Release of Ca^{2+} from intracellular store in smooth muscle cells of rat portal vein by ATP-induced Ca^{2+} entry

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1 The action of adenosine 5'-triphosphate (ATP, 10 μM) was studied in single patch-clamped smooth muscle cells of rat portal vein where the free internal Ca^{2+} concentration in the cell (Ca_i) was estimated by the emission from the dye indo-1.

2 In the presence of 20 μM gallopamil (D600), a blocker of voltage-dependent Ca^{2+} channels, ATP applied to cells held at a holding potential of -60 mV evoked a transient inward current and an increase in Ca_i .

3 The rise in Ca_i evoked by ATP was completely suppressed in the absence of external Ca^{2+} although a transient inward current was still observed.

4 ATP-induced responses were not modified by the addition of the inositol 1,4,5-trisphosphate receptor antagonist, heparin (1 mM) in the pipette solution.

5 In the presence of caffeine (5 mM) or ryanodine (100 μM) in the pipette solution, which deplete the intracellular Ca^{2+} store, the ATP-induced Ca_i rise was greatly reduced.

6 Our results suggest that in single cells from rat portal vein, ATP releases Ca^{2+} from intracellular stores without involving InsP_3 , but via a Ca^{2+} release mechanism activated by Ca^{2+} influx through ATP-gated channels.

Keywords: ATP; intracellular Ca^{2+} store; ryanodine; Ca^{2+} -induced Ca^{2+} release

Introduction

There is considerable evidence that ATP is involved in the control of vascular tone. It mediates both indirect vasodilatation via the endothelium and vasoconstriction by direct stimulation of smooth muscle cells (Kennedy *et al.*, 1985). Sufficient amounts of ATP to activate myocytes could be released locally from damaged endothelial or smooth muscle cells, as well as in the course of intravascular aggregation. In addition, it is well known that ATP is released as an excitatory co-transmitter together with noradrenaline at sympathetic nerve terminals present in smooth muscle tissues (Von Kugelgen & Starke, 1991).

It has been demonstrated in a variety of smooth muscle cells that application of exogenous ATP activates a cation-selective channel (Benham *et al.*, 1987; Benham & Tsien, 1987; Friel, 1988; Xiong *et al.*, 1991; Schneider *et al.*, 1991) and produces a rapid increase in cytosolic Ca^{2+} concentration (Ca_i) (Benham, 1989; Schneider *et al.*, 1991). ATP-induced Ca_i increase could result from stimulation of Ca^{2+} entry either by direct Ca^{2+} permeation through ATP-gated channels (Benham, 1989; Schneider *et al.*, 1991) or indirectly via membrane depolarization and opening of voltage-dependent Ca^{2+} channels (Katsuragi *et al.*, 1990). Furthermore, it has been shown that ATP induces Ca^{2+} release from intracellular stores (Droogmans *et al.*, 1991) probably via the formation of the intracellular second messenger, inositol 1,4,5-trisphosphate (InsP_3).

In the present study, by means of simultaneous measurement of membrane currents and Ca_i , we show that ATP releases Ca^{2+} from an intracellular Ca^{2+} store in smooth muscle cells isolated from rat portal vein, without involving the InsP_3 receptor. The ATP-induced Ca^{2+} release results from a Ca^{2+} -induced Ca^{2+} release (CICR) mechanism, activated by the influx of Ca^{2+} through a cation channel gated by ATP.

Methods

Cell preparation

Wistar rats (150 g) were stunned and then killed by cervical dislocation. The portal vein was cut into several pieces, incubated for 10 min in low- Ca^{2+} (40 μM) physiological saline solution (PSS, composition given below), and then incubated in low- Ca^{2+} PSS containing 1.2 mg ml⁻¹ collagenase, 0.5 mg ml⁻¹ pronase and 1 mg ml⁻¹ bovine serum albumin at 37°C for 20 min. After this, the solution was removed and the pieces of vein were incubated again in a fresh enzyme-free solution and triturated with a fire polished Pasteur pipette to release cells. Cells were stored on glass cover-slips at 4°C in PSS containing 0.8 mM Ca^{2+} at a density of about $1-5 \times 10^4$ cells ml⁻¹ and used on the same day. About 80–90% of the cells excluded trypan blue, and contracted in response to various agonists and membrane depolarization.

Membrane current and fluorescence measurements

Voltage clamp and membrane current recordings were made with standard patch-clamp techniques using a Biologic RK400 patch-clamp amplifier. Whole-cell membrane currents were recorded with borosilicate patch pipettes of 1 to 4 M Ω resistance. The series resistances (5–8 M Ω) were not corrected. The liquid junction potentials were corrected with an offset circuit before each experiment. Membrane potential and current records were stored and analysed with an IBM-PC computer.

Measurement of Ca_i was carried out as described previously (Pacaud *et al.*, 1991). Indo-1 (50 μM) was added to the pipette solution, and so entered cells following establishment of the whole-cell recording mode. Cell fluorescence was then monitored in order to follow dye loading, which usually reached a steady state after 1–3 min and recording was started at this point. Ca_i was estimated from the 405/480 ratio (Gryniewicz *et al.*, 1985).

Results are expressed as the mean $\pm$ s.e.mean with *n* the sample size. Significance was tested by means of Student's *t* test.

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Solutions

The normal physiological saline solution (PSS) contained (in mM): NaCl 130, KCl 5.6, MgCl₂ 1, CaCl₂ 2, glucose 11, N-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulphonic acid] (HEPES) 10, pH 7.4 with NaOH. Ca²⁺-free PSS was prepared by omitting CaCl₂. The basic pipette solution contained (in mM): CsCl 110, NaCl 20, HEPES 10 and was buffered with the addition of approximately 5 mM NaOH. ATP was applied to the recorded cell by pressure ejection from a glass pipette for the period indicated on the records. No change in the holding current was observed during ejection of the physiological saline solution. All experiments were done at room temperature.

Chemicals and drugs

Collagenase was from Worthington, pronase (type E), bovine serum albumin, adenosine 5'-triphosphate disodium salt (ATP), gallopamil hydrochloride (D600) and heparin (from porcine intestinal mucosa; molecular weight, 4000–6000), were purchased from Sigma. Indo-1 (pentasodium salt) and ryanodine were from Calbiochem. Caffeine was from Merck.

Results

ATP induces an inward current and a rise in Ca_i

In the presence of 20 μ M D600, application of exogenous ATP (10 μ M, 5 s) to a single smooth muscle cell held at a potential of -60 mV induced a transient inward current and an increase in Ca_i (Figure 1a). The ATP-induced current reached a peak 0.62 ± 0.08 s ($n = 9$) after the onset of the response. The maximal value of ATP-induced Ca_i rise (533 ± 82 nM, $n = 9$) was reached 3.30 ± 0.64 s ($n = 9$) after the peak of the inward current. The absence of inward currents in response to the voltage pulses from -60 mV to 0 mV attested to the effective blockade of the voltage-dependent Ca²⁺ channels by D600 and thus ruled out the involvement of Ca²⁺ entry through voltage-dependent Ca²⁺ channels in the ATP-induced Ca_i rise.

In nominally Ca²⁺-free solution, application of ATP (10 μ M, 5 s) still induced an inward current whereas no rise in Ca_i was observed (Figure 1b). The absence of inward current and rise in Ca_i in response to membrane depolarization from -60 mV to 0 mV indicated that no external Ca²⁺ entered the cells. The ATP-induced current recorded under these conditions was significantly shorter (2.11 ± 0.17 s, $n = 8$) than that recorded in 2 mM Ca²⁺-PSS (3.04 ± 0.22 s, $n = 9$; $P = 0.02$) although the time to peak was not modified (0.67 ± 0.06 s, $n = 8$; $P > 0.5$). This difference was probably due to the presence of the Ca²⁺-dependent Cl⁻ current component activated by the rise in Ca_i induced by ATP in the presence of external Ca²⁺ (Pacaud *et al.*, 1992).

ATP activates Ca²⁺ release

Experiments shown in Figure 2 were performed in the absence of voltage-dependent Ca²⁺ channel inhibitors at a Cl⁻ equilibrium potential of -45 mV achieved by substituting 105 mM Cs-aspartate for 105 mM CsCl in the pipette solution. In these latter experimental conditions, the Ca²⁺-dependent Cl⁻ current at a holding potential of -60 mV was reduced. Application of ATP to a cell held at -60 mV evoked a transient inward current accompanied by a rise in Ca_i (Figure 2a). The ATP-induced current did not contain a Cl⁻ component as the Ca_i threshold for Cl⁻ current activation was not reached at the time when it was recorded (Pacaud *et al.*, 1992). After the complete decline of the ATP-induced current, Ca_i increased up to a peak and then gradually fell to the resting level. During this delayed Ca_i rise, a small inward Cl⁻ current was activated. Application of

membrane depolarization from -60 mV to 0 mV a few seconds after Ca_i returned to the resting level induced only a small rise in Ca_i which has been shown to correspond to Ca²⁺ entry through voltage-dependent Ca²⁺ channels (Grégoire *et al.*, 1993); 2 min later, a pair of similar depolarizations was applied (Figure 2b). The first one produced a regenerative Ca_i increase which occurred after the end of the voltage pulse and corresponded to Ca²⁺ release induced by Ca²⁺ entry through voltage-dependent Ca²⁺ channels (Grégoire *et al.*, 1993). This rise in Ca_i activated the Ca²⁺-dependent Cl⁻ current. The second depolarization of the pair induced only a small rise in Ca_i which declined at the end of the voltage jump. A similar experiment was performed after replacing external Ca²⁺ by Sr²⁺. Figure 3a clearly shows that under these conditions, the inward current activated by ATP at a holding potential of -60 mV, was accompanied by only a small change in the fluorescence signal. The maximal increase in fluorescence was obtained 2.28 ± 0.74 s ($n = 4$) after the peak of ATP-induced current and was observed together with a large Cl⁻ current. After the fluorescence signal had returned to the resting value, voltage-dependent Ca²⁺ channels, known to be permeable to Sr²⁺ (Hess *et al.*, 1986), were activated by membrane depolarization from -60 mV to 0 mV. This produced a fluorescence increase which declined immediately upon repolarization; 2 min later (Figure 3b), although the Sr²⁺ current evoked by the same depolarization had a similar amplitude, the fluorescence increase was larger and reached a peak 1–2 s after the repolarization. During this phase of regenerative fluorescence rise, ascribed to release from the intracellular Ca²⁺ store (Grégoire *et al.*, 1993), a large Cl⁻ tail current was activated. A second

Figure 1 Effects of application of ATP (10 μ M, 5 s) on membrane current and Ca_i under voltage-clamp at a holding potential of -60 mV. Traces show membrane potential (top), membrane current (middle) and Ca_i (bottom). (a) In normal external PSS with 2 mM Ca²⁺ and 20 μ M D600, ATP induced a transient inward current and an increase in Ca_i. (b) In the absence of external Ca²⁺, ATP induced an inward current but no change in Ca_i. Depolarizations to 0 mV show that no Ca²⁺ entry through voltage-dependent Ca²⁺ channels could be evoked in (a) and (b). Traces in (a) and (b) were obtained from two different cells.

Figure 2 Effects of membrane depolarizations from -60 mV to 0 mV following ATP ($10 \mu\text{M}$) application in 2 mM Ca^{2+} -PSS. Traces show membrane potential (top), membrane current (middle) and Ca_i (bottom). (a) Membrane depolarization applied after ATP application induced a small rise in Ca_i . (b) Two minutes later, the first depolarization of a pair produced a regenerative increase in Ca_i which occurred after the end of the voltage step; the second one induced a small increase in Ca_i , similar to that recorded in (a).

depolarization applied immediately after the membrane current and the fluorescence signal had returned to resting values did not produce a regenerative fluorescence rise but only a small and short increase in fluorescence during the voltage pulse.

Does the ATP-induced Ca_i rise contain an InsP_3 -induced Ca^{2+} release component?

In an attempt to test the possible involvement of InsP_3 -induced Ca^{2+} release in the ATP-evoked Ca_i rise, heparin (1 mM) a competitive antagonist of InsP_3 binding to its receptor (Worley *et al.*, 1987) was added to the pipette solution (Figure 4). This experimental protocol has been shown to inhibit completely the InsP_3 -induced Ca^{2+} release evoked by noradrenaline application, 5 min after breakthrough into the whole-cell recording mode (Pacaud *et al.*, 1991). Under these conditions, ATP still induced a transient inward current at a holding potential of -60 mV and a rise in Ca_i . The peak Ca_i reached $515 \pm 99 \text{ nM}$ ($n = 4$) and thus, was not significantly different from that obtained in the absence of heparin. As described above (Figure 2), only a small and short rise in Ca_i was observed in response to the voltage pulse from -60 mV to 0 mV applied immediately after ATP application; 30 s later, the same voltage pulse produced a regenerative Ca_i rise, attesting that the Ca^{2+} store was at least partially refilled and that the CICR mechanism remained functional in the presence of heparin.

Figure 3 Effects of membrane depolarizations from -60 mV to 0 mV following ATP ($10 \mu\text{M}$) application in 2 mM Sr^{2+} -PSS. Traces show membrane potential (top), membrane current (middle) and indo-1 fluorescence expressed as the 405:480 ratio (bottom). (a) ATP induced a transient inward current and a delayed increase in Ca_i which activated a Ca^{2+} -dependent Cl^- current. The depolarization then applied evoked a rise in fluorescence which decayed upon repolarization. (b) Two minutes later, the first depolarization of a pair produced a larger increase in fluorescence, which was maintained after the repolarization and activated a large Cl^- tail current. The second depolarization evoked a smaller rise in fluorescence, insufficient to activate Cl^- current.

ATP releases Ca^{2+} by activating ryanodine receptor

To gain further insight into the mechanism through which ATP releases Ca^{2+} from intracellular store, ATP responses were evoked in cells treated with caffeine and ryanodine (Figure 5). Caffeine is known to increase the open probability of the Ca^{2+} -activated Ca^{2+} release channel (Herrmann-Frank *et al.*, 1991). When added to the extracellular solution, caffeine (5 mM) causes a large transient increase in Ca_i , which activates the Ca^{2+} -dependent Cl^- current at a holding potential of -60 mV (Pacaud *et al.*, 1989). As in Figure 5a, this Ca_i transient can be followed by smaller oscillations. When membrane current and Ca_i decreased to stable levels, application of ATP still produced a large inward current whereas the rise in Ca_i was strongly reduced in amplitude and duration. The maximal Ca_i ($235 \pm 18 \text{ nM}$, $n = 4$) was reached $0.47 \pm 0.03 \text{ s}$ ($n = 4$) after the peak inward current induced by ATP, i.e. significantly more rapidly than under control conditions. Application of a pair of depolarization pulses to 0 mV from a holding potential of -60 mV evoked small rises in Ca_i that reached a peak at the end of the voltage pulse and decayed immediately upon repolarization. The absence of regenerative Ca_i rise in response to the first depolarization of the pair indicated that treatment with

Figure 4 Effect of ATP ($10\ \mu\text{M}$) in cell held at a holding potential of $-60\ \text{mV}$, in the presence of heparin ($1\ \text{mM}$) in the pipette solution. Traces show membrane potential (top), membrane current (middle) and Ca_i (bottom). The protocol used was similar to that in Figure 2. ATP was applied 5 min after breakthrough into the whole-cell recording mode. ATP application was followed by a depolarization to $0\ \text{mV}$, then approximately 30 s later, a pair of similar depolarizations was applied.

caffeine prevented activation of CICR mechanism (Grégoire *et al.*, 1993).

Ryanodine is also a tool commonly used for the study of CICR and is thought to inhibit CICR by binding to the release channel and stabilizing an open subconductance state (Rousseau *et al.*, 1987). A typical ATP-induced response recorded at a holding potential of $-60\ \text{mV}$ in a cell dialysed with a $100\ \mu\text{M}$ ryanodine-containing pipette solution for 5 min is illustrated in Figure 5b. ATP induced an inward current and an increase in Ca_i similar to those evoked in the presence of caffeine. The maximal Ca_i ($269 \pm 14\ \text{nM}$, $n = 4$) was reached $0.42 \pm 0.03\ \text{s}$ ($n = 4$) after the peak inward current. Then, each depolarization applied from $-60\ \text{mV}$ to $0\ \text{mV}$ induced an identical rise in Ca_i . As previously described (Grégoire *et al.*, 1993), the regenerative CICR evoked by membrane depolarization was abolished in the presence of ryanodine.

Discussion

The experiments described in this paper show that application of extracellular ATP in portal vein smooth muscle cells induces a transient current and an increase in Ca_i . These effects of ATP could be explained either by the activation of a Ca^{2+} permeable conductance which causes a rise in Ca_i , or by a Ca^{2+} release from internal Ca^{2+} store which activates a Ca^{2+} -dependent current.

The ATP-induced current is inward at a holding potential of $-60\ \text{mV}$, is not modified by a shift of the Cl^- equilibrium potential, shows fast activation and inactivation, and allows Ca^{2+} influx. These properties are similar to those reported for the non-selective cation current directly gated by ATP in other smooth muscle cells (Friel 1988; Benham, 1989; Schneider *et al.*, 1991).

The ATP-induced Ca_i rise was resistant to voltage-dependent Ca^{2+} channel block but was completely inhibited in the absence of extracellular Ca^{2+} . The Ca^{2+} release component of the Ca_i rise induced by Ca^{2+} entry through voltage-

Figure 5 Effect of ATP ($10\ \mu\text{M}$) at a holding potential of $-60\ \text{mV}$ in the presence of caffeine or ryanodine. Traces show membrane potential (top), membrane current (middle) and Ca_i (bottom). (a) Extracellular application of caffeine ($5\ \text{mM}$) transiently increased Ca_i and activated the Ca^{2+} -dependent Cl^- current. In the continued presence of caffeine, ATP and a pair of depolarizations to $0\ \text{mV}$ were successively applied to the cell. (b) Recordings showing the effects of ATP and depolarizations to $0\ \text{mV}$ in a cell dialysed with a $100\ \mu\text{M}$ ryanodine-containing pipette solution.

dependent Ca^{2+} channels was abolished when membrane depolarization was applied immediately after the ATP-induced response. A similar result was obtained when extracellular Ca^{2+} was replaced by Sr^{2+} . Because of the indo-1 fluorescence characteristics in the presence of Sr^{2+} (Grégoire *et al.*, 1993), the rise in fluorescence due to Sr^{2+} entry is small and allows a component of Ca^{2+} release, if any, to be observed more easily. Thus, these data suggest that Ca^{2+} or Sr^{2+} entry through ATP-gated cation channels induces a Ca^{2+} release from an intracellular Ca^{2+} store similar to that evoked by Ca^{2+} or Sr^{2+} entry through voltage-dependent Ca^{2+} channels.

In many cell types including smooth muscle cells, the functional effects elicited by exposure to extracellular ATP can be correlated with an ATP-induced mobilization of the intracellular Ca^{2+} store (see Dubyak & El-Moatassim (1993) for review). This Ca^{2+} mobilizing action has been ascribed to ATP-induced stimulation of InsP_3 formation, with the generated InsP_3 mediating the release of Ca^{2+} (Hoiting *et al.*, 1990; Droogmans *et al.*, 1991). In our experiments, although no Ca_i response at all was recorded in the absence of divalent cations in the external solution, it is possible that a release of Ca^{2+} from the internal store induced by the generation of

InsP_3 is involved in the ATP-induced Ca_i rise. It is known that phospholipase C and thus InsP_3 formation can be stimulated by Ca^{2+} (Ryo *et al.*, 1987). Thus, a possible amplification of the Ca^{2+} release initially triggered through CICR mechanism could occur as a consequence of InsP_3 -induced Ca^{2+} release. This hypothesis has been ruled out by the use of heparin: the ATP-induced Ca_i rise was not affected by the presence of heparin in the pipette solution and thus was independent of the activation of the InsP_3 receptors.

Treatment with caffeine or ryanodine, known to deplete intracellular stores, leads to a similar and powerful reduction of the ATP-induced Ca_i rise. This is consistent with the involvement of a CICR component in the Ca_i increase produced by ATP. This component, corresponding to the release of Ca^{2+} from an intracellular store activated by Ca^{2+} influx through an ATP-gated channel constitutes the major part of the ATP-induced Ca_i rise in portal vein smooth muscle cells.

We have recently identified, in this cell type, a CICR mechanism that could be initiated by Ca^{2+} entry through voltage-dependent Ca^{2+} channels (Grégoire *et al.*, 1993). The activation of Ca^{2+} release by Ca^{2+} ions that enter the cell through ATP-activated cation channel suggests that, more generally, every Ca^{2+} entry could be amplified by release of Ca^{2+} from intracellular stores. In the case of ATP, results obtained in the presence of ryanodine or caffeine show that the part of the Ca_i rise due to Ca^{2+} entry was small. As membrane currents and Ca_i could be simultaneously recorded in the same cell in response to application of ATP and membrane depolarization, the contribution of Ca^{2+} to the ATP-activated cation current was evaluated (in the presence of caffeine or ryanodine) by comparing the Ca_i rise due to the ATP-gated current with that due to voltage-dependent Ca^{2+} channel current (Schneider *et al.*, 1991). Based on this comparison, we estimate that approximately 6% of the ATP-

activated cation current is carried by Ca^{2+} ions. This value is very close to that reported by Schneider *et al.* (1991) as they estimated, using the same method, that 5.8% of the ATP-gated channel of myocytes from guinea-pig urinary bladder was carried by Ca^{2+} . This Ca^{2+} entry amplified by a Ca^{2+} release from an intracellular Ca^{2+} store independent of InsP_3 generation constitutes a novel mechanism of action. However, effects of ATP in portal vein myocytes would not be independent of those induced by noradrenaline (NA) which is co-liberated at sympathetic nerve terminals. NA has been shown to release Ca^{2+} from an intracellular Ca^{2+} store via activation of the InsP_3 receptor (Pacaud *et al.*, 1991) but, in portal vein smooth muscle cells, InsP_3 -sensitive and ryanodine-sensitive Ca^{2+} stores seem to overlap widely (Grégoire *et al.*, 1993). Thus, responses to NA and ATP, at least for maximal concentrations, might not be additive. For intermediate concentrations, ATP and NA are expected to act in a synergistic manner, because Ca^{2+} released via InsP_3 receptor activation during NA stimulation could amplify the CICR activated by ATP and conversely, InsP_3 receptor could also be positively regulated by Ca^{2+} released through the ryanodine receptor (Iino & Endo, 1992). Thus, synergistic interaction between different pharmacological receptors can occur not only for agonists that converge on the same intracellular transduction mechanism (Helliwell *et al.*, 1994). This hypothesis remains to be demonstrated; nevertheless, it seems that the vascular neurotransmission is more complex than previously thought.

This work was supported by a grant from the 'Conseil Régional d'Aquitaine' (9203023). We thank Drs R. Marthan and J.P. Savineau for helpful advice in the preparation of this manuscript.

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(Received April, 11, 1994

Revised June 13, 1994

Accepted June 20, 1994)