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Characterization of the P_{2Y}-purinoceptor involved in the ATP-induced rise in cytosolic Ca^{2+} concentration in rat ileal myocytes

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1 The P_2-purinoceptor subtype and the intracellular signalling mechanism(s) involved in the rise in the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by ATP and analogues were analyzed in myocytes isolated from the longitudinal muscle layer of rat ileum by means of molecular and physiological techniques.

2 The P_2-purinoceptor expressed by ileal smooth muscle cells shared 100% amino acid identity with the rat P_{2Y1}-receptor.

3 Short applications of the purinoceptor agonists induced a transient rise in $[Ca^{2+}]_i$ in an all-or-nothing manner. The rank order of potency of the analogues of ATP and ADP, determined by measuring the percentage of responding cells was 2-methylthioATP = 2-chloro-ATP > ADP > ATP, with concentrations giving $[Ca^{2+}]_i$ response in 50% of cells ranging between 3 nM and 0.6 μM. The concentration-response curves to ADP and ATP were shifted to the right by 10 μM pyridoxal phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS).

4 Although the rise in $[Ca^{2+}]_i$ induced by stimulation of the ileal P_{2Y}-purinoceptor was inhibited by heparin (5 mg ml^{-1}), we were not able to detect stimulation of phospholipase C under conditions (37°C) where muscarinic cholinoceptor activation markedly increased inositol phosphate (InsP) accumulation. However, the carbachol (CCh)-induced increase in InsP accumulation was suppressed when the agonist was applied at 20°C while a CCh-induced $[Ca^{2+}]_i$ rise similar to that obtained in response to the P_2-purinoceptor agonist was still observed.

5 Our results indicate that the rat ileal myocytes express a PPADS-sensitive P_2-purinoceptor similar to the P_{2Y1}-receptor subtype. Although there is no detectable increase in InsP production, stimulation of these receptors leads to a rise in $[Ca^{2+}]_i$ by activation of the inositol 1,4,5-trisphosphate receptor-channel of the intracellular Ca^{2+} store, indicating that they couple to phospholipase C.

Keywords: Intestinal smooth muscle; P_2-purinoceptors; ATP; free cytosolic calcium; inositol phosphate

Introduction

The cell surface receptors responsible for the physiological response to ATP and ADP are collectively classed as P_2-purinoceptors. The identification of subclasses of P_2-purinoceptors has proved difficult since no selective P_2-receptor antagonists are available. However, they are separated into several subclasses according to a potency rank order for some agonists (Abbrachio & Burnstock, 1994; Fredholm *et al.*, 1994). Recently, cDNA encoding P_2-purinoceptors coupled with G protein, P_{2Y} and P_{2U} (Webb *et al.*, 1993; Lustig *et al.*, 1993) and those forming non-selective cation channels, P_{2X} (Brake *et al.*, 1994; Valera *et al.*, 1994; Chen *et al.*, 1995; Lewis *et al.*, 1995; Bo *et al.*, 1995) have been cloned and characterized. These data confirm the existence of multiple receptor subtypes within the major classes of P_2-purinoceptors and the availability of the nucleotide sequences should probably facilitate the identification of new members of the P_2-purinoceptor family. Although these subtypes of receptor are expected to possess very different second messenger-coupling specificities, the signalling mechanisms associated with P_2-purinoceptors are only partially understood.

In intestinal smooth muscle, the effects of ATP and analogues as well as the mechanism (or the mechanisms) involved remain unclear. Exogenous ATP has been shown to relax intestinal muscle (Furukawa & Nomoto, 1989; Nicholls *et al.*, 1990; Johnson & Hourani, 1994; Windscheif *et al.*, 1995) by activation of P_2-purinoceptors while in isolated intestinal myocytes, ATP and other P_{2Y} agonists induced a transient rise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Blottière *et al.*, 1996).

The present study was undertaken in order to identify, by means of molecular and physiological techniques, the P_2-purinoceptor and the intracellular signalling mechanism(s) involved in the rise in $[Ca^{2+}]_i$ induced by ATP in myocytes from the longitudinal muscle layer.

Methods

Cell preparation

Wistar rats (150 g) were stunned and then killed by cervical dislocation. The longitudinal muscle layer of ileum was peeled from the underlying circular muscle in the physiological saline solution (PSS), (composition given below) and cut into small pieces. Smooth muscle cells were isolated by use of a protocol derived from that used by Pacaud & Bolton (1991). Fragments were washed for 10 min in Ca^{2+}-free phosphate-buffered saline (PBS), then incubated in PBS containing 1 mg ml^{-1} collagenase, 0.1 mg ml^{-1} pronase and 20 mg ml^{-1} bovine serum albumin at 37°C for 30 min. After this time, the solution was removed and the pieces of tissues were incubated again in a fresh enzyme solution at 37°C for 30 min. Tissues were then placed in enzyme-free solution and triturated by 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+} and used on the same day. About 80-90% of the cells excluded trypan blue, and contracted in response to various agonists (acetylcholine) and membrane depolarization.

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Membrane current measurement and estimation of $[Ca^{2+}]_i$

Voltage-clamp and membrane current recordings were made with standard patch-clamp techniques by a Biologic RK400 patch-clamp amplifier (Biologic CO., Claix, France). Whole-cell membrane currents were recorded with borosilicate patch pipettes of 1 to 4 MΩ resistance. The series resistances (5–8 MΩ) 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 by an IBM-PC computer.

Changes in $[Ca^{2+}]_i$ were monitored fluorometrically by use of the Ca^{2+}-sensitive probe indo-1 as described previously (Pacaud *et al.*, 1991; 1993). Briefly, cells were loaded with indo-1, either by addition of 50 μM indo-1 to the pipette solution, from which it diffused into the cells following establishment of the whole-cell recording mode, or by incubation in PSS containing 1 μM indo-1 penta-acetoxymethyl ester (indo-1/AM) for 25 min at room temperature. The coverslips with attached cells were then mounted in a chamber and were continuously superfused. The recording system included a Nikon Diaphot inverted microscope fitted with an epifluorescence attachment (Nikon France, Charenton-le-pont, France). The cell studied was illuminated at 360 nm. Emitted light from a window slightly larger than the cell was counted simultaneously at 405 nm and 480 nm by two photomultipliers (P1, Nikon). Voltage signals at each wavelength were stored in an IBM-PC computer for subsequent analysis. The ratio (405/480) was calculated on-line and displayed with the two voltage signals on a monitor. Patch-clamped cell fluorescence was monitored in order to follow dye loading, which usually reached a steady-state after 1–3 min and recording was started at this point. The fluorescence signal from cells loaded with indo-1/AM indicated an internal indo-1 concentration in the range 40–70 μM from comparison with cells directly loaded with indo-1 by a patch pipette. $[Ca^{2+}]_i$ was estimated from the 405/480 ratio (Grynkiewicz *et al.*, 1985) by a calibration for indo-1 determined within cells (Pacaud *et al.*, 1991).

Each measurements were made in at least 10 cells in the same batch and the experiment was reproduced in at least 3 different batches of cells. The results obtained in cells from the same batch were pooled into a single data point. Values thereby obtained in different batches of cells were then averaged so that numerical data represent the mean ± s.e. mean with n the number of batches of cells. Significance was tested by means of Student's t test.

Inositol phosphate (InsP) assay

Small pieces (1 mm^2) of the longitudinal muscle layer of ileum were incubated for 5 h in M199 containing 20 μCi ml^{-1} [^{3}H]-inositol. Muscle pieces were then washed in PSS and pre-incubated for 10 min at 37°C in PSS with 20 mM LiCl before challenge with agonists for 7 min. Incubations were terminated by removal of the medium. The muscle was quickly frozen in liquid nitrogen and 500 μl of 10 mM formic acid was added. After sonication, the supernatant was collected and neutralized with 2 ml of 5 mM NH_4OH. InsP were subsequently separated on Dowex (formate) columns as previously described (Frelin *et al.*, 1993).

Reverse transcriptase polymerase chain reaction (PCR) and experiments

The longitudinal muscle layer of the ileum was carefully dissected as described for the cell preparation. Total RNA was isolated by the method described previously (Chirgwin *et al.*, 1979). cDNA was synthesized from 1 μg of total RNA, using 300 ng oligo(dT) primer, 0.4 mM of each dNTP, 40 units of Rnasin and 200 units Moloney Murine Leukaemia Virus (MMLV) reverse transcriptase. After a 5 min denaturation at 85°C, dNTPs and the enzyme were added. The reaction was allowed to proceed for 1 h at 37°C and stopped by heating to 95°C for 5 min.

Amplification was performed with 100 ng of each primer deduced from the sequence of the rat P_{2Y1}-receptor (Tokuyama *et al.*, 1995), 0.2 mM of each dNTP and 0.5 units of Goldstar DNA polymerase by use of a PHC-2 Techne Cycler programmed with the following conditions: 2 min denaturation at 94°C followed by 5 cycles of 30 s at 94°C, 60 s at 50°C, 90 s at 72°C and 30 cycles with an annealing temperature of 60°C. The sense primer was 5'GGAATTCGAGGATGACCGAGGTG3'. It corresponds to the 613–631 sequence of the cloned P_{2Y}-purinoceptor (GenBank/EMBL database, accession number U22830) and included the start codon. The antisense primer was 5'ATCCTCCTGCCTTCACAAACT-TGTG3'. It corresponds to the 1729–1750 sequence of the cloned P_{2Y}-purinoceptor and includes the stop codon. These primers selected selectively the whole coding sequence of the P_{2Y1}-receptor. The PCR products were resolved on a 1% w/v agarose gel by electrophoresis. The bands were excised from the gel and after purification were subcloned into pTAG plasmid. The amplification product was sequenced (Kit Prism DyeDeoxy, Applied Biosytems) by a DNA sequencer (Model 373A, Applied Biosystems).

Solutions

The normal physiological saline solution (PSS) contained (in mM): NaCl 130, KCl 5.6, $MgCl_2$ 1, $CaCl_2$ 2, glucose 11, HEPES 10, pH 7.4 with NaOH. The basic pipette solution contained (in mM): KCl 120, NaCl 10, HEPES 10, brought to pH 7.3 with the addition of approximately 5 mM NaOH. In most experiments, agonists were 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 and $[Ca^{2+}]_i$ was observed during ejection of the physiological saline solution. Except when indicated, all experiments were performed at room temperature (20°C).

Chemicals and drugs

Collagenase (type I, 326 units mg^{-1}) was from Worthington Biochemical Corp. (Freehold, N.J.). PBS was from Biochrom KG (Berlin, Germany). Pronase (type E, 5.3 units mg^{-1})), bovine serum albumin, heparin (from porcine intestinal mucosa; molecular weight, 4000–6000), Pertussis toxin, adenosine 5'-triphosphate (ATP; disodium salt), adenosine 5'-diphosphate (ADP) and oligo(dT) primer were purchased from Sigma (Saint Quentin Fallavier, France). Indo-1 (pentasodium salt) and indo-1/AM were obtained from Calbiochem (France Biochem, Meudon, France). Caffeine was from Merck (Darmstadt, Germany). 2-Chloro-ATP (2-ClATP), 2-methylthioATP (2-MeSATP) and pyridoxal phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) were purchased from Research Biochemical International (Natick, MA). myo-[2-^{3}H]-inositol (19 Ci $mmol^{-1}$) was from Amersham (Les Ulis, France), Rnasin from Promega (Madison, WI), MMLV reverse transcriptase was from GIBCO BRL (Fragny, France) and Goldstar DNA polymerase from Eurogentec (Seraing, Belgium).

Results

Characterization of the P_{2Y}-purinoceptor expressed in the ileal smooth muscle cell

PCR amplification of ileal smooth muscle cDNA generated a product of 1145 nucleotides length which was cloned and sequenced. This sequence encoded a protein which shared 100% amino acid identity with the rat P_{2Y1} receptor recently isolated from an insulinoma cDNA library (Tokuyama *et al.*, 1995).

ATP-induced $[Ca^{2+}]_i$ rise

As previously shown, single ileal myocytes responded to short application of ATP (10 μM) by a rise in $[Ca^{2+}]_i$ (Figure 1a) which corresponded to Ca^{2+} release from the intracellular Ca^{2+} store (Blottière *et al.*, 1996). Up to 10–15 $[Ca^{2+}]_i$ responses of similar amplitude could be evoked in the same cell by repetitive stimulation with ATP (10 μM) separated by a time interval of approximately 40–50 s, suggesting that short agonist applications did not produce receptor desensitization (Figure 1a). As illustrated in Figure 1b, a transient $[Ca^{2+}]_i$ response to short ATP application takes place in an all-or-none fashion. In the same cell, no response was evoked by 0.1 μM ATP but the $[Ca^{2+}]_i$ transients induced by 1 μM, 10 μM and 100 μM ATP were of similar amplitude. All individual cells showed a similar behaviour, only the threshold concentration varied from cell to cell. The mean values of the maximal $[Ca^{2+}]_i$ rise induced by 1 μM, 10 μM and 100 μM ATP corresponded to 699±36 nM, 641±44 nM and 693±27 nM ($n=4$), respectively. The ATP-induced $[Ca^{2+}]_i$ rise was not affected (610±33 nM, $n=3$, $P>0.5$) by treatment with pertussis toxin (0.5 μg ml^{-1} for 16 h). Continuous application of ATP (10 μM) induced a transient rise in $[Ca^{2+}]_i$ that was followed by small $[Ca^{2+}]_i$ oscillations (Figure 2a). In the continuous presence of 10 μM ATP in the experimental chamber, pressure ejection of ATP 10 μM, 100 μM or ADP 10 μM were ineffective. Prolonged application of a lower concentration of ATP (1 μM) produced a transient $[Ca^{2+}]_i$ rise followed by a maintained $[Ca^{2+}]_i$ rise of small amplitude (Figure 2b). In the continuous presence of 1 μM ATP, pressure ejection of ATP 1 μM was without effect whereas ATP 10 μM induced a transient $[Ca^{2+}]_i$ rise (Figure 2b). In some cells, after the transient $[Ca^{2+}]_i$ rise, prolonged application of a low concentration of ATP (0.1 μM) evoked $[Ca^{2+}]_i$ oscillations (Figure 2c). Ejection of a higher ATP concentration (1 μM) then induced an increase in the frequency of the oscillations.

Rank order of potency of ATP and its analogues

A transient rise in $[Ca^{2+}]_i$ could be evoked by ejection of ATP, 2-ClATP, 2-MeSATP and ADP. Concentration-response curves for these agonists were obtained by plotting the percentage of responding cells against the agonist concentration (Figure 3). These curves revealed the following order of potency: 2-MeSATP = 2-ClATP > ADP > ATP. UTP was inactive even at high concentrations. A $[Ca^{2+}]_i$ rise was obtained in 50% of the cells in response to 3 nM, 4 nM, 25 nM and 0.6 μM for 2-MeSATP, 2-ClATP, ADP and ATP, respectively. ATP was the least potent agonist tested.

Sensitivity to PPADS of the ATP- and ADP-induced responses

Recent data have shown that pyridoxal phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) is an antagonist of P_2-purinoceptors that has differential effects on various P_2-purinoceptor subtypes (Boyer *et al.*, 1994; Brown *et al.*, 1995; Windscheif *et al.*, 1995). PPADS inhibits the P_{2X}-purinoceptor-mediated mechanical responses on vas deferens and urinary bladder detrusor muscle (Lambrecht *et al.*, 1992; Ziganshin *et al.*, 1993), competitively antagonizes the P_{2Y}-purinoceptor-stimulated phospholipase C activity but has no effect on the

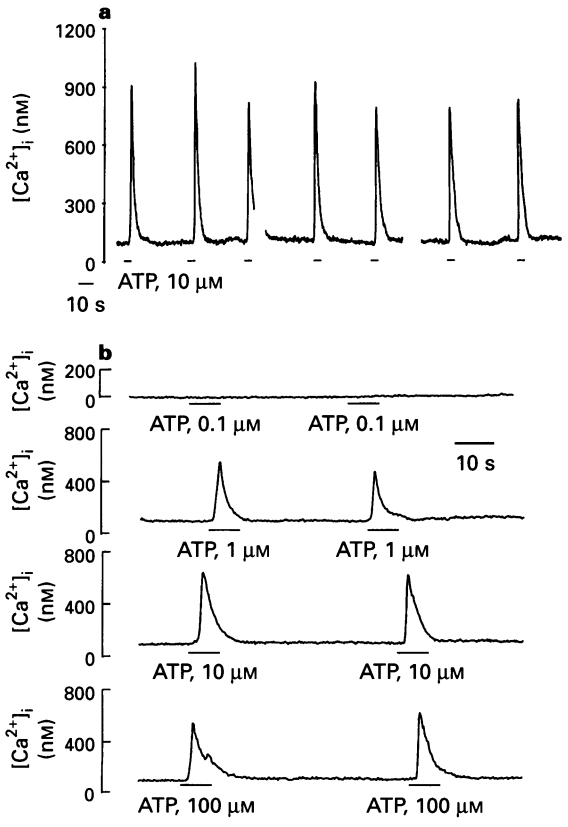

Figure 1 Effect of short ATP application in freshly isolated ileal myocytes. (a), Transient rises in cytosolic Ca^{2+} concentration induced by repetitive applications of ATP (10 μM). Gaps were due to the time (3–4 s) required for the storage of the data. (b), Typical all-or-nothing transient $[Ca^{2+}]_i$ rise induced in the same cell by increasing ATP concentration from 0.1 to 100 μM.

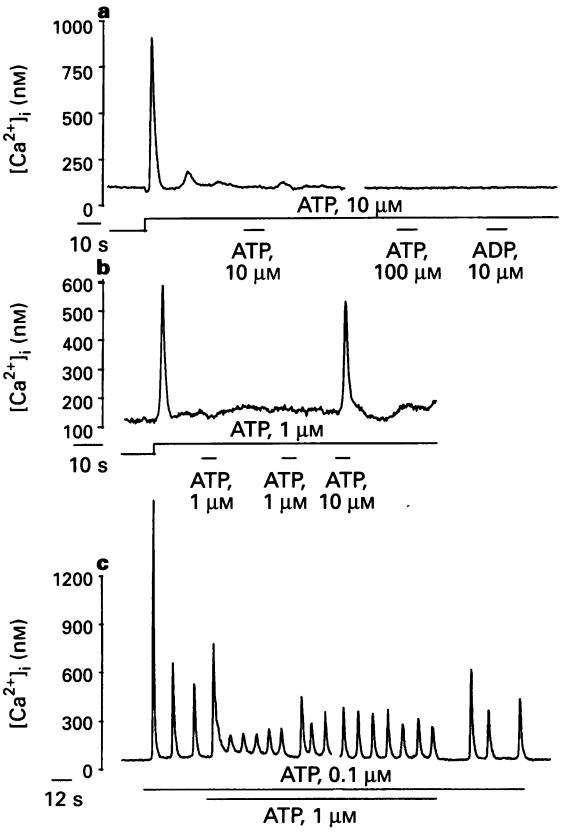

Figure 2 Effect of prolonged application of ATP. (a) Effect of ejection of 10 μM, 100 μM ATP and 10 μM ADP on $[Ca^{2+}]_i$ during prolonged application of ATP (10 μM). (b) Effect of ejection of 1 μM and 10 μM ATP in the continuous presence of ATP (1 μM). (c) Effect of 1 μM ATP during prolonged application of 0.1 μM ATP in cells responding with $[Ca^{2+}]_i$ oscillations. Gaps in the traces correspond to 3–4 s.

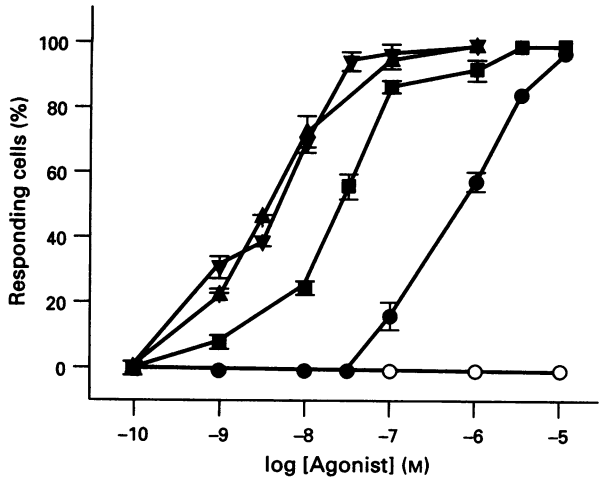

Figure 3 Concentration-dependence of the $[Ca^{2+}]_i$ rise induced by 2-chloro-ATP (▼), 2-methylthioATP (▲), ADP (■), ATP (●) and UTP (○). The % of responding cells was plotted against agonist concentration. Each point was determined by testing cells from 3–8 different batches. Vertical lines show s.e.mean.

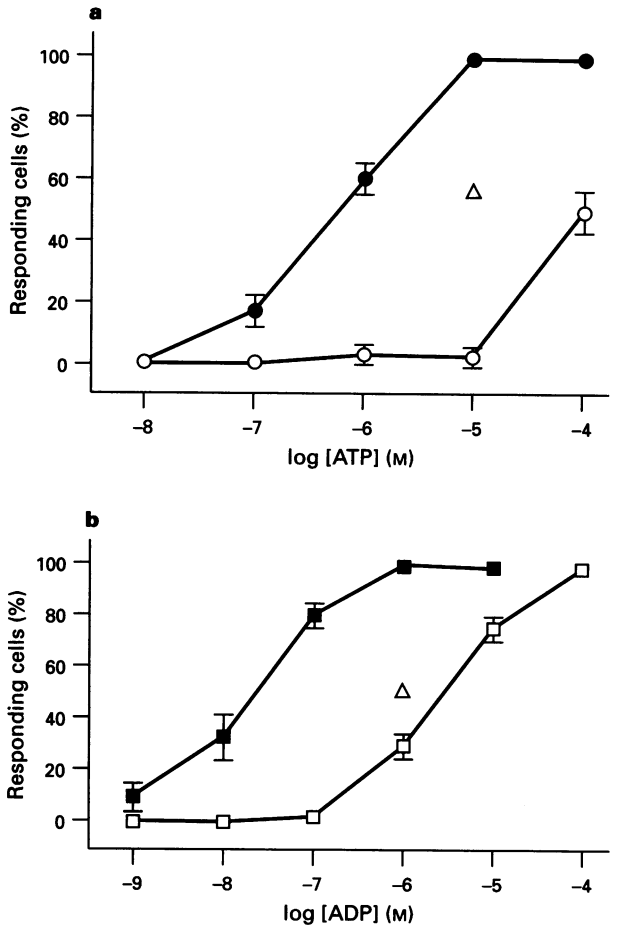

Figure 4 Effect of pyridoxal phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) on ATP- and ADP-induced $[Ca^{2+}]_i$ rise. (a) Concentration-dependence of the $[Ca^{2+}]_i$ rise induced by ATP in the absence (●) and in the presence of PPADS (10 μM, ○). (△) Represents the % of responding cells (from a single batch) to 10 μM ATP in the presence of 5 μM PPADS. (b) Concentration-dependence of the $[Ca^{2+}]_i$ rise induced by ADP in the absence (■) and in the presence of PPADS (10 μM, □). (△) Represents the % of cells (from a single batch) responding to 1 μM ADP in the presence of 5 μM PPADS.

P_{2Y}-mediated inhibition of adenylyl cyclase (Boyer *et al.*, 1994). In the presence of a low concentration of PPADS (10 μM, incubated for 25 min), the dose-response curve to ATP (Figure 4a) and ADP (Figure 4b) were both significantly shifted to the right. Under these conditions, 100 μM ATP and 2.5 μM ADP were necessary to obtain a $[Ca^{2+}]_i$ rise in 50% of the cells, compared to 0.6 μM and 20 nM, respectively in control conditions. This effect was reversed by washout of PPADS (15 min). The mean amplitude of the P_{2Y}-purinoceptor agonist-induced transient $[Ca^{2+}]_i$ rise was not affected by PPADS. The maximal $[Ca^{2+}]_i$ rise in response to 10 μM ADP in the absence and in the presence of 10 μM PPADS was 725 ± 56 nM and 710 ± 5 nM ($n=3$, $P>0.5$), respectively. This result thus suggests that ATP and ADP increased $[Ca^{2+}]_i$ by interacting with the same membrane receptor which was blocked by PPADS.

Effects of purinoceptor agonists on inositol phosphate production

The transient $[Ca^{2+}]_i$ rise induced by stimulation of isolated cells from ileum with P_{2Y}-purinoceptor agonists was not inhibited by the removal of external Ca^{2+} but was suppressed by treatment with thapsigargin (Blottière *et al.*, 1996). This observation seems to be consistent with the stimulation of the G-protein-regulated inositol lipid cascade and Ca^{2+} mobilization that results from P_{2Y}-purinoceptors activation in other cell types (Pirotton *et al.*, 1987; Boyer *et al.*, 1989). We verified this hypothesis by measuring the effects of various P_2-purinoceptor agonists on the production of total inositol phosphates (InsP). Surprisingly, except ADP at high concentration (100 μM, i.e. 100 times the concentration giving maximal $[Ca^{2+}]_i$ response), none of the agonists tested (2-ClATP, 2-MeSATP and ATP) produced a clearly detectable stimulation of InsP production, even at a high concentration (10 or 100 μM) (Figure 5). In agreement with the absence of uridine 5'-triphosphate (UTP)-induced $[Ca^{2+}]_i$ rise, UTP was ineffective on InsP production. This also indicates that the stimulation of InsP production observed with a high ADP concentration did not result from activation of the P_{2U}-purinoceptor. Under the same conditions, carbachol (CCh) (1–10 μM) increased InsP production 1.7 to 3.6 fold. It thus seems that P_{2Y}-purinoceptor agonists increased $[Ca^{2+}]_i$ in the absence of marked InsP production. As a consequence, the involvement of $InsP_3$ receptors of the Ca^{2+} store in the transient $[Ca^{2+}]_i$ rise induced by P_{2Y}-purinoceptor agonists has to be further investigated.

Effect of heparin

Heparin is a well known non-permeable inhibitor of the $InsP_3$ receptor of the Ca^{2+} store membrane which needs to be introduced inside the cell. Therefore, simultaneous recording of membrane current and $[Ca^{2+}]_i$ was performed by use of the whole-cell patch clamp technique to assess the contribution of the $InsP_3$ receptor to the ADP-induced $[Ca^{2+}]_i$ rise. Under control conditions, in a cell maintained at a holding potential of 0 mV, ejections of 10 μM ADP separated by 2 min evoked transient rises in $[Ca^{2+}]_i$ and transient outward currents of similar amplitude (Figure 6a). In the presence of heparin (1 mM) in the patch pipette solution, the first application of 10 μM ADP 1 min after the breakthrough into the whole-cell recording mode evoked a transient rise in $[Ca^{2+}]_i$ and an outward current similar to those obtained under the control conditions but the second application of ADP 4 min later evoked no response (Figure 6b). In freshly isolated ileal myocytes, the intracellular Ca^{2+} store was sensitive to both $InsP_3$ and caffeine (Komori & Bolton, 1991; Komori *et al.*, 1993). The inhibition of the ADP-induced $[Ca^{2+}]_i$ rise was not due to the depletion of the intracellular Ca^{2+} store as caffeine (5 mM) was still able to produce a transient rise in $[Ca^{2+}]_i$ accompanied by a transient outward current. This result is consistent with previous results showing that 1.5 min after the rupture of the

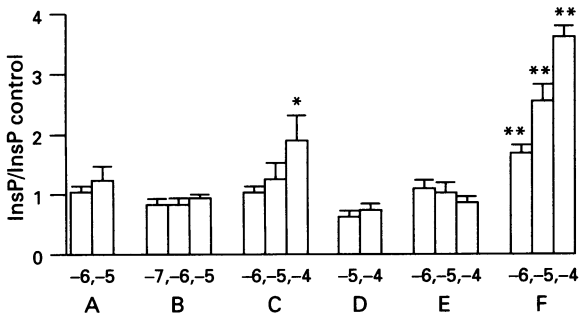

Figure 5 Effect of P_{2Y}-purinoceptor agonists and carbachol (CCh) on inositol phosphate (InsP) formation in ileal cells prelabelled with [^{3}H]-inositol. Results are normalized to control value determined in the absence of stimulation with agonist. Agonist concentration (logM) used is indicated below each column. The agonists used were 2-chloro-ATP (A), 2-methylthioATP (B), ADP (C), ATP (D), UTP (E) and CCh (F). The stimulation with agonists was performed at 37°C. (Results were compared to those obtained under control conditions: $*P<0.01$; $**P<0.003$).

membrane patch, the intracellular concentration of heparin was not sufficient to inhibit the $InsP_3$-induced Ca^{2+} release, but this was achieved 4 min later (Pacaud *et al.*, 1993). This thus suggests that despite the absence of a detectable stimulation of InsP production, the P_{2Y}-purinoceptor agonists-induced $[Ca^{2+}]_i$ rise is mediated by the activation of the $InsP_3$ receptor-channel.

Comparison between the effects of CCh and the P_{2Y}-agonists

The discrepancy between the action of P_{2Y}-purinoceptor agonists and CCh on InsP production was reflected by a difference in the $[Ca^{2+}]_i$ responses when the measurement was performed at 37°C (Figure 7). The amplitudes of the transient rises in $[Ca^{2+}]_i$ were similar: 659 ± 78 nM ($n=5$) and 671 ± 46 nM ($n=5$, $P>0.5$) for 1 μM 2-MeSATP and 10 μM CCh, respectively, but the amplitude of the 2-MeSATP-induced maintained $[Ca^{2+}]_i$ rise (120 ± 5 nM, $n=5$) was significantly smaller than that recorded in response to CCh (226 ± 15 nM, $n=5$, $P<0.0001$) (Figure 7Ab, Bb). In addition, the effect of 2-MeSATP (1 μM) was virtually abolished when it was applied in the presence of CCh (10 μM, Figure 7Ab) whereas, application of CCh (10 μM) in the presence of 2-MeSATP (1 μM) was still able to produce a large transient rise in $[Ca^{2+}]_i$ followed by a maintained phase (Figure 7Bb). These results suggest that 2-MeSATP and CCh release Ca^{2+} from the same intracellular Ca^{2+} store and that CCh activates an additional mechanism.

The difference in the $[Ca^{2+}]_i$ response induced by 2-MeSATP and CCh was suppressed if the measurements were performed at 20°C (Figure 7Aa, Ba). The amplitudes of the $[Ca^{2+}]_i$ transients were not significantly different ($P>0.1$) from those recorded at 37°C: 785 ± 87 nM ($n=3$) for 1 μM 2-MeSATP and 808 ± 41 nM ($n=3$) for 10 μM CCh. The amplitude of the maintained $[Ca^{2+}]_i$ rise induced by 2-MeSATP remained also unchanged (123 ± 9 nM, $n=3$, $P>0.5$) whereas the CCh-induced maintained $[Ca^{2+}]_i$ rise was strongly reduced to a value similar that obtained for 2-MeSATP (125 ± 5 nM, $n=3$, $P>0.5$). Under these conditions, the application of 2-MeSATP (1 μM) in the presence of CCh (10 μM) (Figure 7Aa) or reciprocally, the application of CCh (10 μM) in the presence of 2-MeSATP (1 μM) (Figure 7Ba) had little effect on $[Ca^{2+}]_i$. This suggests that at 20°C, CCh and 2-MeSATP increase $[Ca^{2+}]_i$ via the same pathway and that CCh is not able to activate the mechanism responsible for the additional increase in $[Ca^{2+}]_i$ observed at 37°C.

In agreement with the above results, measurements of InsP production showed that the stimulant effect of CCh is suppressed at 20°C (Figure 8A, C). In the presence of CCh 10 μM,

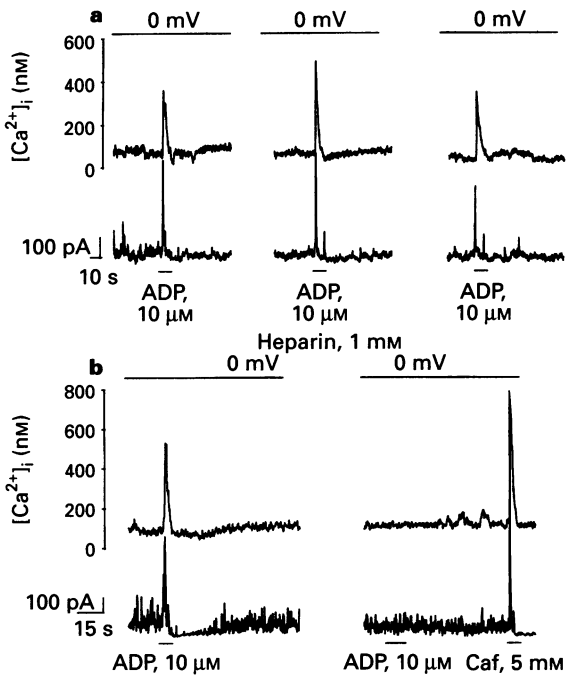

Figure 6 Effect of heparin on the current and the $[Ca^{2+}]_i$ rise induced by ADP (10 μM) at a holding potential of 0 mV. (a) Control experiment in the absence of heparin showing that repetitive applications of ADP separated by 2 min evoked similar $[Ca^{2+}]_i$ and current responses. (b) ADP was applied 1 min (left) and 5 min (right) after breakthrough into the whole-cell recording mode with heparin (1 mM) in the pipette solution. After inhibition of the ADP-induced $[Ca^{2+}]_i$ rise, the effect of caffeine (5 mM) was tested. Traces are representative of 3 independent experiments.

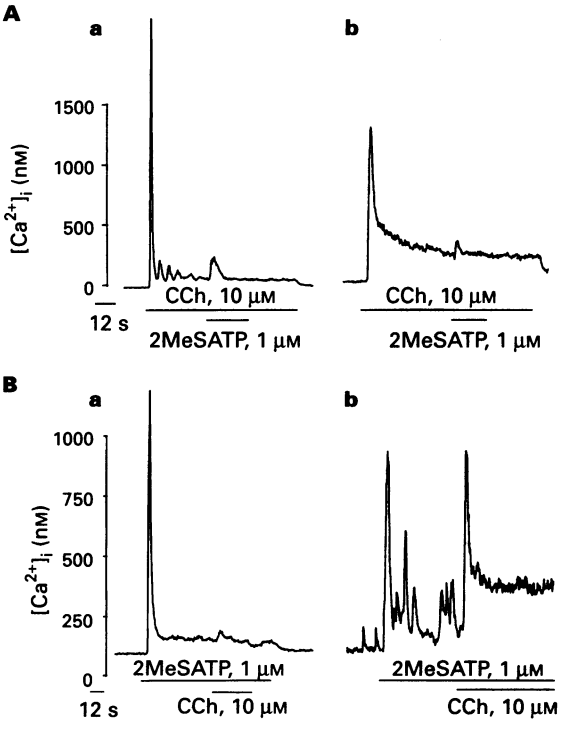

Figure 7 Effect of carbachol (CCh) and 2-methylthioATP (2-MeSATP) on $[Ca^{2+}]_i$ at 20°C and 37°C. (A) 2-MeSATP (1 μM) was applied during the maintained application of CCh (10 μM) at 20°C (a) and 37°C (b). (B) CCh (10 μM) was applied during the maintained application of 2-MeSATP (1 μM) at 20°C (a) and 37°C (b).

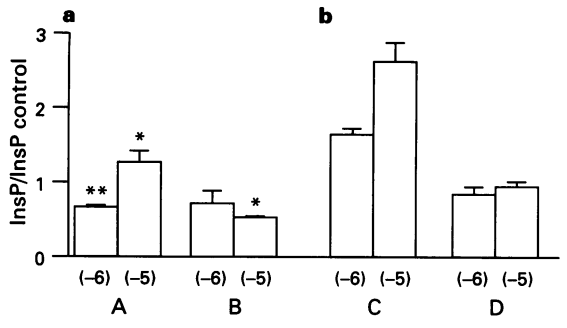

Figure 8 Effect of carbachol (CCh) and 2-methylthioATP (2-MeSATP; 1 and 10 μM) on inositol phosphate (InsP) formation in ileal cells prelabelled with [^{3}H]-inositol at (a) 20°C and (b) 37°C. Results are normalized to control value determined in the absence of stimulation with agonist. Agonist concentration (logM) used is indicated below each column. The agonists used were CCh (A,C) and 2-MeSATP (B,D). Results obtained at 20°C were compared with those obtained at 37°C: $*P<0.01$; $**P<0.003$.

the InsP level was not significantly different from that measured under control conditions ($n=3$, $P>0.1$). On the contrary, the InsP level detected in the presence of 2-MeSATP was only slighty lower than that measured at 37°C (Figure 8B, D).

Discussion

The sequence analysis of the receptor cloned from ileal smooth muscle indicated that it was identical to the rat P_{2Y}-purinoceptor isolated from an insulinoma cDNA library (Tokuyama *et al.*, 1995), which was the homologue of the chicken and turkey P_{2Y1}-receptor previously cloned (Webb *et al.*, 1993; Filtz *et al.*, 1994).

Individual ileal smooth muscle cells responded to a short application of the P_2-purinoceptor agonists by a transient rise in $[Ca^{2+}]_i$ in an all-or-nothing manner. In addition to this transient $[Ca^{2+}]_i$ rise, prolonged application of the P_2-purinoceptor agonists induced $[Ca^{2+}]_i$ oscillations, the frequency of which increased with agonist concentration; at high concentrations, a plateau phase was obtained. The $[Ca^{2+}]_i$ response was inhibited by intracellular application of heparin and probably results from an all-or-nothing mobilization of Ca^{2+} from intracellular stores that involves the Ca^{2+}-dependent feedback control of the $InsP_3$ receptor-channel (Iino *et al.*, 1993). The pharmacological specificity of the receptor involved in the $[Ca^{2+}]_i$ response is similar to that established previously for the P_{2Y}-purinoceptor subtype and markedly differs from those of the P_{2X}- and P_{2U}-receptor subtypes (Fredholm *et al.*, 1994; Abbrachio & Burnstock, 1994). ATP and ADP activated the same membrane receptor as cross-desensitization of the $[Ca^{2+}]_i$ response could be obtained by the consecutive application of both agonists. In addition, $[Ca^{2+}]_i$ rises induced by ATP and ADP were both inhibited by low concentration (5–10 μM) of PPADS. In the same range of concentration, PPADS had no effect on the CCh-induced $[Ca^{2+}]_i$ rise. The rank order of potency of analogues of ATP and ADP is 2-MeSATP = 2-ClATP > ADP > ATP, with concentrations producing a $[Ca^{2+}]_i$ rise in 50% of cells ranging between 3 nM and 0.6 μM. This high potency probably results from the presence of a receptor reserve as it seems that maximal effects could be obtained with the occupancy of only a fraction of the receptors. This suggests that P_{2Y}-purinoceptor agonists induce a $[Ca^{2+}]_i$ response in ileal myocytes by activating a P_{2Y}-purinoceptor on which PPADS acts as an antagonist. In agreement with this, recent findings have shown that PPADS is an effective antagonist at the turkey erythrocyte phospholipase C-linked P_{2Y}-purinoceptor (Boyer *et al.*, 1994) and at the P_{2Y}-purinoceptor of guinea-pig taenia coli and rat duodenum (Windscheif *et al.*, 1995) and bovine aortic endothelial cells (Brown *et al.*, 1995).

The P_{2Y}-purinoceptor agonists-induced $[Ca^{2+}]_i$ rise observed in ileal myocytes is in agreement with the increase in $[Ca^{2+}]_i$ and the activation of a Ca^{2+}-dependent current obtained in response to stimulation of the P_{2Y} receptors previously cloned and expressed in frog or *Xenopus* oocytes (Webb *et al.*, 1993; Tokuyama *et al.*, 1995). In view of the identity of the sequences, the second messenger coupling of these homologues of P_{2Y}-purinoceptors should be the same. The ileal P_{2Y}-purinoceptor is thus expected to activate the inositol lipid signaling cascade through a G protein and phospholipase C. In fact, although the activation of the ileal P_{2Y}-purinoceptor results in the release of intracellular Ca^{2+} by activation of the $InsP_3$ receptor-channel, we were not able to detect stimulation of phospholipase C under conditions where muscarinic cholinoceptor activation markedly increased InsP accumulation. This indicates that for low P_{2Y}-purinoceptor agonist concentrations, a sufficient amount of $InsP_3$ to produce Ca^{2+} store release could be generated without a detectable increase in InsP production. Thus, the absence of a rise in InsP accumulation after stimulation of a membrane receptor does not prove unambiguously, that this receptor does not couple to phospholipase C. Consistent with this, we found that the stimulation of InsP accumulation observed in response to CCh at 37°C was suppressed at 20°C although CCh still induced an $InsP_3$-dependent Ca^{2+} release similar to that obtained in response to P_{2Y}-purinoceptor activation. Such a lack of an inositol lipid response to P_{2Y}-receptor agonists has also been found in C6-2B rat glioma cells (Boyer *et al.*, 1993). These agonists inhibited adenylyl cyclase by activating receptors that expressed the general pharmacological specificity of a phospholipase C-coupled P_{2Y}-purinergic receptor. Therefore, Boyer *et al.* (1993) did not rule out the possibility that a low inositol lipid response to P_{2Y}-receptor agonists occurs in C6-2B glioma cells below the level of detection. The generation of only a small amount of $InsP_3$ in response to P_{2Y}-purinoceptor stimulation is compatible with the observed all-or-nothing Ca^{2+} release as it involves regulation of an $InsP_3$ receptor-channel allowing a low $InsP_3$ concentration to produce a maximal response (Iino *et al.*, 1993).

Our results suggest that the activation of P_{2Y}- and muscarinic receptors, both coupled to phospholipase C leads to different InsP productions. This may indicate that either CCh has an additional positive effect on phospholipase C or P_{2Y}-purinoceptor agonists exert a negative control on phospholipase C activity.

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