

ATP inhibits $\text{Ins}(1,4,5)\text{P}_3$ -evoked Ca^{2+} release in smooth muscle via P2Y_1 receptors

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Summary

Adenosine 5'-triphosphate (ATP) mediates a variety of biological functions following nerve-evoked release, via activation of either G-protein-coupled P2Y- or ligand-gated P2X receptors. In smooth muscle, ATP, acting via P2Y receptors (P2YR), may act as an inhibitory neurotransmitter. The underlying mechanism(s) remain unclear, but have been proposed to involve the production of inositol 1,4,5-trisphosphate [$\text{Ins}(1,4,5)\text{P}_3$] by phospholipase C (PLC), to evoke Ca^{2+} release from the internal store and stimulation of Ca^{2+} -activated potassium (K_{Ca}) channels to cause membrane hyperpolarization. This mechanism requires Ca^{2+} release from the store. However, in the present study, ATP evoked transient Ca^{2+} increases in only ~10% of voltage-clamped single smooth muscle cells. These results do not support activation of K_{Ca} as the major mechanism underlying inhibition of smooth muscle activity. Interestingly, ATP inhibited $\text{Ins}(1,4,5)\text{P}_3$ -evoked Ca^{2+} release in cells that did not show a Ca^{2+} rise in response to purinergic activation. The reduction in $\text{Ins}(1,4,5)\text{P}_3$ -evoked Ca^{2+} release was not mimicked by adenosine and therefore, cannot be explained by hydrolysis of ATP to adenosine. The reduction in $\text{Ins}(1,4,5)\text{P}_3$ -evoked Ca^{2+} release was, however, also observed with its primary metabolite, ADP, and blocked by the P2Y_1 R antagonist, MRS2179, and the G protein inhibitor, GDP β S, but not by PLC inhibition. The present study demonstrates a novel inhibitory effect of P2Y_1 R activation on $\text{Ins}(1,4,5)\text{P}_3$ -evoked Ca^{2+} release, such that purinergic stimulation acts to prevent $\text{Ins}(1,4,5)\text{P}_3$ -mediated increases in excitability in smooth muscle and promote relaxation.

Key words: ATP, Smooth muscle, $\text{Ins}(1,4,5)\text{P}_3$, Calcium

Introduction

The purinergic agonist, adenosine 5'-triphosphate (ATP), is an important and ubiquitous extracellular signalling molecule that mediates diverse physiological effects in numerous cell types and is pivotal in regulating smooth muscle activity, ranging from gastrointestinal motility to vascular tone (Abbracchio et al., 2006; Burnstock, 2006; De Man et al., 2003; Gallego et al., 2006). Alterations in ATP signalling are implicated in several pathological conditions of smooth muscle, including inflammatory bowel disease, partial bladder outlet obstruction and hypertension (Burnstock, 2006; Calvert et al., 2001; Neshat et al., 2009) and there is an emerging role of purinergic receptors as therapeutic targets in such conditions. A major regulator of smooth muscle function is the cytoplasmic Ca^{2+} concentration [Ca^{2+}]_c (Berridge et al., 2003; Himpens et al., 1995). Ca^{2+} release from the intracellular Ca^{2+} store, the sarcoplasmic reticulum (SR), provides a significant mechanism by which agonists such as ATP may regulate [Ca^{2+}]_c and thus, smooth muscle activity. Release occurs via two ligand-gated channel–receptor complexes, the inositol 1,4,5-trisphosphate receptor [$\text{Ins}(1,4,5)\text{P}_3\text{R}$] and the ryanodine receptor (RyR) (Bootman et al., 2001; McCarron et al., 2004). In several cell types, such as smooth muscle, $\text{Ins}(1,4,5)\text{P}_3\text{R}$ is the predominant Ca^{2+} release mechanism and is activated by

$\text{Ins}(1,4,5)\text{P}_3$, generated via G-protein- or tyrosine kinase-linked receptor-dependent activation of phospholipase C (PLC) (Bootman et al., 2001; Iacovou et al., 1990; Marks, 1992).

Smooth muscle cells express ligand-gated P2X receptors (P2XR) and G-protein-coupled P2Y receptors (P2YR). Many of the physiological effects of neuronally released ATP in smooth muscle are influenced by the relaxant actions of P2YR (Abbracchio et al., 2006; Burnstock, 2009; Gallego et al., 2006; King et al., 1998), which are largely coupled to $\text{G}\alpha_{\text{q}}$ proteins and thus to the activation of PLC. Indeed, the direct inhibitory response to ATP on smooth muscle has been proposed to involve PLC-mediated phosphoinositide hydrolysis and the subsequent ATP-dependent production of $\text{Ins}(1,4,5)\text{P}_3$ to evoke local Ca^{2+} release near the plasma membrane via $\text{Ins}(1,4,5)\text{P}_3\text{Rs}$. The [Ca^{2+}]_c rise, it is proposed, may activate Ca^{2+} -activated K^+ (K_{Ca}) channels to hyperpolarize the plasma membrane and decrease bulk average [Ca^{2+}]_c (Bakhramov et al., 1996; Burnstock, 1990; Gallego et al., 2006; Koh et al., 1997; Strøbaek et al., 1996a; Zizzo et al., 2006). Yet, the frequency with which ATP-induced [Ca^{2+}]_c rises are observed varies substantially. For example, a recent study reported that [Ca^{2+}]_c rises to ATP were not observed in colonic smooth muscle cells (Kurahashi et al., 2011) and in other preparations approximately one-third of cells failed to respond to ATP with a rise in [Ca^{2+}]_c (Dockrell et al., 2001; Friel and Bean, 1988; Liu et al., 2002) or ATP-induced Ca^{2+} responses may be either limited or not observed at all (Bardoni et al., 1997; Fukushi, 1999; Kimelberg et al., 1997; Oshimi et al., 1999). These observations suggest that activation of K_{Ca} channels, following Ca^{2+} store

release, may not be a universal mechanism of P2YR-mediated signal transduction.

In the present investigation, the effect of ATP on Ins(1,4,5) P_3 R-mediated Ca^{2+} release was initially examined to characterise the release of Ca^{2+} from the SR by ATP. Freshly isolated single colonic smooth muscle cells were selected, which provide a robust model for studying Ins(1,4,5) P_3 R activity. The use of flash photolysis of caged Ins(1,4,5) P_3 minimised the activation of second messenger systems, to give a clearer understanding of the control of Ca^{2+} release via Ins(1,4,5) P_3 R. The study shows that ATP failed to evoke a rise in $[Ca^{2+}]_c$ in most cells, but instead inhibited Ins(1,4,5) P_3 -evoked Ca^{2+} release. This effect was dependent on G-protein-coupled P2Y $_1$ R activation, but independent of PLC. Thus we propose here, a novel mechanism by which ATP regulates Ins(1,4,5) P_3 -evoked Ca^{2+} release in smooth muscle.

Results

Initial experiments were designed to characterise the release of store Ca^{2+} by ATP. Unexpectedly, however, ATP (1 mM) transiently increased $[Ca^{2+}]_c$ ($\Delta F/F_0 = 2.0 \pm 0.4$) in only ~10% of single smooth muscle cells (Fig. 1A, 9 of 87 cells) voltage-clamped at -70 mV, but did not evoke any resolvable current responses in these cells (Fig. 1A). ATP failed to evoke Ca^{2+} release in the remaining cells (Fig. 1B). The absence of a Ca^{2+} rise in response to ATP cannot be explained by clamping the cells at -70 mV since the agonist also failed to evoke Ca^{2+} release at -30 mV (Fig. 1C, $n=11$).

ATP inhibits Ins(1,4,5) P_3 R-mediated Ca^{2+} release

We hypothesised that ATP may inhibit Ins(1,4,5) P_3 R-mediated Ca^{2+} signalling in cells that did not display an increase in $[Ca^{2+}]_c$ in response to purinergic stimulation. Therefore, the effect of ATP on Ins(1,4,5) P_3 R-mediated Ca^{2+} release from the store was examined. In these experiments, the Ins(1,4,5) P_3 generating agonist, carbachol (CCh), was applied in a Ca^{2+} -free bath solution to prevent Ca^{2+} influx and record purely store Ca^{2+} release. CCh (100 μ M) elicited reproducible transient $[Ca^{2+}]_c$ increases in single myocytes (Fig. 2A) which is routinely observed in ~50% of cells. ATP (1 mM), applied 2 seconds

before CCh, failed to release Ca^{2+} from the store, but significantly ($P < 0.05$) inhibited CCh-evoked $[Ca^{2+}]_c$ increases ($\Delta F/F_0$) by $76 \pm 7\%$ (from 2.28 ± 0.2 to 0.61 ± 0.1 , $n=6$, Fig. 2A). Since ATP did not release Ca^{2+} , neither activation of K_{Ca} channels nor depletion of the store of Ca^{2+} can explain these results. In fact, if the reduction in Ins(1,4,5) P_3 R-mediated Ca^{2+} release arose by depletion of the store of Ca^{2+} , then ATP would be expected to inhibit RyR-mediated Ca^{2+} release, because both Ins(1,4,5) P_3 R and RyR access a single common Ca^{2+} store in this smooth muscle preparation (McCarron and Olson, 2008). Hence, SR Ca^{2+} release was evoked by the RyR activator, caffeine (10 mM), which reproducibly increased $[Ca^{2+}]_c$ (Fig. 2B). ATP (1 mM), applied 2 seconds before caffeine, did not alter caffeine-evoked $[Ca^{2+}]_c$ increases ($[\Delta F/F_0]$ from 2.01 ± 0.4 (control) compared with 2.04 ± 0.3 (following ATP), $n=4$). Together, the results suggest that ATP inhibits Ins(1,4,5) P_3 R-mediated Ca^{2+} signalling.

The inhibition of CCh-evoked Ca^{2+} release by ATP may arise from either inhibition of Ins(1,4,5) P_3 synthesis or alternatively, Ins(1,4,5) P_3 R-mediated Ca^{2+} release. To distinguish between these possibilities, Ins(1,4,5) P_3 R were activated *directly* using caged Ins(1,4,5) P_3 which obviates the synthesis of Ins(1,4,5) P_3 . In these experiments, photo release of caged Ins(1,4,5) P_3 elicited reproducible transient $[Ca^{2+}]_c$ elevations in voltage-clamped single myocytes (Fig. 2C). Again, ATP (1 mM), applied 2 seconds before Ins(1,4,5) P_3 , significantly ($P < 0.05$) decreased Ins(1,4,5) P_3 -evoked $[Ca^{2+}]_c$ increases ($\Delta F/F_0$) by $87 \pm 5\%$ (from 1.17 ± 0.04 to 0.15 ± 0.01 , $n=5$, Fig. 2C). A similar inhibition was observed when ATP was applied at up to 10-fold lower concentrations. Since the $[Ca^{2+}]_c$ increase evoked by photolysis of caged Ins(1,4,5) P_3 does not require Ins(1,4,5) P_3 synthesis, these results suggest that ATP inhibited Ins(1,4,5) P_3 R-mediated Ca^{2+} release.

ATP inhibits Ins(1,4,5) P_3 R-mediated Ca^{2+} release via P2Y $_1$ R

A structural analogue of ATP, adenosine 5'-diphosphate (ADP, 1 mM), also significantly decreased Ins(1,4,5) P_3 -evoked $[Ca^{2+}]_c$ increases ($\Delta F/F_0$) by $76 \pm 7\%$ (from 1.76 ± 0.3 to 0.40 ± 0.1 , $n=3$, $P < 0.05$, Fig. 3A) indicating that the purinergic inhibitory response may be mediated by P2YR. Again, a similar inhibition was observed when ADP was applied at up to 10-fold lower concentrations. In

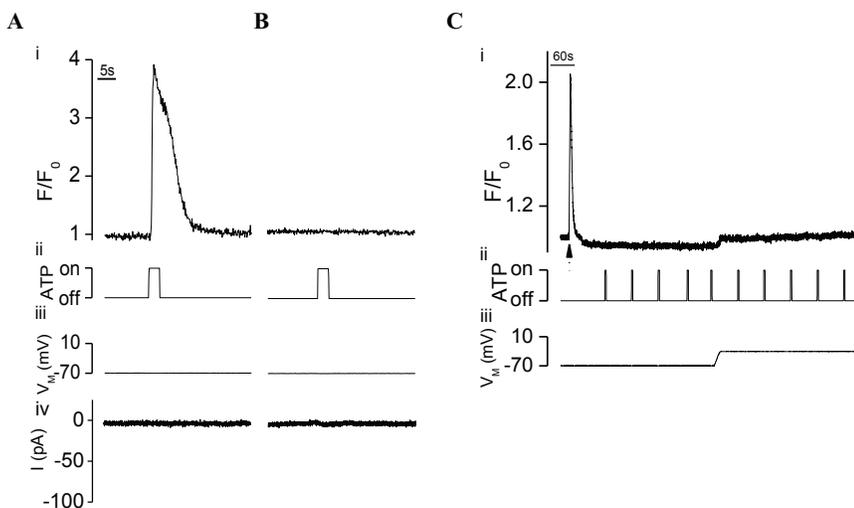


Fig. 1. ATP evoked $[Ca^{2+}]_c$ rises in only a minority of single voltage-clamped myocytes. (A,B) ATP (1 mM by pressure ejection; ii) evoked transient $[Ca^{2+}]_c$ increases in only ~10% of cells voltage-clamped at -70 mV (iii) as indicated by F/F_0 (Ai) and did not elicit any discernible current responses in these cells (iv). ATP failed to evoke Ca^{2+} release in the remaining cells (Bi). (C) Although photolysed caged Ins(1,4,5) P_3 (\blacktriangle) increased $[Ca^{2+}]_c$ (i), ATP (1 mM by pressure ejection; ii), however, failed to evoke $[Ca^{2+}]_c$ rises as indicated by F/F_0 (i) in cells voltage clamped at -30 mV (iii).

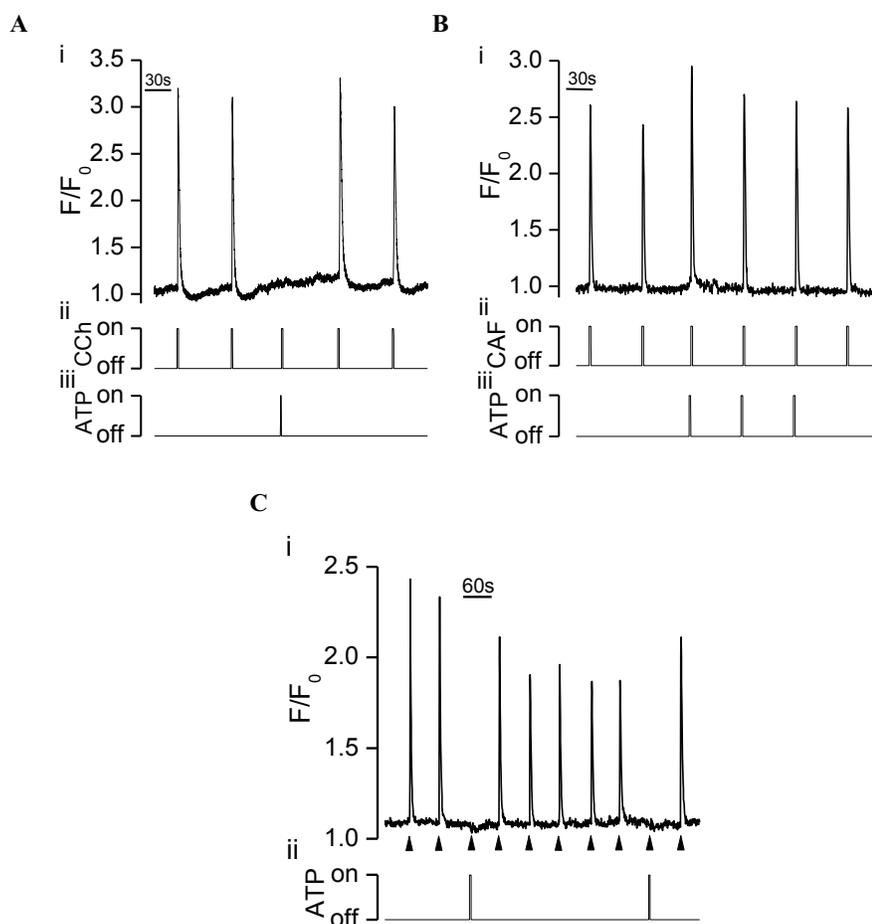


Fig. 2. ATP inhibited Ins(1,4,5) P_3 R- but not RyR-mediated $[Ca^{2+}]_c$ increases in voltage-clamped single myocytes. (A) The Ins(1,4,5) P_3 -generating agonist, CCh (100–250 μ M by pressure ejection; ii), increased $[Ca^{2+}]_c$ (i) as indicated by F/F_0 . CCh was applied in a Ca^{2+} -free bath solution to ensure that $[Ca^{2+}]_c$ rises arose by Ca^{2+} release from the SR. ATP (1 mM), applied by pressure ejection 2 s before CCh (iii), decreased CCh-evoked $[Ca^{2+}]_c$ increases. (B) Ca^{2+} release was evoked using the RyR activator, caffeine. Caffeine (CAF, 10 mM by pressure ejection; ii) increased $[Ca^{2+}]_c$ (i) as indicated by F/F_0 . ATP (1 mM), applied by pressure ejection 2 s before caffeine (iii), did not alter caffeine-evoked $[Ca^{2+}]_c$ increases. (C) Photo release of caged Ins(1,4,5) P_3 (\blacktriangle), which activated Ins(1,4,5) P_3 R directly, increased $[Ca^{2+}]_c$ (i) as indicated by F/F_0 . ATP (1 mM), applied by pressure ejection 2 s before Ins(1,4,5) P_3 (ii), decreased Ins(1,4,5) P_3 -evoked $[Ca^{2+}]_c$ increases ($V_M - 70$ mV).

contrast, adenosine (1 mM), applied 2 seconds before Ins(1,4,5) P_3 , did not however, alter Ins(1,4,5) P_3 -evoked $[Ca^{2+}]_c$ rises ($\Delta F/F_0$ from 1.95 ± 0.2 to 2.02 ± 0.2 , $n=4$, Fig. 3B), excluding a role for adenosine receptors. Consistent with these data, the inhibitory effect of ATP on Ins(1,4,5) P_3 -evoked Ca^{2+} release was blocked by the selective P2Y₁R antagonist, MRS2179 (Boyer et al., 1998). ATP (1 mM), applied 2 seconds before Ins(1,4,5) P_3 , significantly ($P < 0.05$) decreased Ins(1,4,5) P_3 -evoked Ca^{2+} release ($\Delta F/F_0$ from 1.12 ± 0.04 to 0.38 ± 0.09 , $n=5$, Fig. 4). The inhibitory response to ATP was abolished by MRS2179 (10 μ M) ($\Delta F/F_0$ 1.17 ± 0.04 , $n=5$, $P > 0.05$ compared to control, Fig. 4), confirming a role of P2Y₁R in this response.

Role of G proteins in ATP-evoked inhibition of Ins(1,4,5) P_3 R-mediated Ca^{2+} release

Although P2Y₁R classically couple to $G\alpha_{q/11}$ G proteins (Abbracchio et al., 2006), agonist stimulation of P2Y₁R can lead, in some instances, to physiological responses that are independent of G protein activation (Fam et al., 2005; Hall et al., 1998; Lee et al., 2003; O'Grady et al., 1996). The potential role of G proteins in the ATP-mediated inhibition of Ins(1,4,5) P_3 -evoked Ca^{2+} release was therefore, examined using the membrane-impermeable G protein inhibitor, GDP 5'-O-(2-thio-diphosphate) (GDP β S), introduced into the cell via the patch electrode (Fig. 5). GDP β S (10 μ M) abolished the inhibitory effect of ATP on Ins(1,4,5) P_3 -evoked Ca^{2+} release [$\Delta F/F_0$ from 1.79 ± 0.1 (control)

compared with 1.85 ± 0.1 (following ATP), $n=8$], indicating that the inhibitory response to ATP requires G protein activation.

ATP-evoked inhibition of Ins(1,4,5) P_3 R-mediated Ca^{2+} release does not require PLC

G-protein-coupled P2Y₁R-mediated effects are mainly mediated by activation of PLC (Abbracchio et al., 2006). Thus, to investigate the role of PLC in mediating the ATP-dependent inhibition of Ins(1,4,5) P_3 -evoked Ca^{2+} release, we examined the effects of the PLC inhibitor, edelfosine. ATP (1 mM), applied 2 seconds before Ins(1,4,5) P_3 , significantly ($P < 0.05$) decreased Ins(1,4,5) P_3 -evoked $[Ca^{2+}]_c$ increases ($\Delta F/F_0$ from 2 ± 0.39 to 0.14 ± 0.01 , $n=4$, Fig. 6A) and remained effective in decreasing Ins(1,4,5) P_3 -evoked $[Ca^{2+}]_c$ rises in the presence of the PLC inhibitor, edelfosine (10 μ M) ($\Delta F/F_0$ from 2 ± 0.39 to 0.17 ± 0.01 , $n=4$, $P < 0.05$, Fig. 6A). On the other hand, edelfosine significantly inhibited Ca^{2+} release evoked by the Ins(1,4,5) P_3 generating agonist, CCh, ($\Delta F/F_0$) by $93 \pm 2\%$ (from 1.88 ± 0.3 to 0.12 ± 0.03 , $n=6$, $P < 0.05$; Fig. 6B), a result that is consistent with the proposed mechanism of action of the drug (Powis et al., 1992).

Having previously demonstrated that the commonly used PLC inhibitor, U-73122, exerts non-selective effects on SR Ca^{2+} pumps in this tissue (MacMillan and McCarron, 2010), it was necessary to confirm the selectivity of edelfosine as a PLC inhibitor. Edelfosine affected neither Ins(1,4,5) P_3 - [$\Delta F/F_0$ from 1.70 ± 0.3 (control) to 1.73 ± 0.3 (edelfosine), $n=4$, Fig. 7A] or RyR- [$\Delta F/F_0$ from

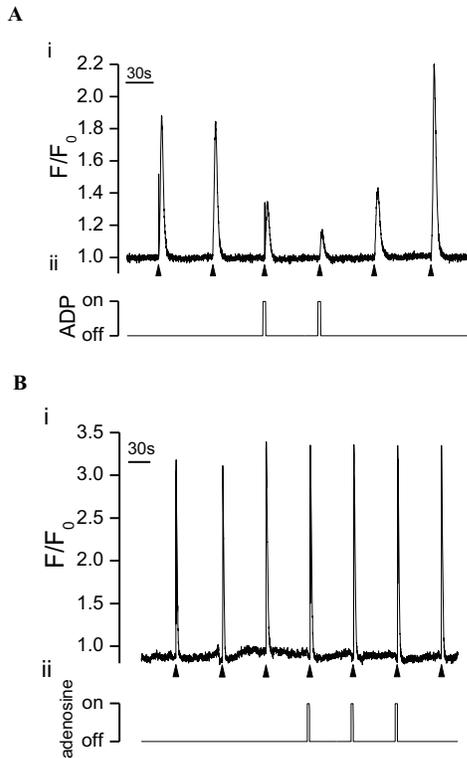


Fig. 3. The inhibitory effect of ATP on Ins(1,4,5) P_3 -evoked $[Ca^{2+}]_c$ increases was mimicked by ADP, but not by adenosine in voltage-clamped single myocytes. At -70 mV photolysed caged Ins(1,4,5) P_3 (\blacktriangle) increased $[Ca^{2+}]_c$ (i) as indicated by F/F_0 . ADP (1 mM; **A**) and adenosine (1 mM; **B**) were each applied by pressure ejection 2 s before Ins(1,4,5) P_3 (ii). ADP decreased, whereas adenosine did not alter Ins(1,4,5) P_3 -evoked $[Ca^{2+}]_c$ increases.

2.84 ± 0.1 (control) to 3.06 ± 0.2 (edelfosine), $n=4$, Fig. 7B] evoked Ca^{2+} release nor the rate of Ca^{2+} removal from the cytoplasm following release. The 80–20% decay interval following Ins(1,4,5) P_3 - and caffeine-evoked Ca^{2+} release was 3.1 ± 0.4 s and

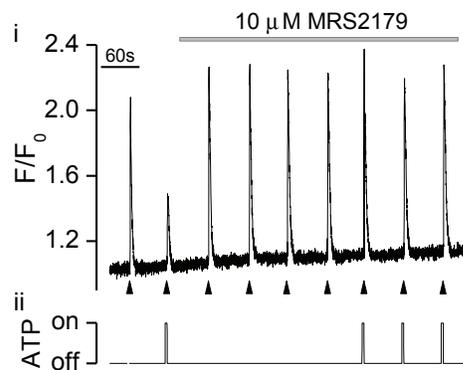


Fig. 4. The inhibitory response to ATP was blocked by the P2Y $_1$ R antagonist, MRS2179, in voltage-clamped single myocytes. At -70 mV photolysed caged Ins(1,4,5) P_3 (\blacktriangle) increased $[Ca^{2+}]_c$ (i) as indicated by F/F_0 . ATP (1 mM), applied by pressure ejection 2 s before Ins(1,4,5) P_3 (ii), decreased Ins(1,4,5) P_3 -evoked $[Ca^{2+}]_c$ increases. The inhibitory effect of ATP on Ins(1,4,5) P_3 -evoked Ca^{2+} release was blocked by MRS2179 (10 μ M). MRS2179 was perfused into the solution bathing the cells.

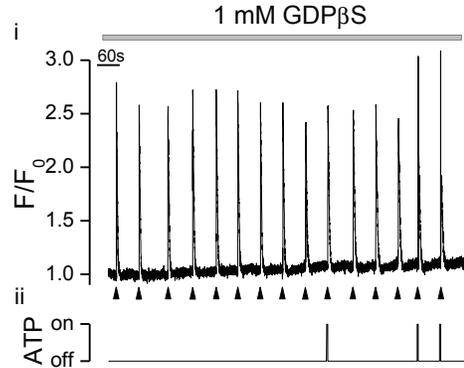


Fig. 5. The G protein inhibitor, GDP β S, blocked the inhibitory effect of ATP on Ins(1,4,5) P_3 -evoked $[Ca^{2+}]_c$ increases in voltage-clamped single myocytes. Photolysed caged Ins(1,4,5) P_3 (\blacktriangle) increased $[Ca^{2+}]_c$ (i) as indicated by F/F_0 . GDP β S (1 mM, introduced via the patch pipette; pretreated for 7–15 mins) blocked the inhibitory effect of ATP (1 mM by pressure ejection, applied 2 s before Ins(1,4,5) P_3 ; ii) on Ins(1,4,5) P_3 -evoked Ca^{2+} release ($V_M -70$ mV).

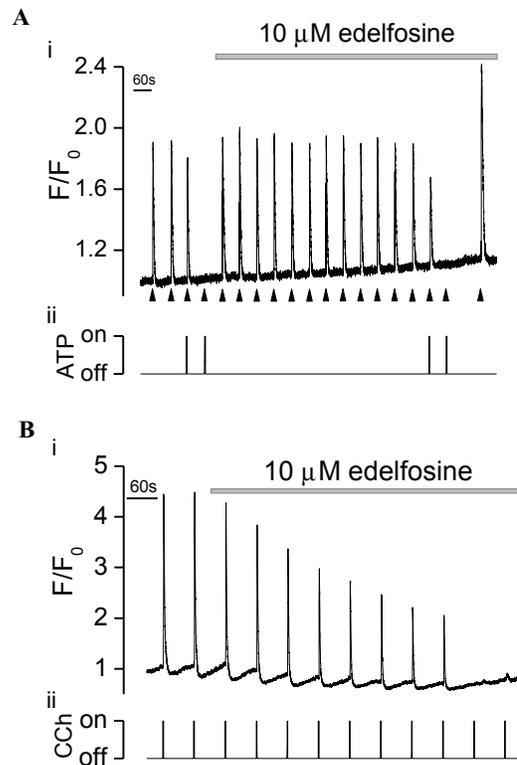


Fig. 6. The PLC inhibitor, edelfosine, did not alter the ATP-mediated reduction in Ins(1,4,5) P_3 -evoked Ca^{2+} release in voltage-clamped single myocytes. (**A**) Photolysed caged Ins(1,4,5) P_3 (\blacktriangle) increased $[Ca^{2+}]_c$ (i) as indicated by F/F_0 . ATP (1 mM by pressure ejection, applied 2 s before Ins(1,4,5) P_3 ; ii) decreased Ins(1,4,5) P_3 -evoked $[Ca^{2+}]_c$ increases. Edelfosine (10 μ M, $n=4$) did not alter the inhibitory effect of ATP on Ins(1,4,5) P_3 -evoked Ca^{2+} release ($V_M -70$ mV). (**B**) The Ins(1,4,5) P_3 -generating agonist, CCh (100–250 μ M by pressure ejection; ii), increased $[Ca^{2+}]_c$ (i) as indicated by F/F_0 . Edelfosine (10 μ M) decreased CCh-evoked $[Ca^{2+}]_c$ increases (i), which is consistent with the proposed mechanism of action of the drug. Edelfosine was perfused into the solution bathing the cells.

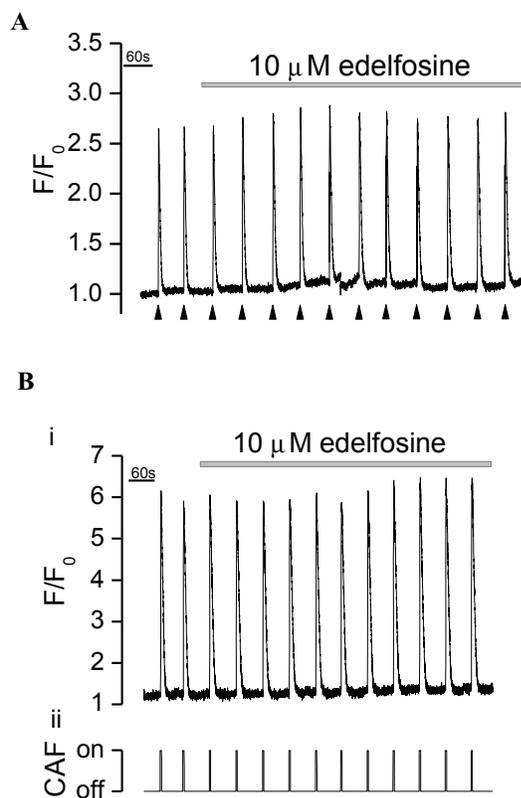


Fig. 7. The PLC inhibitor, edelfosine, neither altered $\text{Ins}(1,4,5)\text{P}_3\text{R}$ - nor RyR-mediated $[\text{Ca}^{2+}]_c$ increases in voltage-clamped single myocytes. Photolysed caged $\text{Ins}(1,4,5)\text{P}_3$ (\blacktriangle ; A) and the RyR activator, caffeine (CAF, 10 mM by pressure ejection; Bii), each increased $[\text{Ca}^{2+}]_c$ as indicated by F/F_0 . Edelfosine ($10 \mu\text{M}$) neither altered $\text{Ins}(1,4,5)\text{P}_3$ -evoked $[\text{Ca}^{2+}]_c$ increases (A) nor caffeine-evoked $[\text{Ca}^{2+}]_c$ increases (Bi; $V_M -70$ mV). Edelfosine was perfused into the solution bathing the cells.

3.6 ± 0.5 s in controls and 3.2 ± 0.5 s and 3.4 ± 0.5 s in edelfosine ($n=4$ and 4), respectively.

In conclusion, these results suggest that $\text{P2Y}_1\text{R}$ inhibition of $\text{Ins}(1,4,5)\text{P}_3$ -evoked Ca^{2+} release is G-protein dependent. A product of the PLC-mediated signalling pathway is not responsible for ATP-dependent inhibition of Ca^{2+} release.

Discussion

At present the mechanism by which ATP mediates smooth muscle relaxation is contentious. The present study demonstrates that ATP induced Ca^{2+} release in only a minority of smooth muscle cells. Instead, in cells that did not show a $[\text{Ca}^{2+}]_c$ rise in response to purinergic stimulation, ATP inhibited $\text{Ins}(1,4,5)\text{P}_3$ -evoked Ca^{2+} release. This response was mediated by $\text{P2Y}_1\text{R}$ and was G protein dependent, but did not involve activation of PLC. Thus we propose a novel mechanism for smooth muscle relaxation whereby stimulation of a G-protein-coupled receptor inhibits $\text{Ins}(1,4,5)\text{P}_3$ -evoked Ca^{2+} release to promote relaxation.

Our initial experiments found that ATP increased $[\text{Ca}^{2+}]_c$ in only $\sim 10\%$ of voltage-clamped single smooth muscle cells. The absence of an increase in $[\text{Ca}^{2+}]_c$ in response to ATP was not influenced by changes in membrane potential and so, cannot be accounted for by voltage control of $\text{Ins}(1,4,5)\text{P}_3$ production or $\text{Ins}(1,4,5)\text{P}_3$ -dependent Ca^{2+} release (Billups et al., 2006; Ganitkevich and Isenberg, 1993; Itoh et al., 1992; Mahaut-

Smith et al., 1999; Martinez-Pinna et al., 2004; Mason et al., 2000). This is intriguing because it is generally believed that purinergic activation leads to the mobilization of $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} stores and hyperpolarization of smooth muscle (Gallego et al., 2006; Hata et al., 2000; Koh et al., 1997; Lecci et al., 2002; Strøbaek et al., 1996a; Strøbaek et al., 1996b; Zizzo et al., 2006). Evidently, a requirement for this mechanism is Ca^{2+} release from the internal store. However, recent evidence from another group, although having previously been in support of this model, also suggest that ATP failed to elicit increased responses in isolated smooth muscle cells (Kurahashi et al., 2011), while the relatively small purinergic K_{Ca} current evoked by ATP or the inability to elicit K_{Ca} currents in smooth muscle (Kurahashi et al., 2011) is inconsistent with previous studies supporting the concept of purinergic signalling via intracellular Ca^{2+} release and subsequent activation of K_{Ca} channels in smooth muscle. Thus, it appears that only a sub-population of smooth muscle cells may be organised to hyperpolarize in response to ATP.

Our results provide an additional mechanism by which ATP may evoke smooth muscle relaxation, that is by modulation of $\text{Ins}(1,4,5)\text{P}_3\text{R}$ -mediated Ca^{2+} signalling. ATP suppressed $[\text{Ca}^{2+}]_c$ increases evoked by CCh ($\text{Ins}(1,4,5)\text{P}_3$ generating agonist) and by photolysis of a caged form of the inositide, to release $\text{Ins}(1,4,5)\text{P}_3$, in all cells that failed to respond to purinergic activation with an increase in $[\text{Ca}^{2+}]_c$. Since ATP did not evoke Ca^{2+} release from the store, neither K_{Ca} channel activation nor Ca^{2+} store depletion can explain the inhibitory response to ATP. Neither can the inhibitory response to ATP be attributed to inhibition of $\text{Ins}(1,4,5)\text{P}_3$ synthesis since $\text{Ins}(1,4,5)\text{P}_3$ was released by photolysis and so synthesis was not required. Consistent with our data, ATP exerted an inhibitory effect on muscarinic-mediated increases in intracellular Ca^{2+} release in platelets and acinar cells (Fukushi, 1999; Hurley et al., 1993; Jørgensen et al., 1995; Métioui et al., 1996; Soslau et al., 1995). These studies also concluded that the mechanism underlying the ATP-dependent inhibition of $\text{Ins}(1,4,5)\text{P}_3\text{R}$ -mediated Ca^{2+} release appears to extend beyond the plasma membrane, but is not due to depletion of the store. ATP, at a concentration which fully inhibits Ca^{2+} store release, affected neither the interaction of agonists with their respective cell surface receptors nor Ca^{2+} store content (Hurley et al., 1993; Jørgensen et al., 1995).

In the present study the ATP-mediated inhibitory response was mimicked by ADP and blocked by the $\text{P2Y}_1\text{R}$ antagonist, MRS2179, in accordance with previous studies which demonstrate that ATP mediates its relaxant actions through the activation of $\text{P2Y}_1\text{R}$ (Brizzolara and Burnstock, 1991; Gallego et al., 2006; Gallego et al., 2008; Mathieson and Burnstock, 1985). Furthermore, ATP was not being hydrolysed to adenosine by ectonucleotidases (Guibert et al., 1998; Kadowaki et al., 2000; Liu et al., 1989; Moody et al., 1984), as demonstrated by the lack of effect of the nucleoside on $\text{Ins}(1,4,5)\text{P}_3$ -evoked Ca^{2+} release. The inability of ATP to elicit inward currents in these cells also excludes a role of P2XR . Thus, it appears that the inhibitory response to ATP may be ascribed to the inhibition of $\text{Ins}(1,4,5)\text{P}_3$ -evoked Ca^{2+} release via $\text{P2Y}_1\text{R}$ activation.

The inhibitory effects of ATP in these experiments were abolished by the G protein inhibitor, $\text{GDP}\beta\text{S}$, indicating that activation of $\text{P2Y}_1\text{R}$ led to downstream stimulation of G proteins. This is important as in some cases $\text{P2Y}_1\text{R}$ stimulation can evoke responses that are G protein independent (Fam et al., 2005; Hall et al., 1998; Lee et al., 2003; O'Grady et al., 1996). However,

P2Y₁R characteristically couple to the G $\alpha_{q/11}$ G proteins (Abbracchio et al., 2006), which in turn stimulate PLC. Thus, we focused on a role of PLC in this inhibition. However, the actions of ATP were unaffected by the PLC inhibitor, edelfosine, at a concentration that inhibited CCh-evoked [Ca²⁺]_c increases. This is consistent with our conclusion that ATP did not act by inhibiting Ins(1,4,5)P₃ synthesis, as has been proposed in glandular cells (Jørgensen et al., 1995; Métioui et al., 1996). We have also demonstrated that the PLC inhibitor used in the former study to confirm a contribution of PLC attenuates Ins(1,4,5)P₃-evoked Ca²⁺ release by depleting the store of Ca²⁺, a mechanism unrelated to inhibition of PLC activity (MacMillan and McCarron, 2010).

A likely candidate is activation of the cAMP second messenger pathway since P2Y₁R can couple to cAMP pathways in addition to phosphoinositide pathways (del Puerto et al., 2012). However, studies carried out previously by us (Flynn et al., 2001) have demonstrated that cAMP does not regulate Ins(1,4,5)P₃R activity in these cells. Elevation of intracellular cAMP levels by either forskolin (which stimulates adenylate cyclase) or IBMX (which inhibits phosphodiesterase) did not significantly affect Ins(1,4,5)P₃-evoked Ca²⁺ release. We also examined the involvement of kinase activation, since Ins(1,4,5)P₃R can be greatly affected by phosphorylation. However, Ins(1,4,5)P₃-evoked Ca²⁺ release was not altered by the broad spectrum kinase inhibitor, H7 (100 μ M; data not shown), and ATP remained effective in decreasing Ins(1,4,5)P₃-evoked Ca²⁺ release in the presence of the broad spectrum PI3K (phosphoinositide 3-kinase) inhibitor, wortmannin (10 μ M; data not shown), which is consistent with our previous work confirming that neither kinase (i.e. PKA, PKG and PKC) inhibition nor activation modulated Ins(1,4,5)P₃-mediated Ca²⁺ release (McCarron et al., 2002; McCarron et al., 2004).

The intracellular signalling cascade downstream of G protein receptor-coupled (GPCR) activation clearly exhibits greater diversity than previously appreciated. Purinergic signalling is further complicated by the fact that P2Y₁R may couple directly to more than one G protein isoform (Hermans, 2003; Ostrom and Insel, 2004; Rashid et al., 2004) and each G protein isoform may also activate multiple signalling pathways (Maudsley et al., 2005; Ostrom, 2002), resulting in a wide range of cellular responses (Boeynaems et al., 2000; Communi et al., 1997; Murthy and Makhlof, 1998; Neary et al., 1999; Qi et al., 2001). This may also be influenced by the physical proximity of the P2Y₁R and its signalling components, which can be partitioned into specialised membrane microdomains. For example, GPCR (Norambuena et al., 2008), G proteins (Oh and Schnitzer, 2001) and G protein effector enzymes (Ostrom et al., 2002) localise to these structures. Moreover, membrane organisation may be critically important for ATP to release store Ca²⁺ in a subpopulation of cells, despite being a potent inhibitor of Ins(1,4,5)P₃-mediated Ca²⁺ release. Thus, the microenvironment may permit selective cellular responses (Delmas et al., 2002; Haley et al., 2000; Segawa et al., 2002; Takemura and Horio, 2005) by providing a mechanism for achieving specificity of the receptor-mediated Ins(1,4,5)P₃ pathway.

The complexity of purinergic signalling pathways constitute a formidable obstacle to the complete understanding of the molecular mechanism of this inhibition. Equally perplexing is the very rapid speed of onset of the inhibition of Ca²⁺ release (i.e. 2 seconds) which likely excludes signalling through slower second messenger systems. We speculate that a such a rapid

time course of inhibition may more likely be explained by the modulation of proteins which interact directly with the Ins(1,4,5)P₃R such as IRBIT [Ins(1,4,5)P₃R binding protein released with Ins(1,4,5)P₃] or Ca²⁺ binding proteins (CaBP), and which can inhibit the Ca²⁺ release activity of the Ins(1,4,5)P₃R channel. Further studies are necessary to characterise the precise molecular mechanism underlying inhibition of Ins(1,4,5)P₃R activity by ATP, but may prove challenging.

In conclusion, purinergic relaxation of smooth muscle has largely been attributed to the mobilization of Ins(1,4,5)P₃-sensitive Ca²⁺ stores to hyperpolarize the cell, but we propose quite the reverse, that P2Y₁R activation at the plasma membrane inhibits direct activation of Ins(1,4,5)P₃R in the SR by Ins(1,4,5)P₃ to inhibit smooth muscle activity. Smooth muscle may be under dual, inhibitory control by purinergic nerves. Thus, we report here for the first time that ATP can regulate the mobilization of Ins(1,4,5)P₃-sensitive Ca²⁺ stores by two diametrically opposed mechanisms which are mediated by the same receptor. Although the biological significance of this divergence in ATP signalling is not yet clear, it is tempting to speculate that this novel mode of regulation may reflect complementary mechanisms of smooth muscle relaxation which have developed to acquire the ability of coordinated contraction and relaxation under a variety of circumstances and in response to a variety of stimuli. The existence of multiple regulatory mechanisms represents an advantageous strategy that allows impairment of one or more of its components. It is therefore understandable that several complementary and cooperating mechanisms are in place to control smooth muscle relaxation. Nevertheless, this study highlights the differential ability of ATP to regulate Ins(1,4,5)P₃-mediated mobilization of intracellular Ca²⁺ which may provide the molecular basis for such heterogeneous responses to ATP which are recognised in a variety of cells.

Materials and Methods

Cell isolation

All animal care and experimental procedures complied with the Animal (Scientific Procedures) Act UK 1986. Male guinea pigs (500–700 g) were sacrificed by cervical dislocation and immediate exsanguination. A segment of distal colon was immediately removed and transferred to an oxygenated (95% O₂, 5% CO₂) physiological saline solution of the following composition (mM): NaCl 118.4, NaHCO₃ 25, KCl 4.7, NaH₂PO₄ 1.13, MgCl₂ 1.3, CaCl₂ 2.7 and glucose 11 (pH 7.4). Following the removal of the mucosa from this tissue, single smooth muscle cells, from circular muscle, were isolated using a two-step enzymatic dissociation protocol (McCarron and Muir, 1999), stored at 4 °C and used the same day. All experiments and loading of cells with fluorescent dyes were conducted at room temperature (20 ± 2 °C).

Electrophysiological experiments

Cells were voltage clamped using conventional tight seal whole-cell recording (MacMillan and McCarron, 2010; Rainbow et al., 2009). The composition of the extracellular solution was (mM): Na glutamate 80, NaCl 40, tetraethylammonium chloride 20, MgCl₂ 1.1, CaCl₂ 3, HEPES 10 and glucose 30 (pH 7.4 adjusted with NaOH 1 M). The Ca²⁺-free extracellular solution additionally contained (mM): MgCl₂ 3 (substituted for Ca²⁺); and EGTA, 1. The pipette solution contained (mM): Cs₂SO₄ 85, CsCl 20, MgCl₂ 1, HEPES 30, pyruvic acid 2.5, malic acid 2.5, KH₂PO₄ 1, MgATP 3, creatine phosphate 5, guanosine triphosphate 0.5, fluo-3 penta-ammonium salt 0.1 and caged Ins(1,4,5)P₃-trisodium salt 0.025 (pH 7.2 adjusted with CsOH 1 M). Whole cell currents were amplified by an Axopatch amplifier (Axon instruments, Union City, CA, USA), low pass filtered at 500 Hz (8-pole Bessel filter; Frequency Devices, Haverhill, MA, USA), and digitally sampled at 1.5 kHz using a Digidata interface, pCLAMP software (version 6.0.1, Axon Instruments) and stored on a personal computer for analysis.

[Ca²⁺]_c measurement

[Ca²⁺]_c was measured as fluorescence using either the membrane-impermeable dye fluo-3 (penta-ammonium salt), introduced into the cell via the patch pipette (MacMillan and McCarron, 2009), or the membrane-permeable dye, fluo-3

acetoxymethyl ester (AM) (McCarron et al., 2004; Rainbow et al., 2009). Where $[\text{Ca}^{2+}]_i$ measurements were made using the AM dye, cells were loaded with fluo-3 AM (10 μM) in the presence of wortmannin (10 μM ; to prevent contraction) for 30 minutes prior to the beginning of the experiment ($n=10$). Fluorescence was quantified using a microfluorimeter which consisted of an inverted microscope (Nikon, Surrey, UK) and a photomultiplier tube with a bi-alkali photo cathode. Fluo-3 was excited at 488 nm (bandpass 9 nm) by a PTI Delta Scan (Photon Technology International Inc., London, UK) through the epi-illumination port of the microscope (using one arm of a bifurcated quartz fibre optic bundle). Excitation light was passed through a field stop diaphragm to reduce background fluorescence and reflected off a 505 nm long-pass dichroic mirror. Emitted light was guided through a 535 nm barrier filter (bandpass 35 nm) to a photomultiplier in photon counting mode (Photon Technology International Inc., London, UK). Interference filters and dichroic mirrors were obtained from Glen Spectra (London, UK).

Caged $\text{Ins}(1,4,5)\text{P}_3$ was photolysed to the uncaged compound by ultraviolet light, which was selected by passing the output of a xenon flash lamp (Rapp Optoelektronik, Hamburg, Germany) through a UG-5 filter to select UV light and merging into the excitation light path of the microfluorimeter using a quartz bifurcated fibre optic bundle. The nominal flash lamp energy was 57 mJ, measured at the output of the fibre optic bundle and the flash duration was ~ 1 ms.

Statistical analysis

Changes in $[\text{Ca}^{2+}]_i$ were expressed as the ratio (F/F_0) of fluorescence counts (F) relative to baseline (control) values (taken as 1) before stimulation (F_0). Summarised data are expressed as mean \pm s.e.m. for n cells. Student's paired t -tests were applied to test and control conditions; a value of $P < 0.05$ was considered significant.

Materials

Caged $\text{Ins}(1,4,5)\text{P}_3$ -trisodium salt and fluo-3 AM were each purchased from Invitrogen (Paisley, UK). Fluo-3 penta-ammonium salt was purchased from TEF labs (Austin, Texas, USA). Edelfosine and MRS2179 were purchased from Tocris Bioscience (Bristol, UK). Papain and collagenase were purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). All other reagents were purchased from Sigma (Poole, Dorset, UK). $\text{Ins}(1,4,5)\text{P}_3$ was released from its caged compound by flash photolysis. ATP (100 μM –1 mM), ADP (100 μM –1 mM), adenosine (1 mM), carbachol (100–250 μM) and caffeine (10 mM) were each applied by hydrostatic pressure ejection using a pneumatic pump (PicoPump PV 820, World Precision Instruments, Stevenage, Herts, UK). With pressure ejection, the concentration of the ejected drug at the cell is unknown, but will be significantly lower than that in the pipette owing to dilution in the bathing solution. Possible ejection artefacts were excluded by pressure ejection of the vehicle solution alone. The concentration of GDP β S and caged, non-photolysed $\text{Ins}(1,4,5)\text{P}_3$ refers to that in the pipette. ATP, ADP, adenosine, carbachol and caffeine were each dissolved in extracellular bathing solution. Edelfosine was dissolved in water and GDP β S was dissolved in pipette solution. MRS2179 was dissolved in DMSO (final bath concentration of the solvent, 0.05%, was by itself ineffective). MRS2179 (10 μM) and edelfosine (10 μM) were each perfused into the solution bathing the cells (~ 5 ml per min).

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