  $\text{Ca}^{2+}$  influx in CK1.4 cells. CK1.4 cells were loaded with fura-2, preincubated for 1 min in Na-PSS + 1 mM  $\text{CaCl}_2$  and diluted 30-fold into cuvettes containing Li- or Na-PSS plus 0.3 mM EGTA. ATP (0.3 mM) and  $\text{CaCl}_2$  (1 mM) were added simultaneously (A) or with a 1 min interval between ATP and  $\text{CaCl}_2$  additions (B). For traces 2 and 4, 100 nM PMA was included in the cuvette solutions. Li-PSS: traces 1 and 2; Na-PSS: traces 3 and 4. In B, the order of the traces before the addition of  $\text{Ca}^{2+}$  are (from top) 1, 2, 3, and 4; the latter two are indistinguishable ( $n = 3-5$ ).

duced in CK1.4 cells treated with 100 nM PMA (trace 2 in each panel); the effect was observed when ATP and  $\text{Ca}^{2+}$  were added simultaneously (Fig. 8 A) but appeared to be more pronounced in experiments where  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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

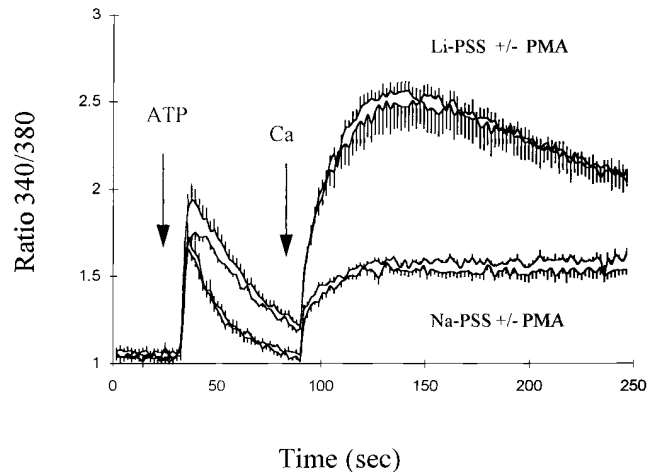


FIGURE 9. Down-regulation of protein kinase C abrogates acute effect of PMA. CK1.4 cells were incubated overnight with 100 nM PMA to down-regulate protein kinase C. The cells were then loaded with fura-2, preincubated for 1 min in Na-PSS + 1 mM  $\text{CaCl}_2$  and diluted 30-fold into cuvettes containing Li- or Na-PSS + 0.3 mM EGTA with or without 100 nM PMA, as indicated. ATP (0.3 mM) and  $\text{CaCl}_2$  were added where indicated ( $n = 3-4$ ).

protein kinase C did not appear to enhance exchange-mediated  $\text{Ca}^{2+}$  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  $\text{Ba}^{2+}$  influx after ATP addition.  $\text{Ba}^{2+}$  influx in Li-PSS was the same whether or not PMA was present, suggesting that the effects of PMA on  $\text{Ca}^{2+}$  influx (Fig. 8) were not brought about by an alteration in exchange activity itself.

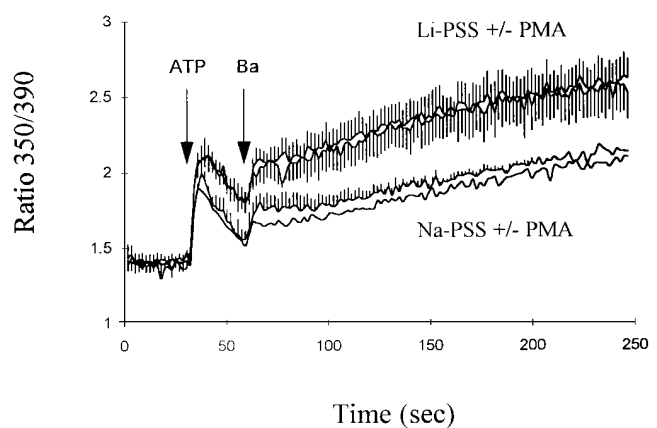


FIGURE 10. Effect of PMA on  $\text{Ba}^{2+}$  influx in CK1.4 cells. CK1.4 cells were loaded with fura-2, preincubated for 1 min in Na-PSS + 1 mM  $\text{CaCl}_2$  and diluted 30-fold into cuvettes containing Li- or Na-PSS plus 0.3 mM EGTA, with or without 100 nM PMA as indicated. Fura-2 fluorescence was monitored (350/390 nm excitation) and ATP (0.3 mM) and  $\text{BaCl}_2$  (1 mM) were added where indicated. ( $n = 2-4$ ; duplicate experiments are shown in the trace without error bars).

## DISCUSSION

ATP interacts with  $P_{2U}$  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  $Ca^{2+}$  efflux 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  $[Ca^{2+}]_i$ . 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  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$ , the rate of  $Ba^{2+}$  influx was independent of  $[Ca^{2+}]_i$  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 re-sequester cytosolic  $Ca^{2+}$ . As in the accompanying manuscript, small increases in  $[Ca^{2+}]_i$  (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  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  which outlasted the corresponding phase of elevated  $[Ca^{2+}]_i$  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,  $Ca^{2+}$  efflux via the exchanger in the CK1.4 cells accelerates the decline in  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  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  $[Ca^{2+}]_i$  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  $^{45}\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  homeostatic mechanisms other than  $\text{Na}^{+}/\text{Ca}^{2+}$  exchange. Recent results with other cell types provide evidence for at least two such mechanisms: protein kinase C activity inhibits influx of  $\text{Ca}^{2+}$  through store-operated  $\text{Ca}^{2+}$  channels (Parekh and Penner, 1995) and accelerates the activity of the plasma membrane  $\text{Ca}^{2+}$  ATPase (see Balasubramanyam and Gardner [1995] and references cited therein).

Our results suggest that  $\text{Na}^{+}/\text{Ca}^{2+}$  exchange activity

in these cells is regulated in a sensitive and dynamic fashion through  $\text{Ca}^{2+}$  uptake and release by intracellular stores. The reduction in exchange activity by intracellular stores as they sequester cytosolic  $\text{Ca}^{2+}$  and the corresponding activation of the exchanger as the stores fill to capacity would act to maintain both  $[\text{Ca}^{2+}]_i$  and the  $\text{Ca}^{2+}$  content of  $\text{InsP}_3$ -sensitive stores at relatively constant levels (Condrescu et al., 1997). Factors which modulate the driving force for  $\text{Na}^{+}/\text{Ca}^{2+}$  exchange and/or the affinity of the exchanger's regulatory sites for  $\text{Ca}^{2+}$  would therefore be expected to exert corresponding influences on the  $\text{Ca}^{2+}$  content of the stores. For cells which exhibit both  $\text{Na}^{+}/\text{Ca}^{2+}$  exchange and  $\text{InsP}_3$ -sensitive  $\text{Ca}^{2+}$  stores (e.g., vascular smooth muscle cells, chromaffin cells, neutrophils, pancreatic  $\beta$  cells) these feedback relations are likely to be important components of the  $\text{Ca}^{2+}$  signalling process.

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