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P_2-purinoceptor-mediated formation of inositol phosphates and intracellular Ca^{2+} transients in human coronary artery smooth muscle cells

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1 The effects of extracellular adenosine 5'-triphosphate (ATP) on smooth muscles are mediated by a variety of purinoceptors. In this study we addressed the identity of the purinoceptors on smooth muscle cells (SMC) cultured from human large coronary arteries. Purinoceptor-mediated increases in $[Ca^{2+}]_i$ were measured in single fura-2 loaded cells by applying a digital imaging technique, and the formation of inositol phosphate compounds was quantified after separation on an anion exchange column.

2 Stimulation of the human coronary artery SMC (HCASMC) with extracellular ATP at concentrations of 0.1–100 μM induced a transient increase in $[Ca^{2+}]_i$ from a resting level of 49 ± 21 nM to a maximum of 436 ± 19 nM. The effect was dose-dependent with an EC_{50} value for ATP of 2.2 μM.

3 The rise in $[Ca^{2+}]_i$ was independent of the presence of external Ca^{2+}, but was abolished after depletion of intracellular stores by incubation with 100 nM thapsigargin.

4 $[Ca^{2+}]_i$ was measured upon stimulation of the cells with 0.1–100 μM of the more specific P_2-purinoceptor agonists α,β-methyleneadenosine 5'-triphosphate (α,β-MeATP), 2-methylthioadenosine 5'-triphosphate (2MeSATP) and uridine 5'-triphosphate (UTP). α,β-MeATP was without effect, whereas 2MeSATP and UTP induced release of Ca^{2+} from internal stores with 2MeSATP being the most potent agonist ($EC_{50} = 0.17$ μM), and UTP having a potency similar to ATP. The P_1 purinoceptor agonist adenosine (100 μM) did not induce any changes in $[Ca^{2+}]_i$.

5 Stimulation with a submaximal concentration of UTP (10 μM) abolished a subsequent ATP-induced increase in $[Ca^{2+}]_i$, whereas an increase was induced by ATP after stimulation with 10 μM 2MeSATP.

6 The phospholipase C (PLC) inhibitor U73122 (5 μM) abolished the purinoceptor-activated rise in $[Ca^{2+}]_i$, whereas pretreatment with the G_i protein inhibitor pertussis toxin (PTX, 500 ng ml^{-1}) was without effect on ATP-evoked $[Ca^{2+}]_i$ increases.

7 Receptor activation with UTP and ATP resulted in formation of inositol phosphates with peak levels of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) observed 5–20 s after stimulation.

8 These findings show, that cultured HCASMC express G protein-coupled purinoceptors, which upon stimulation activate PLC to induce enhanced $Ins(1,4,5)P_3$ production causing release of Ca^{2+} from internal stores. Since a release of Ca^{2+} was induced by 2MeSATP as well as by UTP, the data indicate that P_{2Y}- as well as P_{2U}-purinoceptors are expressed by the HCASMC.

Keywords: Vascular smooth muscle; coronary artery; P_2-purinoceptor; nucleotide receptor; internal calcium; inositol phosphates; inositol 1,4,5-trisphosphate

Introduction

Extracellular adenosine 5'-triphosphate (ATP) is a physiological regulator of vascular tone acting both as an intraluminal and as a neuronal messenger (reviewed by Burnstock, 1993). The responses to ATP are mediated by specific receptors called P_2-purinoceptors as opposed to the receptors for adenosine, which are called P_1-purinoceptors. The identification of purinoceptors is mainly based on potency orders of purinoceptor agonists, since specific and selective antagonists are lacking. Within the P_2-purinoceptor family at least 5 different receptors (P_{2X}, P_{2Y}, P_{2T} P_{2Z}, P_{2U}) have been characterized pharmacologically, and several of these P_2-purinoceptor subtypes are expressed on smooth muscles (Dubyak & El-Moatassim, 1993).

P_{2X} receptors are ATP gated ion channels with two membrane spanning segments (Valera *et al.*, 1994; Brake *et al.*, 1994). Originally a P_{2X}-purinoceptor with a potency order of agonists of α,β-methylene ATP (α,β-MeATP) $\geq \beta,\gamma$-MeATP > ATP $\geq$ ADP > 2-methylthio ATP (2MeSATP) > > uridine 5'-triphosphate (UTP) (Fredholm *et al.*, 1994) was functionally identified mainly on the basis of experiments with vas deferens, in which the fast initial transient contraction was found to be due to activation of receptors by ATP, that had been co-released with noradrenaline upon stimulation of sympathetic nerves (Burnstock & Kennedy, 1985). Patch-clamp studies revealed that P_{2X}-purinoceptors on rabbit ear artery smooth muscle cells (SMC) and rat vas deferens were ligand-gated channels permeable to mono- and divalent cations (Benham & Tsien, 1987; Friel, 1988). Activation of these P_{2X} channels leads to depolarization of the cell and may increase $[Ca^{2+}]_i$ through direct receptor-operated influx of Ca^{2+} combined with an influx through depolarization-activated Ca^{2+} channels.

P_{2Y}- as well as P_{2U}-purinoceptors belong to the family of receptors with 7 transmembrane-spanning domains. These receptors are coupled to G proteins, and their stimulation may lead to activation of phospholipase C (PLC), formation of inositol (1,4,5)-trisphosphate and subsequent release of Ca^{2+} from internal stores (Webb *et al.*, 1993; Erb *et al.*, 1993; Barnard *et al.*, 1994). The agonist potency order at the P_{2Y} receptor is considered to be 2MeSATP > ATP = ADP > > α,β-

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MeATP>>UTP, whereas the P_{2U}-purinoceptors are characterized by being sensitive to pyrimidine as well as purine nucleotides with an agonist potency order of UTP≥ATP =ATPγS>>2MeSATP=α,β-MeATP (Fredholm *et al.*, 1994).

ATP-induced accumulation of inositol phosphates (Phaneuf *et al.*, 1987) and release of Ca^{2+} from internal stores have been demonstrated in aortic SMC (Phaneuf *et al.*, 1987; Tawada *et al.*, 1987; Droogmans *et al.*, 1991; Kalthof *et al.*, 1993; von der Weid *et al.*, 1993). These responses were due to activation of P_2 purinoceptors, but the P_2-purinoceptor subtypes were not characterized in these studies. UTP has been shown to mediate release of intracellular Ca^{2+} with subsequent activation of a Na^+-permeable channel as well as Ca^{2+}-activated K^+ channels in SMC from thoratic aorta (Sanchez-Fernandez *et al.*, 1993).

In order to characterize the signaling processes activated by purinoceptor stimulation in human coronary artery smooth muscle cells (HCASMC) we identified the purinoceptor subtypes by measuring receptor-activated changes in inositol phosphates as well as changes in the $[Ca^{2+}]_i$. We found, that stimulation with purinoceptor agonists activated P_2-purinoceptors inducing activation of PLC, inositol phosphate formation and release of Ca^{2+} from thapsigargin-sensitive stores, and that the responses could be induced by activation of P_{2Y}- as well as P_{2U}-purinoceptors.

Methods

Preparation and culture of human coronary artery smooth muscle cells

Human coronary arteries were obtained from 4 patients, who were receiving heart transplantation due to nonvascular-related diseases such as cardiomyopathia and inborn heart malfunction. Small pieces were dissected from the left anterior descending coronary artery immediately after removal of the heart, and they were brought to the laboratory in a physiological saline at 4°C. The study protocol was approved by the local ethical committee, and informed consent was obtained from each patient. SMC were obtained by a modification of the explant method (Ross, 1971). Briefly, the arteries were cleaned from fat and adventitia and opened longitudinally. The intima was removed by scraping with a razorblade, and small pieces of media were stripped with a forceps. The smooth muscle tissue was cut into 1 mm^3 pieces with a tissue chopper, placed in culture flasks and covered with Dulbecco's modified eagles medium (DMEM) supplemented with 10% heat inactivated foetal calf serum, 2 mM glutamine, penicillin (100 u ml^{-1}) and streptomycin (100 mg ml^{-1}). The SMC migrated out from the explants and a confluent cell layer was obtained within 5–6 weeks displaying the characteristic SMC 'hill-and-valley' pattern (Chamley-Campbell *et al.*, 1979). The SMC were subsequently subcultured once a week by mild treatment with trypsin/EDTA and cells from one patient between passage levels 4 and 10 were used in the experiments presented. We did not observe variations in the responses between different patients or passage levels of cells.

Measurements of inositol phosphates

The agonist-induced production of inositol phosphates was assayed in cells grown in 6-well plates (Nunc Inc., U.S.A.). The cells were prelabelled with 0.5 μM myo-[2-^{3}H]-inositol (10 μCi ml^{-1} for 24 h in an inositol- and glucose-free DMEM. Before agonist application the cells were washed 3 times, at room temperature, in the standard extracellular solution consisting of (in mM): NaCl 140, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, glucose 5, HEPES 10, with pH adjusted to 7.4. In these washing steps 2 mM unlabelled *myo*-inositol was added to the extracellular solution in order to remove excessive myo-[2-^{3}H]-inositol. The cells were incubated in 400 μl extracellular solution with or without agonist for given time periods. The incubation was terminated by adding 200 μl ice cold trichloroacetic acid to a final concentration of 0.7 M. The well content was centrifuged, and the trichloroacetic acid in the supernatants was extracted twice with diethyl ether. Inositol phosphate contents were determined by separatin on an anion exchange column (DOWEX, Vydak 303NT405 Rainin Instrument Co. U.S.A.) according to Gromada *et al.* (1995b).

$[Ca^{2+}]_i$ measurements

$[Ca^{2+}]_i$ was measured in fura-2 loaded single cells seeded in Coverglass Chambers (Nunc Inc., U.S.A.). The cells were loaded with 2.5 μM fura-2/AM in DMEM for 30 min in the incubator, then washed 3 times in extracellular solution and placed on top of a fluorescence microscope (Zeiss IM35). Images of $[Ca^{2+}]_i$ in single cells were obtained by excitation with a 75 W xenon lamp with a UG5 filter inserted after the light source. A filter selector was used to alternate between 340 and 380 nm excitation filters (10 nm half-width; Optisk Lab., Lyngby Denmark). Emission was measured with a 530 nm cut off filter in the emission path. Images were captured with a Nikon 40x, 1.3 NA objective, a silicone intensified target camera (SIT, series 66; Dage-MTI, U.S.A.), and a digital image-processing and analysis system (Universal Corporation, U.S.A.). Ratioed images were formed with a frequency of 0.2 Hz and $[Ca^{2+}]_i$ was calculated from the ratioed fluorescence intensities according to the equation:

$$[Ca^{2+}]_i = K_d \cdot [(R - R_{min})(R_{max} - R)] \cdot (S_{f2}/S_{b2})$$

where K_d is the dissociation constant (224 nM) (Grynkiewicz *et al.*, 1985), and R_{max} and R_{min} are ratios (340 and 380 nm excitation) of fluorescence intensities of fura-2 at Ca^{2+}-saturating and Ca^{2+}-free conditions. R_{max} amounted to 250 gray values, after addition of 500 μM UTP and 5 μM ionomycin. R_{min} was obtained by suspending cells in a Ca^{2+}-free extracellular solution with 100 μM EGTA and 5 μM ionomycin. The gray values were recorded after several minutes, and they amounted to 42. The proportionality constant (S_{f2}/S_{b2}) is the fluorescence intensity at wavelength 2 (380 nm) of the free (S_{f2}) and Ca^{2+}-bound (S_{b2}) dye at low fura-2 concentration. S_{f2}/S_{b2} amounted to 2.65 when obtained from high K^+ solutions one of which was Ca^{2+} free and contained 2 mM EGTA and one which contained 2 mM Ca^{2+}. The images were stored on a video tape recorder (Sony, Umatic SP), and the analysis of changes in $[Ca^{2+}]_i$ was made by playing the images back through the image processing system (Image-1) and measuring the changes in gray values at a particular location in the cell as a function of time.

Drugs

Fura-2 acetoxymethylester (Fura-2/AM) was from Molecular Probes, OR, U.S.A.; myo-[2-^{3}H]-inositol from NEN Research Products, MA, U.S.A.; U73122 (1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione) and U73343 (1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]-amino]hexyl]-1*H*-pyrrolidine-2,5-dione) from Biomol, PA, U.S.A.; and 2MeSATP from RBI, MA, U.S.A. Thapsigargin was from Alomone lab, Israel. Dulbecco's modified Eagle's medium (DMEM) was from Biochrom KG, Germany; foetal calf serum from Sera-Lab, U.K.; and trypsin/EDTA and penicillin/streptomycin from Gibco BRL, MD, U.S.A. All other chemicals were from Sigma, MO, U.S.A.

Results

Time course and concentration-dependence of the ATP-induced rise in $[Ca^{2+}]_i$

ATP in the concentration range 0.1–100 μM induced transient increases in $[Ca^{2+}]_i$ from a resting level of 49±21 nM (n=225)

to a maximal value of 436±19 (n=91) when added to the solution bathing the HCASMC. The time courses of the responses shown in Figure 1 are the mean values of $[Ca^{2+}]_i$ calculated from 4–6 cells in 3–15 experiments. As often observed in single cell measurements, there was a considerable variation from cell to cell in the ATP-induced response both with respect to the amplitude of the transient and to the occurrence of oscillations. At the lowest ATP concentrations tested (0.1–1 μM) the upstroke of the transient was slower, and not every cell responded, whereas at intermediate ATP concentrations (3–30 μM) oscillations occurred in 20–40% of the cells with an interspike interval of 8–20 s. At higher concentrations of ATP (300–500 μM) all cells responded with a single transient (data not shown).

The maximal rise in $[Ca^{2+}]_i$, which was defined as the difference between the value at the peak of the response and the unstimulated value, is plotted as a function of the ATP concentration in Figure 1b. The dose-response relation is a saturating function, and the curve was obtained by fitting the data to a Michaelis-Menten type equation:

$$\Delta[Ca^{2+}]_i = (\Delta[Ca^{2+}]_i^{max}[ATP])/([ATP] + EC_{50})$$

From the fit an EC_{50} value of 2.2±1.9 μM was obtained for the ATP-induced rise in $[Ca^{2+}]_i$.

Effect of removal of extracellular Ca^{2+} on the ATP-induced rise in $[Ca^{2+}]_i$

We tested to what extent the increase in $[Ca^{2+}]_i$ was due to a Ca^{2+} release from internal stores and to a Ca^{2+} influx from the extracellular solution. The results are shown in Figure 2, where the SMC were stimulated by addition of 100 μM ATP to the bath solution either in the presence or in the absence of external Ca^{2+}. Removal of extracellular Ca^{2+} did not affect the magnitude of the peak of the $[Ca^{2+}]_i$ transient, revealing that the initial Ca^{2+} rise was due to Ca^{2+} release from internal stores. We also tested, whether the internal Ca^{2+} pools were affected by removal of extracellular Ca^{2+} and could be depleted by the presence of the inhibitor of intracellular Ca^{2+}-ATPases, thapsigargin (Thastrup *et al.*, 1990). After the first ATP application the cells were washed and left for 5 min either in the Ca^{2+}-free solution, in extracellular solution containing 100 nM thapsigargin, or in control extracellular solution (Figure 2). The cells in the Ca^{2+}-containing control solution displayed a rise in $[Ca^{2+}]_i$ in response to a second stimulation by 100 μM ATP, although it was of smaller magnitude than the first transient. When refilling of the internal stores was prevented by keeping the cells in the solution without extracellular Ca^{2+} or incubating them with thapsigargin, the $[Ca^{2+}]_i$ rise in response to a second stimulation by ATP was abolished, indicating that the intracellular Ca^{2+} pools responsible for the ATP-induced $[Ca^{2+}]_i$ rise were depleted by these treatments. The declining phase of the first $[Ca^{2+}]_i$ transient was faster when Ca^{2+} was omitted from the bath, indicating that an influx component was responsible for maintaining a more sustained Ca^{2+} response in the Ca^{2+}-containing extracellular solution. This influx component was not inhibited by incubating the cells with the Ca^{2+} channel blockers nifedipine (2.5 μM, n=25, not shown) or Cd^{2+} (10 μM, n=25, not shown).

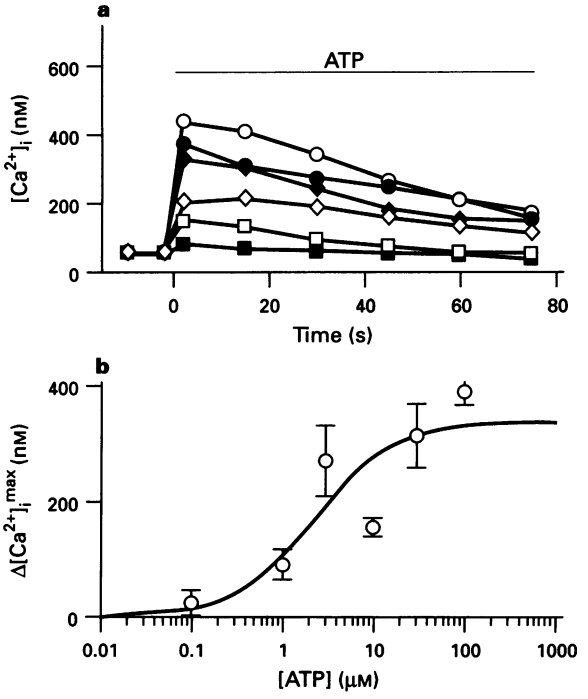

Figure 1 ATP-stimulated changes in $[Ca^{2+}]_i$ in single human coronary artery smooth muscle cells: time course and concentration-dependence. (a) Time courses of the mean $[Ca^{2+}]_i$ level in cells stimulated with ATP at the following concentrations (μM): 100 (○, n=91); 30 (●, n=19); 10 (◇, n=35); 3 (◆, n=19); 1 (□, n=17); or 0.1 (■, n=12). ATP was present during the time indicated by the bar. Error bars are omitted for clarity. (b) Dose-response relationship for ATP. The change in $[Ca^{2+}]_i$ at the peak of the ATP-responses shown in (a) ($\Delta[Ca^{2+}]_i^{max}$) is depicted as a function of the ATP concentration. The Michaelis-Menten fit to the data gives an EC_{50} value of 2.2 μM. Vertical lines show s.e.mean.

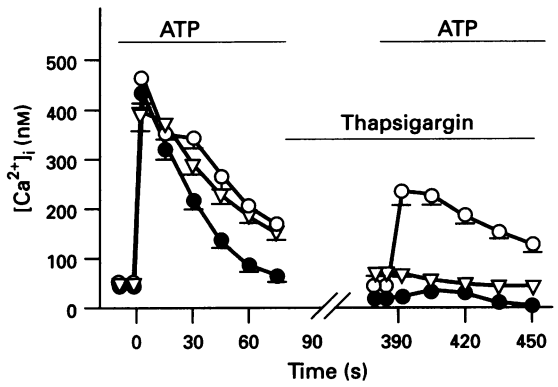

Figure 2 Contribution of internal and external Ca^{2+} to the ATP-induced response. The Ca^{2+}-rise induced by 100 μM ATP was measured in a Ca^{2+}-containing extracellular solution (○, n=18) and in a nominal Ca^{2+}-free (●, n=19) extracellular solution containing 100 μM EGTA. The contribuiton of internal stores was further elucidated by incubating cells in the Ca^{2+}-containing extracellular solution with thapsigargin (100 nM) (▽, n=18) for 5 min before a second ATP exposure. The presence of ATP and of thapsigargin is indicated by the bars. Vertical lines show s.e.mean.

Stimulation of the HCASMC by different purinoceptor agonists

The ability of several purinoceptor agonists to cause a rise in $[Ca^{2+}]_i$ were measured in order to evaluate the purinoceptor subtype involved in the ATP-induced increase in $[Ca^{2+}]_i$. Figure 3a, b, and c show original tracings of responses to 10 μM of ATP, UTP and 2MeSATP, respectively. The responses to ATP and UTP at all concentrations displayed similar magnitudes and time courses, whereas the initial increase in $[Ca^{2+}]_i$ in response to 2MeSATP was of greater magnitude and was characterized by a peak and then a plateau. Dose-response relationships for 2MeSATP, UTP and α,β-MeATP were compared to that obtained for ATP by plotting the mean maximum rise in $[Ca^{2+}]_i$ as a function of the agonist concentration. Figure 3d shows that with regard to the initial Ca^{2+} transient, 2MeSATP induced the largest change in $[Ca^{2+}]_i$ and had the highest potency. In order to get a better fit to the 2MeSATP data we applied a Michaelis-Menten fitting proce-

dure with two binding constants. We obtained EC_{50}-values of 0.3 μM and 9.7 mM, but since the second binding constant is very high, we have only used the high affinity binding constant as evidence for expression of P_{2Y} purinoceptors. UTP induced an increase in $[Ca^{2+}]_i$ with a potency similar to that of ATP, whereas the cells did not respond to stimulation with α,β-MeATP with an increase in $[Ca^{2+}]_i$. Neither the responses to 2MeSATP (17 cells) or UTP (9 cells) were dependent on the presence of Ca^{2+} in the extracellular solution, revealing that the responses to these agonists were due to release from intracellular stores (not shown). To rule out the possibility that the responses were due to the nucleotides being metabolized by extracellular ectonucleotidases and thereby mediating their effects via P_1-purinoceptors, we added adenosine (100 μM) to the cells, which, however, did not induce an increase in $[Ca^{2+}]_i$ (12 cells, not shown).

2MeSATP and UTP are believed to mediate release of Ca^{2+} through P_{2Y}- and P_{2U}-purinoceptors, respectively, and the data therefore suggest that the HCASMC express both of these receptor subtypes. We tested this possibility further by stimulating the cells with submaximal concentrations of 2MeSATP and UTP 100 s before 10 μM ATP was added to investigate whether a further increase in $[Ca^{2+}]_i$ could be elicited. An ATP-induced increase was obtained in the cells prestimulated with 2MeSATP but not in those stimulated with UTP (Figure 4). The lack of response to ATP after prior stimulation with UTP is unlikely to be due to depletion of the stores by the first stimulation, since a response was obtained after stimulation with 2MeSATP, which induced the largest release of Ca^{2+}.

Coupling of receptors to phospholipase C via a pertussis toxin-insensitive mechanism

P_{2Y}- and P_{2U}-purinoceptors are G protein-coupled, and in order to gain more information about the transduction pathway we tested whether the increase in $[Ca^{2+}]_i$ could be inhibited either by the G_i protein inhibitor pertussis toxin (PTX) or by the inhibitor of the PLC reaction, U73122. Figure 5a shows the increase in $[Ca^{2+}]_i$ after addition of 100 μM ATP to cells pretreated with PTX at 500 ng ml^{-1} for up to 24 h at 37°C compared to the response in untreated cells. Treatment with PTX did not significantly affect the magnitude of peak of the $[Ca^{2+}]_i$ transient induced by 100 μM ATP. The declining phase seemed to be faster in the PTX-treated cells, but this was not investigated further. In Figure 5b the effects of U73122 and its inactive structural analogue U73343 are shown. The cells were all stimulated once with 100 μM ATP, then they were washed and left for 5 min in either control extracellular solution, extracellular solution containing 5 μM U73122, or 5 μM U73343. Incubation with U73122 completely abolished the response to a second ATP exposure (99% inhibition, $n=25$)

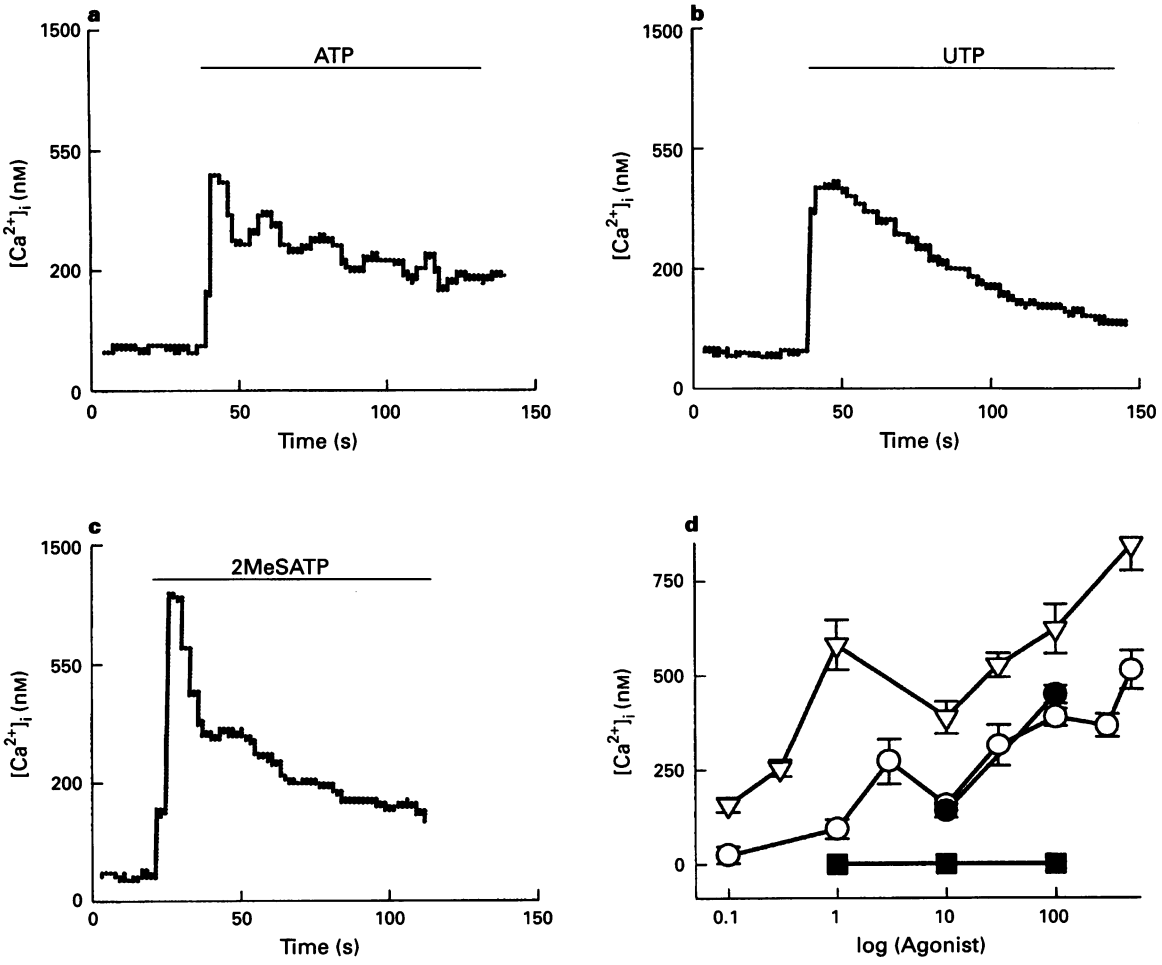

Figure 3 $[Ca^{2+}]_i$ responses induced by various P_2 receptor agonists. (a) (b) and (c): Examples of the time course of the increase in $[Ca^{2+}]_i$ induced by addition of 10 μM ATP, UTP, and 2MeSATP, respectively. (d) Dose-response relationships for ATP (○), UTP (●), 2MeSATP (▽), and α,β-MeATP (■) in the concentration range 0.1–500 μM. The magnitude of the maximal increase in $[Ca^{2+}]_i$ (i.e. peak value minus unstimulated value) is depicted as a function of agonist concentration. The values are means of 6–91 cells and vertical lines show s.e.mean.

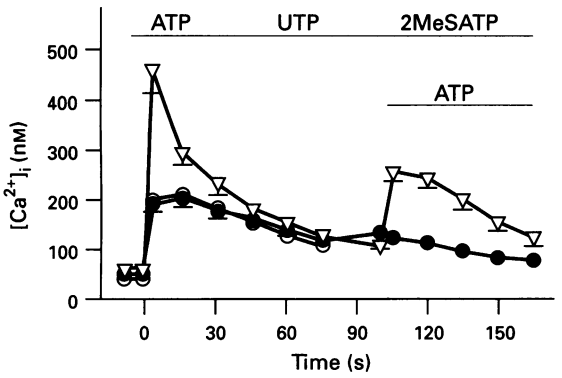

Figure 4 Cross-desensitization between responses to ATP and UTP. Mean time courses of the increase in $[Ca^{2+}]_i$ in cells stimulated with 10 μM of ATP (○, $n=35$); UTP (●, $n=29$); or 2MeSATP (▽, $n=18$). When exposed to an additional 10 μM ATP at time 100 s the cells bathed in 2MeSATP responded to ATP by a $[Ca^{2+}]_i$ rise. The presence of agonists is indicated by the bars. Vertical lines show s.e.mean.

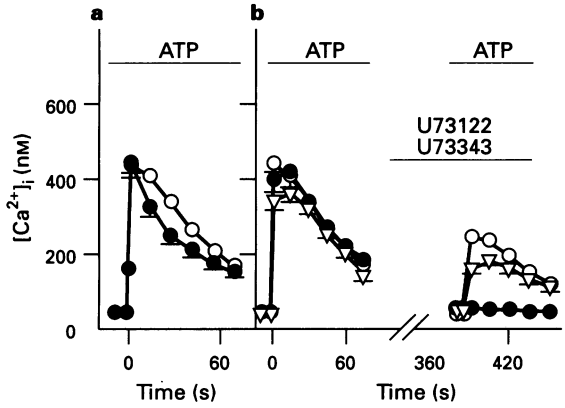

Figure 5 Effects of signal transduction blockers. (a) The $[Ca^{2+}]_i$ transient induced by stimulation with 100 μM ATP was determined in cells preincubated with (●, $n=19$) or without (○, $n=91$) pertussis toxin (500 ng ml^{-1}) for 24 h in the incubator. (b) The involvement of phospholipase C in the signal transduction pathway was tested by measuring the $[Ca^{2+}]_i$ transient induced by a second addition of 100 μM ATP during incubation of the cells with extracellular solution (○, $n=35$); 5 μM U73122 (●, $n=25$); or 5 μM U73343 (▽, $n=25$). Vertical lines show s.e.mean. ATP was present during the periods indicated by the bars.

in contrast to incubation with the inactive analogue U73343 (45% inhibition, $n=25$). The responses to 2MeSATP (15 cells) were also not blocked by PTX but U73122 abolished the 2MeSATP-induced increase in $[Ca^{2+}]_i$ (16 cells, not shown).

Activation of inositol phosphate production through P_{2U}-purinoceptors

As an additional approach in evaluating signaling processes underlying the Ca^{2+} release we also directly measured the purinoceptor-induced increase in cellular inositol phosphate content. Phosphatidyl inositol 4,5-bisphosphate ($PtInsP_2$) of cells growing under conditions similar to those under which the measurements of $[Ca^{2+}]_i$ were performed was prelabelled with [^{3}H]-myo-inositol before the cells were stimulated for different periods of time. The individual inositol phosphate compounds were separated on an anion exchange column, and Figure 6a shows the elution profiles for unstimulated cells as well as for cells stimulated with 100 μM UTP for 60 s. Addition of UTP (100 μM) induced a rapid production of inositol phosphates, and Figure 6b and c illustrates the changes in the concentration of 4 inositol phosphates. The % of total inositol phosphates expressed on the ordinate scale is proportional to the concentration of the individual inositol phosphates. The $Ins(1,4,5)P_3$ appeared within 5–10 s after stimulation (Figure 6b), and the $Ins(1,4)P_2$ and $Ins(1,3,4,5)P_4$ compounds, which are formed by either dephosphorylation or phosphorylation of $Ins(1,4,5)P_3$ appeared very rapidly (Figure 6c). These data are consistent with a relatively large $Ins(1,4,5)P_3$ synthesis taking place right at the onset of stimulation which subsequently is converted to $Ins(1,4)P_2$ or $Ins(1,3,4,5)P_4$. The $Ins(1,3,4)P_3$ compound is formed by dephosphorylation of $Ins(1,3,4,5)P_4$ and appeared with a slower rate (Figure 6b). The large increase in $Ins(1,4)P_2$ and the small increase in $Ins(1,3,4)P_3$ reveal that the major part of the inositol phosphate metabolism passed through the pathway involving dephosphorylation of $Ins(1,4,5)P_3$ to $Ins(1,4)P_2$ and not through phosphorylation to $Ins(1,3,4,5)P_4$. In five experiments we found that increases in the amount of inositol phosphates upon stimulation with 100 μM ATP had the same magnitudes and the same time courses as those observed for UTP (data not shown).

In order to test whether inositol phosphates were produced subsequent to an increase in $[Ca^{2+}]_i$ we measured the inositol phosphates under conditions that prevented a rise in $[Ca^{2+}]_i$. We found that incubation with the Ca^{2+} chelator BAPTA/AM (50 mM for 30 min), which completely prevented the ATP-induced Ca^{2+} rise, did not abolish the ATP induced inositol phosphate synthesis (data not shown).

Discussion

In this study we have cultured SMC from human coronary arteries and identified the purinoceptors expressed as well as the signal transduction cascade activated by these. The responses to various purinoceptor agonists were followed by measuring $[Ca^{2+}]_i$ and formation of inositol phosphates. The cells responded dose-dependently to extracellular applied ATP with increases in $[Ca^{2+}]_i$ from a basal level of 49±21 nM to 436±19 nM. An EC_{50} value of 2.2±1.9 μM was found for the ATP-induced increase in $[Ca^{2+}]_i$. We generally found, that the rise in $[Ca^{2+}]_i$ returned to the basal level within 1–2 min even in the continued presence of ATP although considerable variations were observed in the amplitude and the duration of the $[Ca^{2+}]_i$ transients even in cells lying next to each other in the recording chamber. Some cells displayed oscillations, and at the onset of stimulation Ca^{2+} waves could occasionally be seen. At the single cell level we observed that the nuclei appeared to have a lower resting $[Ca^{2+}]_i$ than the cytosol, but often reached the highest value after stimulation, which is in accordance with a previous finding in cultured SMC (Himpens *et al.*, 1994). The declining phase of the $[Ca^{2+}]_i$ transient was faster, and a second response to ATP could not be obtained, when extracellular calcium was omitted. An influx therefore appears to be necessary in order to refill the stores, but this influx of external Ca^{2+} did not occur through voltage-operated Ca^{2+} channels, since it could not be inhibited by Cd^{2+} or nifedipine. Further, in patch-clamp recordings we have been unable to measure L-type Ca^{2+} channels in these cells (unpublished), which seems to be a characteristic feature of cultured SMC (Kalthof *et al.*, 1993). The nature of the mechanism behind the calcium influx is unknown but several influx pathways, which are activated upon store depletion have been suggested (reviewed by Fasolato *et al.*, 1994). The insensitivity of the initial peak to external Ca^{2+} revealed, that the rapid increase in $[Ca^{2+}]_i$ was due to Ca^{2+} release from intracellular stores consistent with $Ins(1,4,5)P_3$ acting as the Ca^{2+} releasing factor. Furthermore, the ATP-induced $[Ca^{2+}]_i$ transient was abolished after incubation with thapsigargin, which is known to deplete internal stores (Thastrup *et al.*, 1990).

Various classes of purinoceptor have been described and classified according to their sensitivity to adenosine, ATP and ATP analogues. In addition to ATP we also measured a release of intracellular Ca^{2+} upon stimulation with 2MeSATP as well

as with UTP, and since these compounds are believed to activate P_{2Y}- and P_{2U}-purinoceptors, respectively (O'Connor *et al.*, 1991; Webb *et al.*, 1993; Erb *et al.*, 1993; Fredholm *et al.*, 1994) these receptor subtypes seem to be co-expressed on the HCASMC.

We did not find evidence for an expression of P_{2X}-purinoceptors on the HCASMC, since the P_{2X}-purinoceptor specific agonist α,β-MeATP was without effect on $[Ca^{2+}]_i$ and the increases in $[Ca^{2+}]_i$ induced by 2MeSATP and ATP were due to release from internal stores and not an influx of calcium through P_{2X} channels. In addition we found that the only inward current activated by purinoceptor agonists in these cells was a chloride current and not a non-selective cation current as the one carried by P_{2X} channels (unpublished data).

Lately it has become apparent that the presence of ectonucleotidases on various cells may blunt the 'true' agonist potency order at the purinoceptor since analogues such as ATP and 2MeSATP are more susceptible to breakdown than e.g. α,β-MeATP (Crack *et al.*, 1994; 1995; Trezise *et al.*, 1994). These findings are of special importance in relation to experiments performed in whole-tissue since ATP is more susceptible to break-down in the poorly stirred interstitium. In the present study, the compounds were added to a small number of cells in a volume of 1 ml, and within the short time-interval for measuring $[Ca^{2+}]_i$ (1 min) there should therefore be insignificant degradation of the nucleotides. Further, we tested whether some of the measured responses could be due to activation of P_1-purinoceptors by adding the P_1-purinoceptor agonist adenosine, which did not induce any changes in $[Ca^{2+}]_i$.

Following stimulation with UTP we found a rapid increase in Ins(1,4)P_2 and Ins-(1,3,4,5)P_4, and since both of these compounds are products of Ins(1,4,5)P_3, the data reveal, that there has been a considerable synthesis of Ins(1,4,5)P_3 at the onset of stimulation. The peak concentration of Ins(1,4,5)P_3 was observed 5–20 s after stimulation, but large amounts of Ins(1,4,5)P_3 can pass through its metabolic pathway without the metabolite building up at considerable concentration if the rate constant for emptying the PtInsP_2 pool is small, compared to the sum of the rate constants converting Ins(1,4,5)P_3 to Ins(1,4)P_2 and Ins(1,3,4,5)P_4. The finding that Ins(1,4,5)P_3 rapidly appears upon receptor activation with UTP is consistent with this compound being responsible for the release of Ca^{2+} from internal stores. Inositol phosphates were formed in cells loaded with BAPTA, which prevents a rise in $[Ca^{2+}]_i$, and this excludes the possibility that Ca^{2+} was released from internal pools by a different signaling process causing the synthesis of insositol phosphates by a Ca^{2+}-activated PLC reaction as previously described by Gromada *et al.* (1995a,b).

The involvement of PLC and inositol phosphate accumulation in the signal transduction pathway was also measured at the $[Ca^{2+}]_i$ level. Stimulation of the purinoceptors activated PLC, since the ATP-induced increase in $[Ca^{2+}]_i$ could be inhibited by the aminosteroid U73122, which has been shown to inhibit a variety of PLC mediated events (Smallridge *et al.*, 1992; Vickers, 1993; Jin *et al.*, 1994; Willems *et al.*, 1994; Gromada *et al.*, 1995a). The responses to UTP and 2MeSATP were also abolished by U73122 indicating that both P_{2Y}- and P_{2U}-receptors couple to PLC. When we added U73122 to the

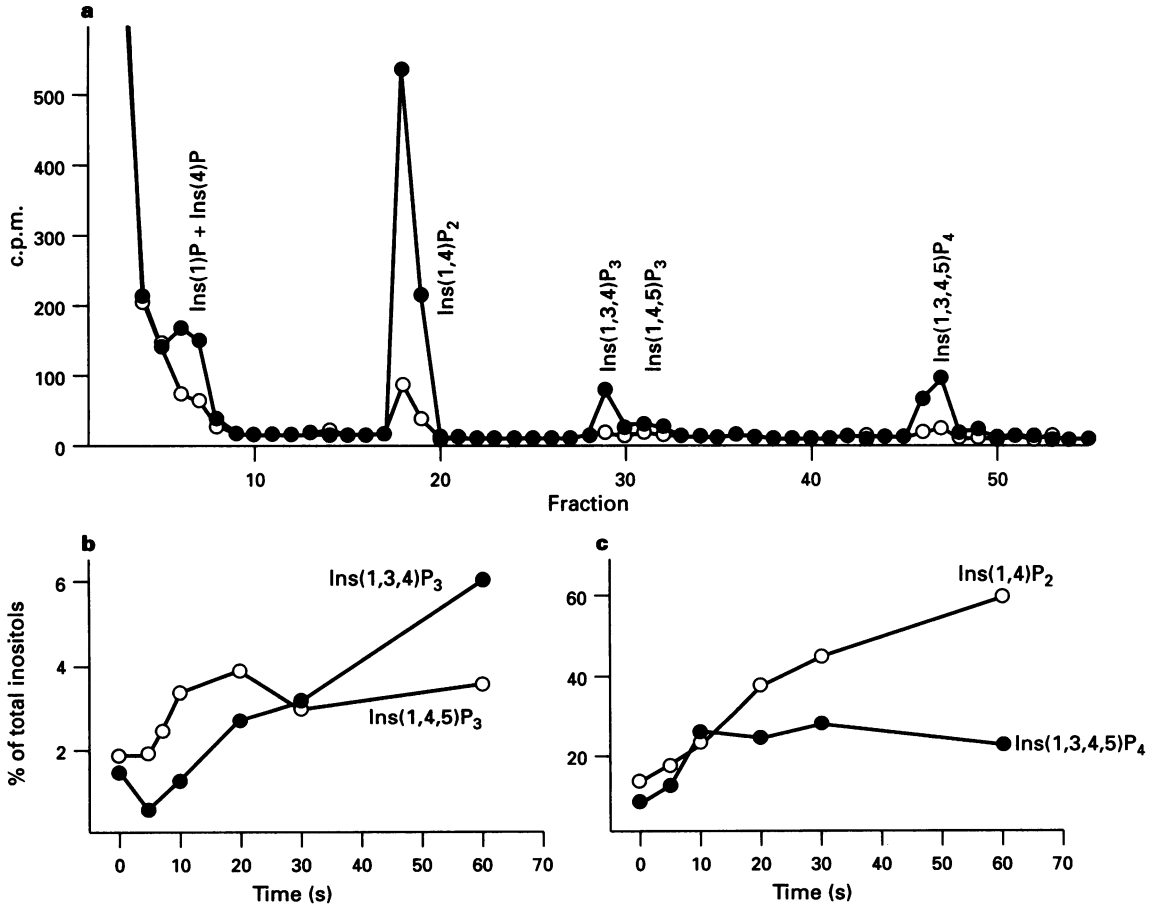

Figure 6 Time course of inositol phosphate formation after stimulation with 100 μM UTP. (a) The amount of ^{3}H-label in each of the 55 fractions collected after separation on the anion exchange column in unstimulated (○) cells and in cells incubated with 100 μM UTP for 60 s (●). (b and c) Time course of inositol phosphate formation after stimulation with 100 μM UTP. Each of the inositol phosphates Ins(1,4)P_2 (c,○), Ins(1,3,4)P_3 (b,●), Ins(1,4,5)P_3 (b,○) and Ins(1,3,4,5)P_4 (c,●) is expressed as % of the total amount of inositol phosphates measured at 60 s after stimulation. The data points are averages from five (c) and two (b) experiments.

cells it did not induce an increase in $[Ca^{2+}]_i$ by itself, and inhibition of the agonist-induced $[Ca^{2+}]_i$ transient was much smaller with the structural analogue U73343 indicating that the observed effect is not due to non-specific interactions of U73122 with membranes or to U73122 working as a Ca^{2+}-releasing agent.

The ability of P_2 purinoceptor agonists to induce formation of inositol phosphates and Ca^{2+} mobilization is attenuated or inhibited by pertussis toxin in some cell types (see Dubyak & El-Moatassim, 1993). In the HCASMC we did not find an inhibition of the initial peak of the Ca^{2+}-transient by pretreatment with PTX. This is compatible with the release being induced by Ins(1,4,5)P_3 generated from activation of PLCβ by a member of the $G_{q/11}$ class of G proteins which is PTX-insensitive (Hepler & Gilman, 1992).

Our data are thus consistent with the expression of both P_{2U}- and P_{2Y}-purinoceptors on the human coronary artery SMC. A release of intracellular calcium was induced subsequent to receptor-activated stimulation of G proteins (presumably of the $G_{q/11}$ subtype), which activated PLC to produce Ins(1,4,5)P_3. ATP-induced release of Ca^{2+} from internal stores has also been described in cultured aortic SMC (Phaneuf *et al.*, 1987; Tawada *et al.*, 1987; Droogmans *et al.*, 1991; Kalthof *et al.*, 1993; von der Weid *et al.*, 1993), and P_{2U}-purinoceptors have been demonstrated in bovine thoracic aorta SMC (Sanchez-Fernandez *et al.*, 1993). In many vessels ATP induces contraction via P_{2X}-purinoceptors located on the SMC and relaxation via P_{2Y}-purinoceptors located on the endothelium (Burnstock & Kennedy, 1986; von Kügelgen & Starke, 1991). However, in rabbit coronary arteries ATP-induced relaxation was mediated by P_{2Y}-purinoceptors located on the SMC (Corr & Burnstock, 1991). The lack of P_{2X} receptors in this study could therefore be due to coronary arteries responding to ATP in a different manner. It is, however, also possible that proliferale SMC, which specialize in synthesis and migration, do not express P_{2X}-purinoceptors.

The present culture of human coronary artery SMC appears to be a useful model for characterizing the intracellular signals following receptor stimulation. The cells express P_{2Y}- and P_{2U}-purinoceptors, that mediate their responses through the activation of PLC resulting in liberation of inositol phosphates and release of intracellular Ca^{2+}.

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References

BARNARD, E.A., BURNSTOCK, G. & WEBB, T.E. (1994). G protein-coupled receptors for ATP and other nucleotides: a new receptor family. *Trends Pharmacol. Sci.*, **15**, 67–70.

BENHAM, C.D. & TSIEN, R.W. (1987). A novel receptor-operated Ca^{2+}-permeable channel activated by ATP in smooth muscle. *Nature*, **328**, 275–278.

BRAKE, A.J., WAGENBACH, M.J. & JULIUS, D. (1994). New structural motif for ligand-gated ion channels defined by a ionotropic ATP receptor. *Nature*, **371**, 519–523.

BURNSTOCK, G. (1993). Physiological and patophysiological roles of purines: An update. *Drug Dev. Res.*, **28**, 195–206.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, **16**, 433–440.

BURNSTOCK, G. & KENNEDY, C. (1986). A dual function for adenosine 5'-triphosphate in the regulation of vascular tone. *Circ. Res.*, **58**, 319–330.

CHAMLEY-CAMPBELL, J., CAMPBELL, G.R. & ROSS, R. (1979). The smooth muscle cell in culture. *Physiol. Rev.*, **59**, 1–61.

CORR, L. & BURNSTOCK, G. (1991). Vasodilatory response of coronary smooth muscle to the sympathetic co-transmitters noradrenaline and adenosine 5'-triphosphate. *Br. J. Pharmacol.*, **104**, 337–342.

CRACK, B.E., BEUKERS, M.W., MCKECHNIE, K.C.W., IJZERMAN, A.P. & LEFF, P. (1994). Pharmacological analysis of ecto-ATPase inhibition: evidence for combined enzyme inhibition and receptor antagonism in P_{2X}-purinoceptor ligands. *Br. J. Pharmacol.*, **113**, 1432–1438.

CRACK, B.E., POLLARD, C.E., BEUKERS, M.W., ROBERTS, S.M., HUNT, S.F., INGALL, A.H., MCKECHNIE, K.C.W., IJZERMAN, A.P. & LEFF, P. (1995). Pharmacological and biochemical analysis of FPL 67156, a novel, selective inhibitor of ecto-ATPase. *Br. J. Pharmacol.*, **114**, 475–481.

DROOGMANS, G., CALLEWAERT, G., DECLERCK, I. & CASTEELS, R. (1991). ATP-induced Ca^{2+} release and Cl^- current in cultured smooth muscle cells from pig aorta. *J. Physiol.*, **440**, 623–634.

DUBYAK, G.R. & EL-MOATASSIM, C. (1993). Signal transduction via P_2-purinergic receptors for extracellular ATP and other nucelotides. *J. Physiol.*, **34**, C577–C606.

ERB, L., LUSTIG, K.D., SULLIVAN, D.M., TURNER, J.T. & WEISMAN, G.A. (1993). Functional expression and photoaffinity labeling of a cloned P_{2U} purinergic receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 10449–10453.

FASOLATO, C., INNOCENTI, B. & POZZAN, T. (1994). Receptor-activated Ca^{2+} influx: how many mechanisms for how many channels? *Trends Pharmacol. Sci.*, **15**, 77–83.

FREDHOLM, B.B., ABBRACCHIO, M.P., BURNSTOCK, G., DALY, J.W., HARDEN, T.K., JACOBSEN, K.A., LEFF, P. & WILLIAMS, M. (1994). Nomenclature and classification of purinoceptors. *Pharmacol. Rev.*, **46**, 143–156.

FRIEL, D.D. (1988). An ATP-sensitive conductance in single smooth muscle cells from the rat vas deferens. *J. Physiol.*, **401**, 361–380.

GROMADA, J., JØRGENSEN, T.D. & DISSING, S. (1995a). Role of protein kinase C in the regulation of inositol phosphate production and Ca^{2+} mobilization evoked by ATP and acetylcholine in rat lacrimal acini. *Pflügers Arch.*, **429**, 578–586.

GROMADA, J., JØRGENSEN, T.D. & DISSING, S. (1995b). The release of intracellular Ca^{2+} in lacrimal acinar cells by α,β-adrenergic and muscarinic cholinergic stimulation: the roles of inositol trisphosphate and cyclic ADP ribose. *Pflügers Arch.*, **429**, 751–761.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

HEPLER, J.R. & GILMAN, A.G. (1992). G. proteins. *Trends Pharmacol. Sci.*, **17**, 383–387.

HIMPENS, B., DE SMEDT, H. & CASTEELS, R. (1994). Relationship between $[Ca^{2+}]$ changes in nucleus and cytosol. *Cell Calcium*, **16**, 239–246.

JIN, W., LO, T.-M., LOH, H. & THAYER, S.A. (1994). U73122 inhibits phospholipase C-dependent calcium mobilization in neuronal cells. *Brain Res.*, **642**, 237–243.

KALTHOF, B., BECHEM, M., FLOCKE, K., POTT, L. & SCHRAMM, M. (1993). Kinetics of ATP-induced Ca^{2+} transients in cultured pig aortic smooth muscle cells depend on ATP concentration and stored Ca^{2+}. *J. Physiol.*, **466**, 245–262.

O'CONNOR, S.E.. DAINTY, I.A. & LEFF, O. (1991). Further subclassification of ATP receptors based on agonist studies. *Trends Pharmacol. Sci.*, **12**, 137–141.

PHANEUF, S., BERTA, P., CASANOVA, J. & CAVADORE, J.-C. (1987). ATP stimulates inositol phosphates accumulation and calcium mobilization in a primary culture of rat aortic myocytes. *Biochem. Biophys. Res. Commun.*, **143**, 454–460.

ROSS, R. (1971). The smooth muscle cell. II. Growth of smooth muscle in culture and formation of elastic fibers. *J. Cell. Biol.*, **50**, 172–186.

SANCHEZ-FERNANDEZ, M., KATZ, G.M., SUAREZ-KURTZ, G., KACZOROWSKI, G.J. & REUBEN, J. (1993). Mobilization of intracellular calcium in cultured vascular smooth muscle cells by uridine triphosphate and the calcium ionophore A23187. *J. Membr. Biol.*, **135**, 273–287.

SMALLRIDGE, R.C., KIANG, J.G., GIST, I.D., FEIN, H.G. & GALLOWAY, R.J. (1992). U-73122, an aminosteroid phospholipase C antagonist, noncompetitively inhibits thyrotropin-releasing hormone effects in GH_3 rat pituitary cells. *Endocrinology*, **131,** 1883–1888.

TAWADA, Y., FURUKAWA, K.-I. & SHIGEKAWA, M. (1987). ATP-induced calcium transient in cultured rat aortic smooth muscle cells. *J. Biochem.*, **102,** 1499–1509.

THASTRUP, O., CULLEN, P.J., DRØBAK, B.K., HANLEY, M.R. & DAWSON, A.P. (1990). Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc. Natl. Acad. Sci. U.S.A.*, **87,** 2466–2470.

TREZISE, D.J., BELL, N.J., KENNEDY, I. & HUMPHREY, P.P.A. (1994). Effects of divalent cations on the potency of ATP and regulated agonists in the rat isolated vagus nerve: implications for P_2 purinoceptor classification. *Br. J. Pharmacol.*, **113,** 463–470.

VALERA, S., HUSSY, N., EVANS, R.J., ADAMI, N., NORTH, R.A., SURPRENANT, A. & BUELL, G. (1994). A new class of ligand-gated ion channel defined by P_{2X} receptor for extracellular ATP. *Nature*, **371,** 516–519.

VICKERS, J.D. (1993). U73122 affects the equilibria between the phosphoinositides as well as phospholipase C activity in unstimulated and thrombin-stimulated human and rabbit platelets. *J. Pharmacol. Exp. Ther.*, **266,** 1156–1162.

VON DER WEID, P.Y., SEREBRYAKOV, V.N., ORALLO, F., BERGMANN, C., SNETKOV, V.A. & TAKEDA, K. (1993). Effects of ATP on cultured smooth muscle cells from rat aorta. *Br. J. Pharmacol.*, **108,** 638–645.

VON KÜGELGEN, I. & STARKE, K. (1991). Noradrenaline-ATP co-transmission in the symphathetic nervous system. *Trands Pharmacol. Sci.*, **12,** 319–324.

WEBB, T.E., SIMON, J., KRISHEK, B.J., BATESON, A.N., SMART, T.G., KING, B.I., BURNSTOCK, G. & BARNARD, E.A. (1993). Cloning and functional expression of a brain G-protein-coupled ATP receptor. *FEBS*, **324,** 219–225.

WILLEMS, P.H.G.M., VAN DE PUT, F.H.M.M., ENGBERSEN, R., BOSCH, R.R., VAN HOOF, H.J.M. & DE PONT, J.J.H.H.M. (1994). Induction of Ca^{2+} oscillations by selective, U73122-mediated, depletion of inositol-trisphosphate-sensitive Ca^{2+} stores in rabbit pancreatic acinar cells. *Pflügers Arch.*, **427,** 233–243.

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