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Extracellular ATP-dependent activation of plasma membrane Ca^{2+} pump in HEK-293 cells

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1 It is well known that extracellular ATP (ATP_o) elevates the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by inducing Ca^{2+} influx or mobilizing Ca^{2+} from internal stores *via* activation of purinoceptors in the plasma membrane. This study shows that ATP_o also activates the plasma membrane Ca^{2+} pumps (PMCPs) to bring the elevated $[Ca^{2+}]_i$ back to the resting level in human embryonic kidney-293 (HEK-293) cells.

2 The duration of ATP_o-induced intracellular Ca^{2+} transients was significantly increased by PMCP blockers, La^{3+} or orthovanadate. In contrast, replacement of extracellular Na^+ with $NMDG^+$, a membrane-impermeable cation, had no significant effect on duration, thus suggesting that Na^+/Ca^{2+} exchangers do not participate in the ATP_o-induced Ca^{2+} transient.

3 A rapid and significant decrease in $[Ca^{2+}]_i$, which was not dependent on extracellular Na^+, was induced by ATP_o in cells pretreated with thapsigargin (TG). This decrease was blocked by orthovanadate, indicating that it was caused by PMCPs rather than sarco/endoplasmic reticulum Ca^{2+} pumps (SERCPs).

4 UTP and ATPγS also caused a decrease in $[Ca^{2+}]_i$ in cells pretreated with TG, although they were less effective than ATP. The effect of UTP implies the involvement of both $P2Y_1$ and $P2Y_2$ receptors, while the effect of ATPγS implies no significant role of ectophosphorylation and agonist hydrolysis in the agonist-induced $[Ca^{2+}]_i$ decreases.

5 These results point to a role of PMCPs in shaping the Ca^{2+} signal and in restoring the resting $[Ca^{2+}]_i$ level to maintain intracellular Ca^{2+} homeostasis after agonist stimulation.

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Abbreviations: ATP_o, extracellular ATP; ATPγS, adenosine 5′-O-(3-thiotriphosphate); $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; HEK-293 cell, human embryonic kidney-293 cell; PMCP, plasma membrane Ca^{2+} pump; SERCP, sarco/endoplasmic reticulum Ca^{2+} pump; TG, thapsigargin; UTP, uridine 5′-triphosphate

Introduction

Extracellular ATP (ATP_o) is a potent signal that modulates a variety of cellular functions through activation of plasma membrane P_2-type purinoceptors (Ralevic & Burnstock, 1998). The P_2-type purinoceptors have been subdivided into ATP_o-gated ion channels and G protein-coupled receptors. Activation of the latter may trigger phosphoinositide-specific phospholipase C, which catalyzes production of inositol 1,4,5-trisphosphate (IP_3) from the membrane lipid, phosphatidylinositol 4,5-bisphosphate. As a Ca^{2+} mobilizing messenger, IP_3 diffuses into the cytoplasm and releases Ca^{2+} from intracellular stores to generate a Ca^{2+} signal (Ralevic & Burnstock, 1998). This Ca^{2+} signal is composed of two phases, one rising and one declining. The rising phase reflects activation of Ca^{2+} mobilizing systems, which in turn activate intracellular signalling mechanisms to induce a proper cellular response, while the declining phase reflects activation of Ca^{2+} sequestration systems, to avoid high and potentially toxic $[Ca^{2+}]_i$ levels. For successful Ca^{2+} signalling, it is therefore essential that $[Ca^{2+}]_i$ increase and reduction are closely integrated.

Increasing evidence has recently suggested that plasma membrane Ca^{2+} pumps (PMCPs) activated by Ca^{2+} mobilizing agonists play a significant role in controlling the Ca^{2+} signal. For example, an increase in Ca^{2+} extrusion *via* PMCP activation upon agonist stimulation has been demonstrated in human platelets (Rink & Sage, 1987), mouse pancreatic acinar cells (Tepikin *et al*., 1992), rat pancreatic acinar cells (Zhang *et al*., 1992), A7r5 smooth muscle cells (Broad *et al*., 1999), human U373 MG astrocytoma cells (Young *et al*., 1998), and GH3 pituitary cells (Nelson & Hinkle, 1994). These results imply that an agonist which causes an increase in $[Ca^{2+}]_i$ may also accelerate Ca^{2+} extrusion by activating PMCPs (Broad *et al*., 1999).

In the present study, several lines of evidence are presented to show that ATP_o, a potent purinoceptor agonist, can strongly and rapidly activate PMCPs in human embryonic kidney-293 (HEK-293) cells. The duration of ATP_o-induced intracellular Ca^{2+} transients became longer in the presence of PMCP blockers. In the presence of a sarco/endoplasmic reticulum Ca^{2+} pump (SERCP) inhibitor, thapsigargin (TG), ATP_o caused a rapid decrease in $[Ca^{2+}]_i$. A similar decrease in $[Ca^{2+}]_i$ was also caused by UTP and ATPγS in TG-treated cells. These results suggest that agonist-activated PMCPs contribute significantly to terminating the initial $[Ca^{2+}]_i$ increase and to rapidly bringing the increased $[Ca^{2+}]_i$ back to the resting level to maintain intracellular Ca^{2+} homeostasis.

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Methods

HEK-293 cells were grown on fibronectin-coated coverslips at 37°C in a humidified 5% CO_2 atmosphere in DMEM-L (Dulbecco's modification of Eagle's medium with low glucose) culture medium supplemented with 10% foetal calf serum, 100 units ml^{-1} penicillin, and 100 $\mu g\ ml^{-1}$ streptomycin (GIBCO BRL). After 2–4 days, the cells were transferred to a 'standard' solution containing (mM): $CaCl_2$ 1.3, $MgCl_2$ 1.0, KC1 5.4, NaCl 140, HEPES 10.0 (*p*H adjusted to 7.4 with NaOH). For Ca^{2+}-free solution, 0.1 mM EGTA was added to nominally Ca^{2+}-free standard solution. For Na^+-free solution, $NMDG^+$ (N-methyl-D-glutamine) was used to replace Na^+ in the standard solution. The osmolality of the solutions was adjusted to that of the culture medium (320 mOsm $litre^{-1}$) using 40 mM D-glucose. Cells were incubated in the culture medium containing 3 μM fluo-3/AM and 0.02% Pluronic F-127 (Sigma Chemical Co.) for 30 min. Fluo-3 intensity (F, 480 ± 15 nm excitation, 530 ± 30 nm emission) was monitored every 2 s by a real time laser confocal microscope (Insight-IQ, Meridian Instruments, Inc.) and plotted as relative intensity with respect to that of a basal calcium level (F_0). The results shown were representative of over 30 cells from at least three independent experiments. Each agonist was dissolved in the bath solution and drawn into a glass pipette (G-1, Narishige, Japan) with a tip diameter of *ca.* 2 μm. The pipette was placed at adjacence of approximately 70 μm from the closest cells for local application of agonist. The flow rate of the agonist through the pipette was controlled by a microinjection system (Eppendorf, Germany), and was adjusted to a value below the threshold for cell detachment from the coverslip. To prevent cells being influenced by agonist leakage, the pipette was raised 250 μm after each application. Experiments were carried out at 37°C.

Results and Discussion

It is generally accepted that PMCPs play a role in Ca^{2+} extrusion from the cytosol, particularly in non-excitable cells (Carafoli, 1994; Guerini, 1998). To evaluate the effect of PMCPs on ATP$_o$-induced intracellular Ca^{2+} transients, we measured the duration of ATP$_o$-induced intracellular Ca^{2+} transients in the presence of PMCP blockers. In preliminary experiments, we observed that the time courses of intracellular Ca^{2+} transients were quite variable among individual cells upon ATP$_o$ application at low concentrations (1–10 μM). To synchronize the time course of the intracellular Ca^{2+} transient, a relatively high concentration of ATP$_o$ (50 μM) was applied to the cells. Approximately 2 s after an ATP$_o$ application, a transient increase in $[Ca^{2+}]_i$ was observed (Figure 1). This Ca^{2+} transient was not affected by the presence or absence of extracellular Ca^{2+} (data not shown), suggesting that the Ca^{2+} transient was caused by the mobilization of Ca^{2+} from internal stores, presumably *via* activation of purinoceptors in the plasma membrane. The duration of the ATP$_o$-induced $[Ca^{2+}]_i$ transient was measured in the absence and presence of orthovanadate and La^{3+}, two widely used PMCP blockers (Carafoli, 1994; Guerini, 1998). Both blockers increased the duration of the ATP$_o$-induced Ca^{2+} transient (Figure 1a,b). This increase can be seen clearly in the furthest right panel of each trace, where paired time courses of the ATP$_o$ induced Ca^{2+} transients respectively in the absence and presence of the blockers are overlapped. It is obvious that neither La^{3+} nor orthovanadate had any significant effect on the initial rising phase of the Ca^{2+} transient in response to ATP$_o$, whereas both drugs significantly increased duration and slowed down the declining phase. It is well known that both La^{3+} and orthovanadate are blockers not only of PMCPs but also of other P-type pumps, including SERCPs (Carafoli, 1994). When more than 200 μM La^{3+} was contained in the Ca^{2+}-free perfusing solution, the $[Ca^{2+}]_i$ level increased without any stimulation. Furthermore, a much smaller increase in $[Ca^{2+}]_i$ was evoked by ATP$_o$ after perfusion (data not shown). These observations imply that SERCPs were inhibited by 200 μM La^{3+}. However, we observed no increase in $[Ca^{2+}]_i$ in the above blocker-containing perfusing solutions and nearly the same increase in the ATP$_o$-induced $[Ca^{2+}]_i$ before and after perfusion. This suggests that the Ca^{2+} content of internal stores was not affected by the blockers, suggesting that these blockers had a negligible effect on SERCPs. Indeed, it has been previously shown that SERCPs are less sensitive to orthovanadate than PMCPs (Marin *et al*., 1999). Thus, the similar effect of these two blockers on the duration of the Ca^{2+} transient strongly suggests that the elongation of the Ca^{2+} transient is due to the blockade of PMCPs, because these two blockers inhibit PMCPs by completely different mechanisms (Carafoli, 1994; Guerini, 1998). In addition, the effect of orthovanadate can be partially recovered after a 15 min rinse (Figure 1a,d), providing further evidence that PMCPs participate in the ATP$_o$ induced Ca^{2+} transient. A similar conclusion, i.e. that La^{3+} increases the duration of the TRH (thyrotropin-releasing hormone)-evoked Ca^{2+} transient by inhibiting PMCP, was reached in GH3 pituitary cells (Nelson & Hinkle, 1994). Moreover, there was no significant change in the duration of the Ca^{2+} transient when the experiment was performed in Na^+-free solution (Figure 1c,f). This result indicates that Ca^{2+} extrusion by Na^+/Ca^{2+} exchangers has little effect on the ATP$_o$-induced Ca^{2+} decrease in HEK-293 cells. It has been reported that overexpressing PMCPs in the Chinese hamster ovary cell line significantly decreases the activity of endogenous sarcoplasmic reticulum Ca^{2+}-ATPase (Guerini *et al*., 1995). This result suggests that there is a compensation effect between PMCPs and SERCPs: i.e., inhibiting one will make the other more active. Therefore, the elongated Ca^{2+} transient caused by inhibiting PMCPs may have been underestimated in the above experiment, because SERCPs might have been activated more intensively to restore the basal $[Ca^{2+}]_i$ level due to the blockade of PMCPs.

To evaluate the contribution of ATP$_o$-activated PMCPs to the reduction of ATP$_o$-stimulated cytoplasmic Ca^{2+} level, experiments were carried out using the following procedure (Figure 2a). First, a $[Ca^{2+}]_i$ transient was induced by a puff application of ATP$_o$ (50 μM, 20 s) in a Ca^{2+}-free solution to evaluate the level of ATP$_o$-dependent $[Ca^{2+}]_i$ increase. Then, a Ca^{2+}-free solution containing 200 nM TG, a highly selective and irreversible inhibitor of SERCPs. (Thastrup *et al*, 1990), was continuously perfused for 15 min to completely inhibit SERCPs. $[Ca^{2+}]_i$ was then elevated through capacitative Ca^{2+} entry triggered by restoration of extracellular $[Ca^{2+}]$ to 1.3 mM. Finally, after the elevated $[Ca^{2+}]_i$ level had become stable, the same amount of ATP$_o$ as that used in the first step was applied to the cells. As shown in Figure 2a, the second application of ATP$_o$ rapidly decreased $[Ca^{2+}]_i$. It has already been shown that $[Ca^{2+}]_i$ elevated by capacitative Ca^{2+} entry can activate PMCPs (Snitsarev & Taylor, 1999). This explains the stable level of high $[Ca^{2+}]_i$, which may represent a balance between Ca^{2+} entry and extrusion. The Ca^{2+} entry is through the capacitative pathway, while the Ca^{2+} extrusion is due to PMCP activation. However, this PMCP activation due to elevated $[Ca^{2+}]_i$ only contributes to keeping the balance between the Ca^{2+} entry and extrusion, whereas it may not

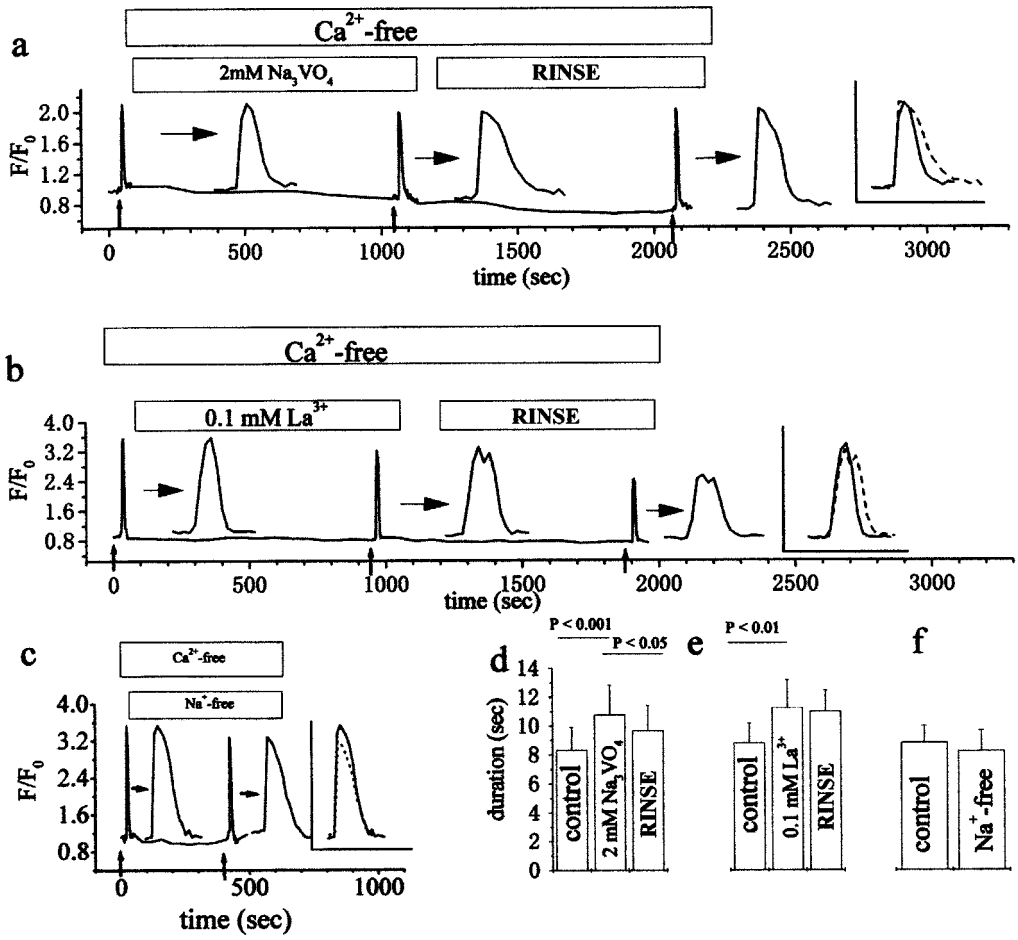

Figure 1 Effect of PMCP blockers and extracellular Na^+ depletion on the duration of Ca^{2+} transients induced by ATP_o. Comparison of intracellular Ca^{2+} transients induced by ATP_o in the absence and presence of 2 mM orthovanadate (a), 100 μM La^{3+} (b) or Na^+ depletion (c), respectively. For greater clarity, corresponding Ca^{2+} transients are expanded 10 times, as indicated by horizontal arrows (a, b and c). An overlap of paired Ca^{2+} transients induced by ATP_o in the absence and presence of each corresponding blocker is shown in the furthest-right panel of a, b and c. Small vertical arrows indicate the time of a puff application of ATP_o (50 μM, 5 s). Statistical results on the duration of the Ca^{2+} transient in a, b and c, which were obtained from at least three independent experiments over 40 cells, are summarized as means $\pm$ s.d. in d, e and f, respectively. The duration of the Ca^{2+} transient was calculated as the mean time between the start of the $[Ca^{2+}]_i$ increase (defined as the point when $[Ca^{2+}]_i$ has increased 50% above the average basal $[Ca^{2+}]_i$) and the point where $[Ca^{2+}]_i$ had returned halfway from the peak to the basal.

contribute to the rapid decrease in $[Ca^{2+}]_i$. The observation that the decrease may be inhibited by 2 mM orthovanadate (Figure 2b) suggests that the rapid decrease in $[Ca^{2+}]_i$ is due to the ATP_o-activated PMCPs. The trace in Figure 2a suggests that a large fraction of the increase in $[Ca^{2+}]_i$ evoked by ATP_o could be removed by ATP_o activated PMCPs. This result is in good agreement with an early report that PMCPs can rapidly eject large amounts of Ca^{2+} during maximal receptor activation (Tepikin *et al*., 1992). We did not use La^{3+} to block PMCPs, because it also blocks the capacitative Ca^{2+} entry in HEK-293 cells as it does in other cells (Klishin *et al*., 1998). These results indicate that the ATP_o activated PMCPs contribute significantly to the clearance of ATP_o-induced $[Ca^{2+}]_i$ increase.

To evaluate whether the Na^+-Ca^{2+} exchanger contributes to the rapid decrease in the ATP_o induced $[Ca^{2+}]_i$ increase, experiments were carried out in Na^+-free solutions under the same experimental condition in Figure 2a. After the $[Ca^{2+}]_i$ increase elevated by capacitative Ca^{2+} entry had reached a stable level, a rapid decrease in $[Ca^{2+}]_i$ was induced by ATP_o application to the TG pretreated cells (Figure 3a). This decrease was not affected by replacement of extracellular Na^+ with $NMDG^+$, a membrane-impermeant cation. Therefore, under a condition where the Na^+-Ca^{2+} exchanger and SERCPs cannot be activated, ATP_o can still stimulate rapid Ca^{2+} extrusion. Besides, Figure 3b shows that the decrease in $[Ca^{2+}]_i$ by ATP_o is dose-dependent, implying that PMCPs are activated by ATP_o.

At least two types of P_2 receptors, i.e., $P2Y_1$ and $P2Y_2$, are endogenously expressed in HEK-293 cells (Gao *et al*., 1999; Schachter, 1997). It is well known that ATP_o is a potent agonist at these two receptors (Ralevic & Burnstock, 1998). To investigate the involvement of $P2Y_1$ and $P2Y_2$ receptors in PMCP activation by ATP_o, 50 μM UTP which is equipotent as ATP at $P2Y_2$ (Janssens *et al*., 1999) but has almost no effect on $P2Y_1$, was applied to the TG-treated cells. Figure 4a shows that UTP can also cause a decrease in $[Ca^{2+}]_i$, but its effect is smaller than that caused by the same amount of ATP. This result suggests that not only $P2Y_2$ but also $P2Y_1$ is involved in the activation of PMCPs by ATP_o. To check if ectophosphorylation and ATP hydrolysis are involved in PMCP activation, ATPγS which is resistant to hydrolytic enzymes was applied to the TG-treated cells. Figure 4b shows that ATPγS also caused significant decrease in $[Ca^{2+}]_i$, although to a lesser extent. This result suggests that ectophosphorylation, the effect of which has been shown in T-lymphocyte (Redegeld *et al*.,

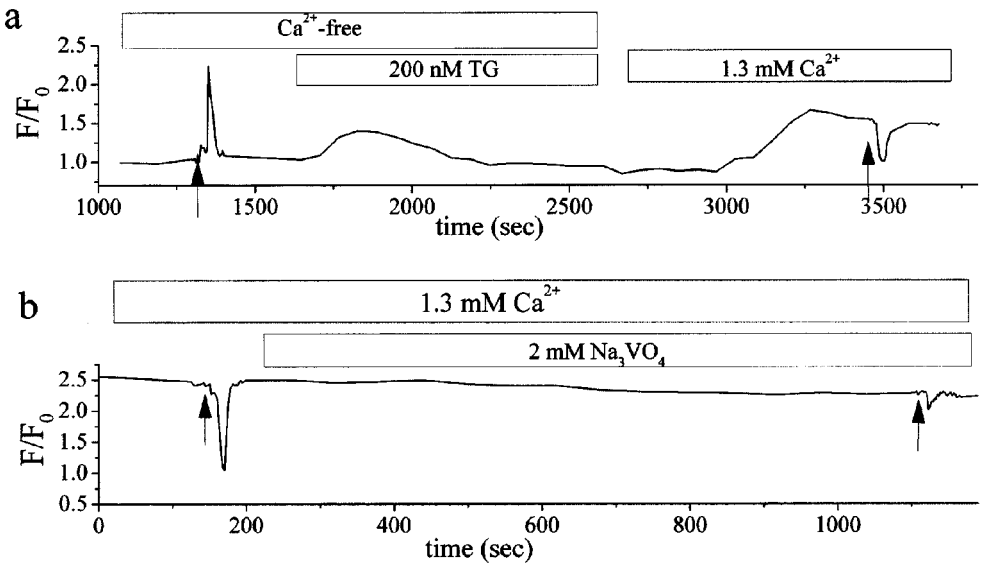

Figure 2 (a) Comparison of the amount of $[Ca^{2+}]_i$ changes between ATP_o (arrows: 50 μM, 20 s)-induced $[Ca^{2+}]_i$ increase and decrease (see text for details). (b) Inhibition of ATP_o-induced decrease in $[Ca^{2+}]_i$ by 2 mM orthovanadate. Cells were incubated in culture medium containing 200 nM TG during 30-min Fluo-3 loading before rinsing with the Ca^{2+}-free solution. $[Ca^{2+}]_i$ was elevated with the standard solution containing 1.3 mM Ca^{2+} like that in (a). ATP_o (arrows: 50 μM, 20 s) was applied to the cells once the $[Ca^{2+}]_i$ had reached a steady-state level.

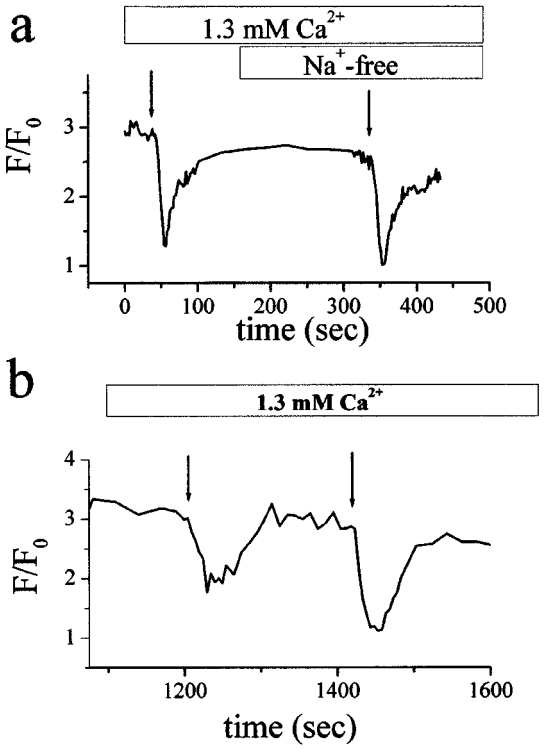

Figure 3 Reduction of steady-state $[Ca^{2+}]_i$ by ATP_o in the cells pretreated with TG. (a) Comparison of steady-state $[Ca^{2+}]_i$ decreases by ATP_o in the presence and absence of Na^+. Each arrow indicates the time of a puff application of ATP_o (50 μM, 10 s). (b) Dose dependence of ATP_o-induced decrease in $[Ca^{2+}]_i$. First arrow: 6 μM ATP_o, 20 s; second arrow: 50 μM ATP_o, 20 s. Experimental procedures in both (a) and (b) as in Figure 2b.

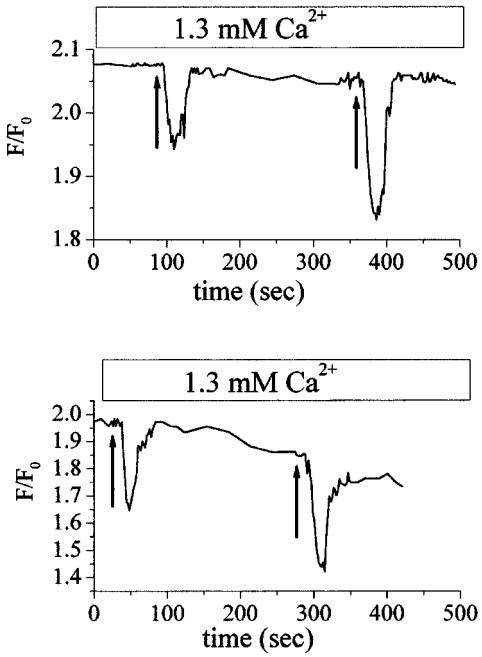

Figure 4 Reduction of steady-state $[Ca^{2+}]_i$ by UTP and ATPγS in the cells pretreated with TG. (a) Comparison of steady-state $[Ca^{2+}]_i$ decreases by UTP and ATP_o. First arrow: 50 μM UTP, 20 s; second arrow: 50 μM ATP, 20 s. (b) Comparison of steady-state $[Ca^{2+}]_i$ decreases by ATPγS and ATP. First arrow: 50 μM ATPγS, 20 s. Second arrow: 50 μM ATP, 20 s. Experimental procedures in both (a) and (b) as in Figure 2b.

1997) or ATP hydrolysis, plays an insignificant role in PMCP activation by ATP_o. ATPγS is a less potent agonist at both $P2Y_1$ (Schachter *et al.*, 1996) and $P2Y_2$ (Janssens *et al.*, 1999) receptors. If the $P2Y_2$ receptor alone is involved in PMCP activation by ATP_o, then ATPγS will cause a much smaller decrease in $[Ca^{2+}]_i$ than UTP. Consequently, the present result implies that both $P2Y_1$ and $P2Y_2$ receptors are involved in PMCP activation by ATPγS. These results strongly suggest that PMCPs are activated by ATP_o through activation of P2Y-type receptors.

It has been indicated that Ca^{2+}-calmodulin, protein kinase A and C, acidic phospholipids, and proteases (for review see Carafoli, 1994 and Monteith & Roufogalis, 1995) could be involved in stimulating PMCPs. It has also been shown in human erythrocyte membranes that the activity of calmodulin-

dependent PMCPs is Ca^{2+}-dependent, i.e. the higher the $[Ca^{2+}]_i$, the higher the activity of the calmodulin-dependent PMCP (Scharff & Foder, 1982). Since calmodulin-binding sites are highly conserved among PMCPs (Carafoli, 1994), it is possible that calmodulin is involved in the activation of PMCPs by ATP_o if the ATP_o application can increase $[Ca^{2+}]_i$ before ATP_o-dependent PMCP activation. However, this possibility might be ruled out, because PMCPs were activated even without any trend toward an increase in $[Ca^{2+}]_i$ (Figures 2 and 3). Other pathways for agonist activation of PMCPs remain contradictory. For example, in A7r5 cells, Arg^8-vasopressin stimulates Ca^{2+} extrusion by a phospholipase C (PLC) and protein kinase C (PKC)-independent mechanism (Broad *et al*., 1999), while in U373 MG astrocytoma cells, PKC is involved in histamine stimulation of PMCPs (Young *et al*., 1998). Further investigations are required to gain insight into the mechanism of PMCP activation by ATP_o.

In summary we have demonstrated that, in HEK-293 cells, ATP_o has a dual effect on $[Ca^{2+}]_i$, evoking Ca^{2+} releases from intracellular stores through activation of purinoceptors and extrusion of Ca^{2+} by activating PMCPs. Whilst this extrusion process may be considered as a safety mechanism preventing excessive increases in $[Ca^{2+}]_i$, it might also be important in shaping the intracellular Ca^{2+} signal. These results, together with previous observations on agonist-dependent activation of PMCPs in both excitable and non-excitable cells, suggest that agonist-activated Ca^{2+} extrusion may be a universal component of the agonist-activated Ca^{2+} response. Therefore, agonist-activated PMCP plays an important role in generating a variety of Ca^{2+} signals, the most widely used means of controlling cellular activity (Berridge *et al*., 1998).

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