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Effects of purinoceptor agonists on cytosolic Ca^{2+} concentration in swine tracheal smooth muscle cells in culture

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1 The effects of various purinoceptor agonists on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in swine tracheal smooth muscle cells in primary culture were examined to investigate the subtype of purinoceptors in these cells.

2 ATP (1 μM to 1 mM) concentration-dependently increased $[Ca^{2+}]_i$ which was measured by monitoring the fluorescence signal of fura2.

3 α,β-Me ATP at concentrations higher than 10 μM increased $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}. Responses to the drug were 12 ± 5 and 61 ± 4% of responses to ATP (100 μM) at 100 μM and 1 mM, respectively ($n=7$). The response to 100 μM ATP was inhibited by 62% in the presence of 1 mM α,β-Me ATP ($n=8$), though the drug at concentrations lower than that did not affect the response to ATP.

4 ATP increased $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. The response to ATP in this condition was 40% of that in the presence of extracellular Ca^{2+} ($n=8$).

5 Neither cibacron blue 3GA (10 μM) ($n=8$) nor suramin (10 and 100 μM) ($n=10$) affected the response to ATP (1 μM to 100 μM).

6 The rank order of potency in the absence of extracellular Ca^{2+} was UTP > ATP > adenosine 5'-o-(3-thiotriphosphate) > > ADP = α,β-methylene adenosine 5'-triphosphate > 2-(methylthio)-adenosine 5'-(tetrahydrogen triphosphate).

7 UTP (1 μM to 100 μM) concentration-dependently increased inositol 1,4,5-triphosphate (IP_3) production.

8 These results suggest that the increase in $[Ca^{2+}]_i$ induced by purinoceptor agonists is mediated mainly via a nucleotide receptor in swine tracheal smooth muscle cells in primary culture.

Keywords: Nucleotide receptor; cultured tracheal smooth muscle cells; UTP; ATP; suramin; inositol 1,4,5-triphosphate

Introduction

Adenosine and adenine nucleotides mediate either relaxation or contraction of tracheal smooth muscles via P_1- or P_2-purinoceptors (Fredholm *et al.*, 1979; Advenier *et al.*, 1982; Welford & Anderson, 1988; Candenas *et al.*, 1992; Aksoy & Kelsen, 1994). P_1-purinoceptors, which primarily respond to adenosine, were subclassed into A_1 A_2 and A_3, while P_2-purinoceptors, which primarily respond to adenosine 5'-triphosphate (ATP), were subclassed into P_{2X}, P_{2Y}, P_{2U}, P_{2T}, P_{2Z} and P_{2D} according to different agonist pharmacology (Fredholm *et al.*, 1994).

The relaxation due to P_1 stimulation by adenosine has been shown in guinea-pig and rabbit tracheal smooth muscles (Fredholm *et al.*, 1979: Aksoy & Kelsen, 1994). In guinea-pig trachea it was also found that the intact ATP molecule could itself induce relaxation of the muscle and this relaxant effect of the drug was more evident than that of adenosine (Welford & Anderson, 1988), suggesting the existence of the pathway which mediated relaxation via P_2-purinoceptors in this tissue.

Adenine nucleotides have been shown to induce a contraction in guinea-pig tracheal smooth muscle. There seems to be at least two different purinoceptors mediating contraction in this tissue. One might be the P_{2X}-receptor, since the application of α,β-methylene adenosine 5'-triphosphate (α,β-Me ATP) most potently induced contraction (Candenas *et al.*, 1992). The other type of receptor might be P_{2Y}, since the contractile effect of ATP was suggested to be coupled to the release of arachidonic acid (Advenier *et al.*, 1982). In human airway smooth muscle cells in primary culture ATP was shown to induce Ca^{2+} transients, though the type of receptor mediating the ATP response was not investigated (Panettieri *et al.*, 1989). Recently, we have also found that in swine tracheal smooth muscle cells (TSMCs) in primary culture ATP increased the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) as observed in human airway smooth muscle cells in culture, although in freshly isolated TSMCs it did not significantly affect $[Ca^{2+}]_i$. In this study we examined the effects of various purinoceptor agents on $[Ca^{2+}]_i$ in swine TSMCs in primary culture in order to investigate the subtype of purinoceptor in these cells.

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Methods

Cell culture

Swine trachea were obtained from the slaughter house. After removal of connective tissue, the membranaceous portion was cut into smaller pieces in ice-cold Ca^{2+}-Mg^{2+}-free Tyrode solution (for composition see below). The tissue pieces were stored for 20 h at 4°C in 6 ml Ca^{2+}-Mg^{2+}-free Tyrode solution containing 35 mg collagenase, 10 000 PU disperse and 200 mg albumin. Thereafter they were agitated at 37°C for about 10 min, and TSMCs were mechanically dispersed with a pipette. Tissue debris was removed by filtering the solution through nylon mesh (diameter of 200 μm). The isolated cells were pelleted by centrifugation and then suspended in culture medium at a density of 1.3×10^6 cells per dish (diameter of 100 mm; Corning). After confluence (7-9 days in culture), cells were treated with phosphate buffer containing 0.1% trypsin and 0.02% EDTA for 4 min at 37°C, and were suspended in 10 ml of culture medium. Cells were then pelleted, resuspended in Tyrode solution at a density of 3.0×10^6 cells ml^{-1}, and used for experiments. When we performed experiments with freshly isolated cells, cells were also suspended in Tyrode solution at the same density.

In order to exclude the possibility of contamination of fibroblasts in cultured cells prepared by the method described above, we used indirect immunofluorescence with antibodies directed against smooth muscle actin (1A4, DAKO). Cells obtained from muscle layer and cultured as mentioned above were strongly stained with these antibodies, whereas fibroblasts which were proliferated from small pieces of connective tissue from trachea during tissue culture were not stained. Furthermore, with indirect immunofluorescence with antikeratin (DAKO) and antihuman cytokeratin (MWF 116, DAKO), staining was not seen in cells used in this study but was seen in fibroblasts. Therefore, cells used in this study were revealed to be smooth muscle cells.

Measurement of $[Ca^{2+}]_i$

Fura2 was loaded into cells by incubating them in Tyrode solution containing 2 μM acetoxymethyl ester of fura2 (fura2-AM) for 50 min at room temperature. Extracellular fura2-AM was removed by centrifuging the solution, and cells were suspended at a density of 3.0×10^6 cells ml^{-1} in either Tyrode or Ca^{2+}-free Tyrode solution according to the experimental condition. For the fluorescence measurements 0.5 ml cell suspension was used. Fura2 fluorescence (excitation wave length of 340 and 380 nm, emission wave length of 510 nm) was measured at 37°C by use of a fluorescence spectrophotometer (HITACHI F2000). $[Ca^{2+}]_i$ was calculated by the usual equation (Grynkiewicz *et al.*, 1985) with $K_d = 224$ nM. F_{max} was obtained by adding 5 μl 10% triton X-100, whereas F_{min} was obtained by adding 100 mM EGTA solution after F_{max} had been measured. The change in $[Ca^{2+}]_i$ induced by agonists was expressed as $\triangle[Ca^{2+}]_i$ which was obtained by subtracting resting $[Ca^{2+}]_i$ from maximum $[Ca^{2+}]_i$ obtained by agonist stimulation.

In the experiments examining the responses to ATP and carbachol (CCh) in the presence of α,β-Me ATP, ATP and CCh were added in the presence of α,β-Me ATP when $[Ca^{2+}]_i$ returned to the resting level after its transient increase induced by pretreatment with α,β-Me ATP.

When the change in $[Ca^{2+}]_i$ was measured in the absence of extracellular Ca^{2+}, we added 0.5 mM EGTA 30 s before the agonist application. In these experiments F_{max} was measured by adding 2.3 mM $CaCl_2$ after disruption of cell membrane with triton X-100.

Measurements of inositol 1,4,5-triphosphate (IP_3)

IP_3 was extracted by essentially the same method as previously described by Downes *et al.* (1986). Two hundred and forty μl of cell suspensions were incubated at 37°C for 10 min, and 60 μl of the agonist solution (37°C) containing uridine 5'-triphosphate (UTP) of various concentrations was added. The reaction times used in the experiments were 0, 2, 5, 10, 20 and 30 s. The reaction was terminated by adding 100 μl ice-cold 10% (v/v) $HClO_4$. After 20 min on ice, the solution was centrifuged for 10 min at 2000 *g*, and 350 μl of the supernatant was transferred to a separate tube. The samples were neutralized by adding 200 μl of 1:1 (v/v) mixture of trichlorotrifluoroethane (Freon) and tri-n-octylamine. After centrifugation for 1 min at 2000 *g*, a 250 μl portion of the upper phase which contained neutralized sample and all water-soluble components was removed for the measurement of IP_3.

IP_3 was measured with the inositol 1,4,5-triphosphate ^3H radioreceptor assay kit (Dupont). In preliminary experiments, the maximum production of IP_3 was observed within 10 s of reaction at the latest: in this study the production of IP_3 by agonist stimulations was measured after 10 s of the reaction.

Solutions

Composition of Tyrode solution (mM): NaCl 136.9, KCl 5.4, $MgCl_2$ 0.5, NaH_2PO_4 0.33, $CaCl_2$ 1.8, HEPES 5 and glucose 5 (pH = 7.4). Ca^{2+}-free and Ca^{2+}-Mg^{2+}-free Tyrode solutions were made by omitting $CaCl_2$ and both $CaCl_2$ and $MgCl_2$ from Tyrode solution, respectively. Culture medium was 1:1 (v/v) mixture of Dulbecco's modified Eagle's medium and Ham's medium F12 with 10% foetal calf serum (FCS), penicillin (50 iu ml^{-1}), streptomycin (50 μg ml^{-1}) and amphotericin B (2.5 μg ml^{-1}). Composition of phosphate buffer (mM): NaCl 171.12, KCl 3.35, Na_2HPO_4 10.12, KH_2PO_4 1.84 (pH = 7.4).

Drugs

ATP, ADP, uridine 5'-triphosphate (UTP), adenosine 5'-o-(3-thiotriphosphate) (ATPγS), 2-(methylthio)-adenosine 5'-(tetrahydrogen triphosphate) (2-MeSATP), α,β-methylene adenosine 5'-triphosphate (α,β-Me ATP), histamine (His), carbachol (CCh) and cibacron blue 3GA (CB3GA) were purchased from Sigma. Germanin (suramin) was a gift from Bayer. Culture mediums, FCS, penicillin, streptomycin and amphotericin B were purchased from Flow laboratories. Freon and tri-*n*-octylamine were obtained from Nakarai Tesque.

Data analysis

Results are expressed as mean ± s.e.mean. Statistical significance was tested by analysis of variance (ANOVA). A probability less than 0.05 was considered significant.

To investigate the potency order of various purinoceptor agonists, we estimated the half-maximal effective concentration (EC_{50}) of each agonist by non-linear regression analysis.

Results

The effects of ATP on $[Ca^{2+}]_i$ in cultured TSMCs

Figure 1a shows the effects of various agonists on $[Ca^{2+}]_i$ of freshly isolated ($n = 5$) and cultured ($n = 10$) TSMCs. Cells were obtained from the same animal for the comparison. The concentration of agonists was 100 μM. In freshly isolated TSMCs neither ATP nor adenosine significantly increased $[Ca^{2+}]_i$, though CCh and histamine increased $[Ca^{2+}]_i$ by 207 ± 48 and 62 ± 17 nM, respectively. In contrast to freshly isolated cells, in cultured cells ATP increased $[Ca^{2+}]_i$ by 333 ± 26 nM, though adenosine did not significantly affect $[Ca^{2+}]_i$. CCh increased $[Ca^{2+}]_i$ by 153 ± 24 nM in these cells which did not significantly differ from the CCh response of freshly isolated cells, whereas histamine increased $[Ca^{2+}]_i$ by 260 ± 25 nM in cultured cells which did significantly differ from the histamine response in freshly isolated cells ($P < 0.01$).

The increasing effect of ATP on $[Ca^{2+}]_i$ in cultured TSMCs was concentration-dependent as shown in Figure 1b. The change in $[Ca^{2+}]_i$ induced by the application of ATP was 27 ± 2, 152 ± 35, 346 ± 77 and 460 ± 133 nM, at 1 μM, 10 μM, 100 μM and 1 mM ATP, respectively ($n = 8$ for each concentration).

The effect of α,β-Me ATP on $[Ca^{2+}]_i$

Since P_{2X}-receptors have been suggested to exist in tracheal smooth muscle (Candenas *et al.*, 1992), we initially investigated whether α,β-Me ATP, a specific P_{2X}-agonist, was more potent than ATP in increasing $[Ca^{2+}]_i$ in cultured TSMCs. (Figure 2a). As shown in Figure 2a, a significant increase in $[Ca^{2+}]_i$ was induced by the drug at 100 μM and 1 mM. The responses at 100 μM and 1 mM were 12 ± 5% and 61 ± 4% of the response to 100 μM ATP ($n = 7$). The rise in $[Ca^{2+}]_i$ at 1 mM was significantly larger than that at 100 μM ($P < 0.01$). Although ATP significantly raised $[Ca^{2+}]_i$ at 1 μM, α,β-Me ATP did not cause a significant increase in $[Ca^{2+}]_i$ at concentrations lower than 100 μM, indicating that ATP was more potent than α,β-Me ATP in increasing $[Ca^{2+}]_i$ in these cells.

Figure 2b shows the effects of pretreatment of cultured TSMCs with α,β-Me ATP on the response to ATP and CCh. ATP (100 μM) and CCh (100 μM) were applied in the presence

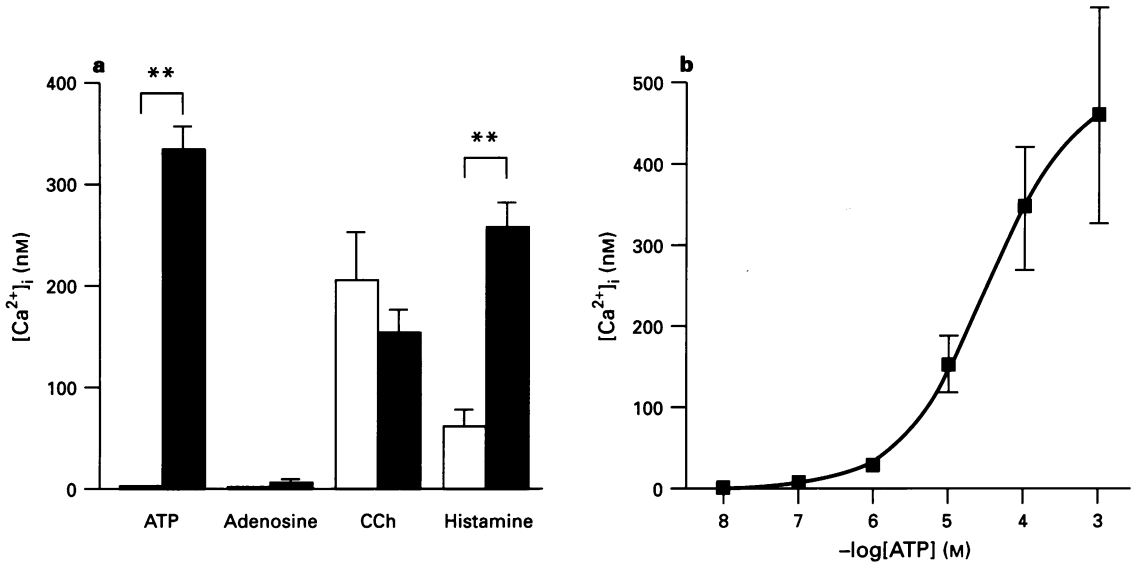

Figure 1 (a) The effects of various agonists on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in freshly isolated and cultured tracheal smooth muscle cells (TSMCs). The concentration of agonists was 100 μM. Ordinate scale indicates the maximum change in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) induced by agonists. Open and solid columns indicate $\Delta[Ca^{2+}]_i$ in freshly isolated ($n=5$) and cultured TSMCs ($n=10$), respectively. Each column indicates mean and s.e.mean. $^{**}P<0.01$ (b) The increase in $[Ca^{2+}]_i$ induced by ATP at various concentrations. Ordinate scale indicates $\Delta[Ca^{2+}]_i$ in nM, whereas abscissa scale indicates the concentration of ATP. Symbols and vertical lines indicate mean and s.e.mean of eight experiments. The solid curve was drawn by fitting data with non-linear regression analysis.

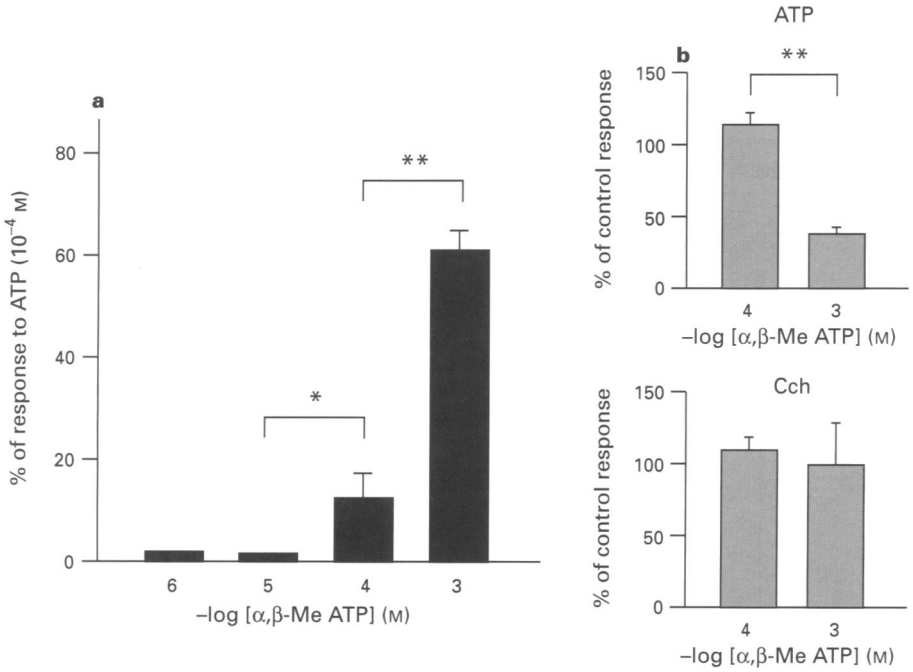

Figure 2 (a) The effects of α,β-Me ATP on $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+}. The response was expressed as percentage of response to 100 μM ATP. Abscissa scale indicates the concentration of α,β-Me ATP. Each column indicates mean and s.e.mean of seven experiments. $^{*}P<0.05$; $^{**}P<0.01$ (b) The increase in $[Ca^{2+}]_i$ induced by ATP (upper panel) and carbachol (CCh, lower panel) in the presence of α,β-Me ATP (100 μM and 1 mM). The response is expressed as percentage of control response obtained in the absence of α,β-Me ATP. Each column indicates mean and s.e.mean from eight (upper panel) or five (lower panel) experiments.

of 100 μM and 1 mM α,β-Me ATP. In the presence of 100 μM α,β-Me ATP the response to ATP did not significantly differ from that in the absence of α,β-Me ATP, and it was $113\pm10\%$ of the control response ($n=8$). In the presence of 1 mM α,β-Me ATP it decreased significantly ($P<0.01$) and was $38\pm3\%$ of the control response ($n=8$). This decrease was not attributable to deterioration of cells by being exposed to a high concentration (ie 1 mM) of the drug, since the response to CCh was not significantly altered in the presence of 1 mM α,β-Me ATP, as shown in the lower panel of Figure 2b. The response to CCh was $112\pm9\%$ and $102\pm28\%$, in the presence of 100 μM and 1 mM α,β-Me ATP, respectively ($n=5$). Although the inhibition of the response to ATP by α,β-Me ATP was consistent with the existence of P_{2X}-purinoceptors, the concentration of α,β-Me ATP needed to inhibit the response to ATP in the present study nearly 100 times higher than that causing desensitization of typical P_{2X}-receptors (O'Connor *et al.*, 1990). These results suggest that in swine TSMCs in primary culture the ATP-induced increase in $[Ca^{2+}]_i$ is unlikely to be mediated via P_{2X}-purinoceptors.

The response to ATP in the absence of extracellular Ca^{2+} ions and the effects of P_{2Y}-antagonists

We next examined whether the response to ATP is dependent on extracellular Ca^{2+} ions (Figure 3a and b), as it is known that the P_{2Y}-purinoceptor-mediated response shown to exist in tracheal smooth muscle is mediated by Ca^{2+} release from intracellular Ca^{2+} store sites. Figure 3a shows the change in fluorescence intensity of fura2 induced by the application of ATP (100 μM) in the presence and absence of extracellular Ca^{2+}. In both conditions ATP increased $[Ca^{2+}]_i$. In this particular experiment, $[Ca^{2+}]_i$ before the application of ATP was 122 and 73 nM in the presence and the absence of extracellular Ca^{2+}: the peak values of $[Ca^{2+}]_i$ obtained after application of ATP were 505 and 230 nM in the presence and the absence of extracellular Ca^{2+} ions, respectively. In 8 experiments the change in $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} was 346 ± 77 nM which was significantly more than that in the absence of Ca^{2+}, 139 ± 25 nM ($P<0.01$). The ratio of the change in $[Ca^{2+}]_i$ in the absence to that in the presence of extracellular Ca^{2+} was 0.40. Similar experiments were performed with CCh (100 μM); in 4 experiments the ratio of the change in $[Ca^{2+}]_i$ in the absence to that in the presence of extracellular Ca^{2+} was 0.56.

Figure 3c shows the effects of P_{2Y}-antagonists on the response to ATP. Neither CB3GA (10 μM) ($n=8$) nor suramin (10 and 100 μM) ($n=10$) affected the response to ATP, 1 to 100 μM, in cultured TSMCs. Higher concentrations affected the fluorescence signals of fura2, and therefore we could not examine the effects of these drugs at any higher concentrations than those used in this study. We also examined the effect of indomethacin on the increase in $[Ca^{2+}]_i$ induced by ATP, and found that in four experiments indomethacin at concentrations lower than 100 μM did not affect the response to ATP (100 μM) (data not shown).

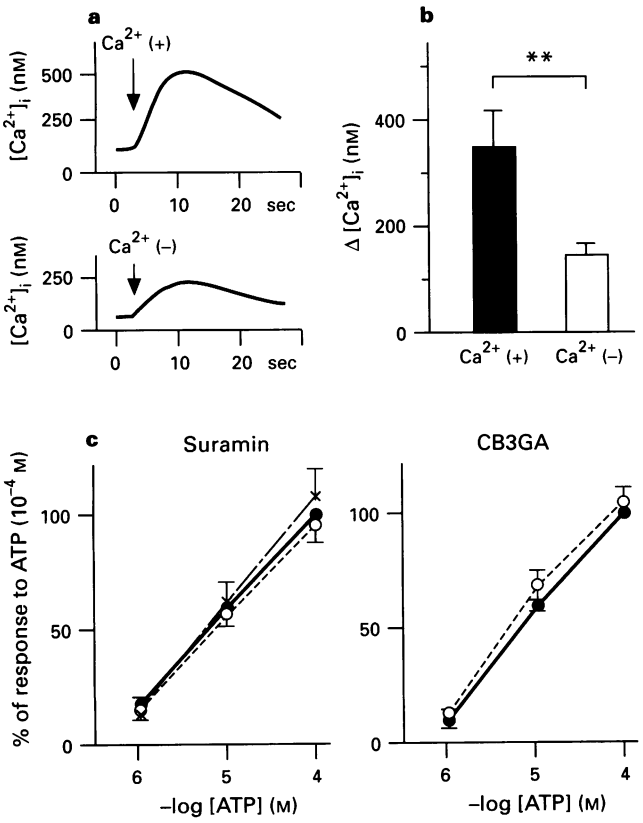

Figure 3 (a) The change in fluorescence intensity of fura2 induced by the application of ATP (100 μM) in the presence (upper panel) and absence (lower panel) of extracellular Ca^{2+}. Arrows in each panel indicate the application of ATP. (b) The increase in $[Ca^{2+}]_i$ induced by 100 μM ATP in the presence (solid column) and absence (open column) of extracellular Ca^{2+}. Columns indicate mean and s.e.mean from eight experiments. $^{**}P<0.01$ (c) Effects of the P_2-antagonists, cibacron blue (CB3GA; $n=8$) and suramin ($n=10$), on the increase in $[Ca^{2+}]_i$ induced by ATP 1, 10 and 100 μM. Responses in the absence and presence of antagonists are expressed as percentage of the response to 100 μM ATP (ordinate scale). (●——●) Response in the absence of antagonists; (○ - - -○) and (× ····· ×) indicate the response in the presence of 10 and 100 μM of antagonists, respectively. Symbols indicate mean and vertical lines show s.e.mean.

The increase in $[Ca^{2+}]_i$ induced by various purinoceptor agonists

Figure 4 shows responses of cultured TSMCs to various purinoceptor agonists obtained in the absence of extracellular Ca^{2+} ions ($n=8$ in all agonists). UTP concentration-dependently increased $[Ca^{2+}]_i$ 16 ± 2, 32 ± 3, 40 ± 5, 44 ± 6 and 68 ± 6% of the response to ATP (100 μM) obtained in the presence of extracellular Ca^{2+}, with 0.1 μM, 1 μM, 10 μM, 100 μM and 1 mM UTP, respectively ($P<0.05$ between 0.1 μM and 1 μM, $P<0.01$ between 100 μM and 1 mM). Although ATP did not cause a significant increase in $[Ca^{2+}]_i$ at 0.1 μM, it concentration-dependently increased it at concentrations higher than 0.1 μM; 5 ± 1, 23 ± 1, 41 ± 4 and 48 ± 5% of the response to ATP (100 μM) obtained in the presence of extracellular Ca^{2+}, at 1 μM, 10 μM, 100 μM and 1 mM, respectively ($P<0.01$: between 1 μM and 10 μM, and between 10 μM and 100 μM). Responses to ATPγS were relatively similar to those to ATP. ATPγS increased $[Ca^{2+}]_i$ to 2 ± 1, 16 ± 1, 31 ± 1 and 44 ± 3% of the response to ATP (100 μM) obtained in the presence of extracellular Ca^{2+}, at 1 μM, 10 μM, 100 μM and 1 mM, respectively ($P<0.01$ between each concentration). ADP, a specific P_{2T} agonist, did not cause a significant increase in $[Ca^{2+}]_i$ at concentrations lower than 100 μM. Responses to ADP were 12 ± 1 and 54 ± 8% of the response to ATP (100 μM) obtained in the presence of extracellular Ca^{2+}, at 100 μM and 1 mM, respectively ($P<0.01$). Responses to α,β-Me ATP were 3 ± 1 and 65 ± 5% of the response toATP (100 μM) obtained in the presence of extracellular Ca^{2+}, at 100 μM and 1 mM respectively ($P<0.01$), which were similar to those obtained in the presence of extracellular Ca^{2+} (Figure 2a). The specific P_{2Y} agonist, 2-MeSATP significantly increased $[Ca^{2+}]_i$ only at 1 mM (16 ± 2% of the response to ATP (100 μM) obtained in the presence of extracellular Ca^{2+}).

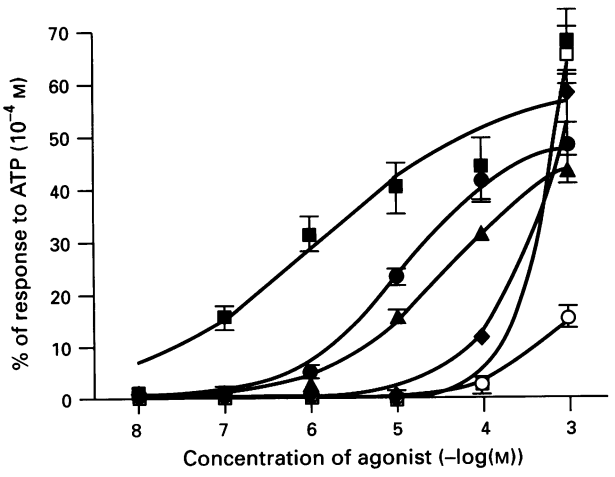

Figure 4 The increase in $[Ca^{2+}]_i$ induced by various purinoceptor agonists in the absence of extracellular Ca^{2+}. Agonists were UTP (■), ATP (○), ATPγS (▲), ADP (◆), α,β-Me ATP (□) and 2-MeSATP (○). Symbols and vertical lines indicate mean and s.e.mean of eight experiments. Solid curves were drawn by fitting the data with non-linear regression analysis. Responses are expressed as percentage of response to 100 μM ATP in the presence of extracellular Ca^{2+}.

Responses to UTP were significantly larger than those to any other agonists at concentrations lower than 100 μM ($P<0.01$). Responses to ATP were significantly larger than those to ATPγS at 10 and 100 μM ($P<0.05$). Responses to ATPγS wee significantly larger than those to α,β-Me ATP and ADP at concentrations lower than 1 mM ($P<0.01$). At 1 mM the response to 2-MeSATP was significantly smaller than those to any other agonists ($P<0.01$). The order of agonist potency for the increase in $[Ca^{2+}]_i$ was UTP ($EC_{50}=1$ μM)>ATP ($EC_{50}=12$ μM)>ATPγS ($EC_{50}=41$ μM)>>ADP=α,β-Me ATP>2-MeSATP.

IP_3 production by the application of UTP

Since the agonists increased $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} ions, we examined the effects of UTP (1, 10 and 100 μM) on the production of IP_3 in cultured TSMCs. Figure 5 shows the increased production of IP_3 induced by UTP. The production of IP_3 was concentration-dependently increased by UTP ($n=5$ for each concentration): 2.3 ± 0.5, 4.1 ± 0.9 and 6.6 ± 1.4 pmol ml^{-1}, at 1, 10 and 100 μM, respectively, though a significant difference was only observed between 1 and 100 μM ($P<0.05$). Consistent with the increased production of IP_3 by the UTP, $[Ca^{2+}]_i$ was also increased concentration-dependently by UTP (inset of Figure 5). $[Ca^{2+}]_i$ was measured in the presence of extracellular Ca^{2+} in cultured cells obtained from the same animals used for measuring IP_3. The change in $[Ca^{2+}]_i$ was 227 ± 31, 496 ± 83 and 668 ± 87 nM at 1, 10 and 100 μM UTP, respectively. UTP responses were significantly different between 1 and 10 μM ($P<0.05$), though no significant difference was observed between 10 and 100 μM. This finding was consistent with that obtained in the absence of extracellular Ca^{2+} (Figure 4).

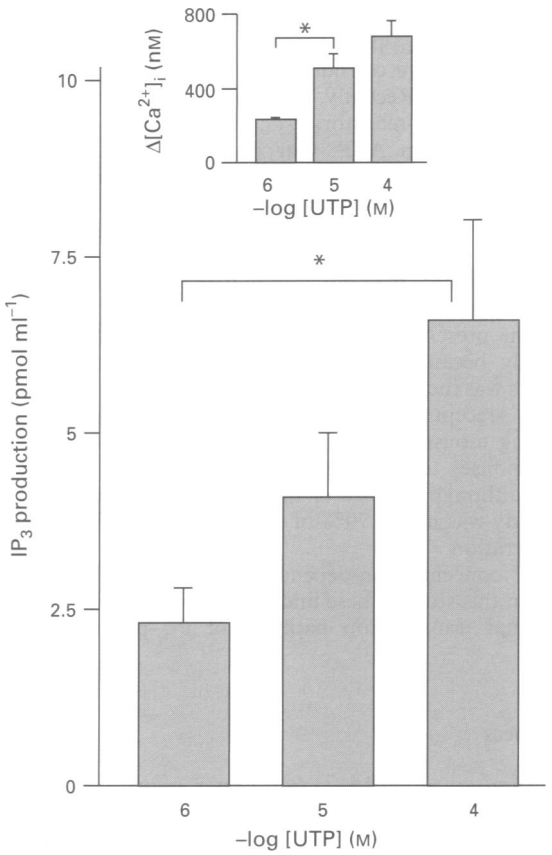

Figure 5 The effects of UTP on the production of inositol 1,4,5-triphosphate (IP_3). The production of IP_3 by the application of 1, 10 and 100 μM UTP was plotted against the drug concentration (−log[UTP] (M)). Columns indicate mean and s.e.mean of five experiments. Data were obtained in the presence of extracellular Ca^{2+}. The inset shows the change in $[Ca^{2+}]_i$ in response to the application of 1, 10 and 100 μM UTP in the presence of extracellular Ca^{2+}. Data were obtained from the same animals used for the measurement of the production of IP_3. Each column indicates mean and s.e.mean of seven experiments. *$P<0.05$.

Discussion

This study shows that swine TSMCs in primary culture preserve the physiological responses to both CCh and histamine. Although in our study the response to CCh in cultured cells was relatively but not significantly smaller than that in freshly isolated cells, the response to histamine was significantly enhanced in cultured cells compared to freshly isolated cells. The response to histamine has been extensively studied in guinea-pig TSMCs in long-term subculture (Devore-Carter *et al.*, 1988). Although Devore-Carter *et al.* described the morphological changes in cultured cells induced by histamine in depth, no quantitative comparison of the histamine response between cultured and freshly isolated cells was performed. The smaller response to histamine than to CCh in freshly isolated cells is consistent with the finding in a tissue preparation that the contraction induced by histamine was 2 to 3 times smaller than that induced by CCh (Sparrow & Mitchell, 1990). One possible explanation for the enhanced response to histamine in cultured cells is that TSMCs express a high level of surface histamine receptors in our culture conditions.

The increase in $[Ca^{2+}]_i$ induced by ATP in cultured TSMCs was first demonstrated in human TSMCs (Panettieri *et al.*, 1989). In the present study, ATP concentration-dependently increased $[Ca^{2+}]_i$, indicating that the effect of ATP on $[Ca^{2+}]_i$ may be via P_2-purinoceptors. Although ATP has been demonstrated to contract tracheal smooth muscle of guinea-pig via P_2-purinoceptors (Advenier *et al.*, 1982; Candenas *et al.*, 1992), in freshly isolated swine TSMCs we observed no significant increase in $[Ca^{2+}]_i$ on application of ATP. These findings suggest that the increase in $[Ca^{2+}]_i$ induced by ATP is undetectably small in freshly isolated cells due to the low density of P_2-purinoceptors and during the culture period the cells express high levels of these receptors, similar to histamine receptors, or that P_2-purinoceptors are newly expressed on the cell surface only during the culture period.

Which subtype of P_2-purinoceptor mediates the increase in $[Ca^{2+}]_i$ in swine TSMCs in primary culture? Since P_{2X}- and P_{2Y}-purinoceptors have been suggested to be present in guinea-pig tracheal smooth muscle (Advenier *et al.*, 1982; Cadenas *et al.*, 1992), we first examined whether the receptor mediating the increase in $[Ca^{2+}]_i$ by ATP belonged to one of these subtypes. We then found that α,β-Me ATP was less potent than ATP and that α,β-Me ATP could not inhibit the response to ATP at the concentration (30 μM) shown previously to desensitize P_{2X}-receptors (O'Connor *et al.*, 1990). We also found that the response to ATP was observed in the absence of extracellular Ca^{2+}. These results suggest that P_{2X}-purinoceptors are unlikely to be involved in the response to ATP. In addition, the finding that 2-MeSATP was the least potent of the purinoceptor agonists examined, together with the fact that neither CB3GA nor suramin inhibited the response to ATP, suggest that the response of cultured TSMCs to ATP is mediated via P_2-purinoceptors other than the P_{2Y}-subtype.

The purinoceptor investigated in this study responded mainly to UTP, ATP and ATPγS. In addition, IP_3 production was concentration-dependently increased by the application of UTP. These results suggest that the purinoceptor in swine tracheal smooth muscle cells in culture has relatively similar characteristics to those of the P_{2U}-subtype (O'Connor, 1992). The potency order of agonists generally obtained at P_{2U}-purinoceptors is UTP$\geqslant$ATP>ATPγS>>2-MeSATP$\geqslant\alpha,\beta$-Me ATP (O'Connor, 1992; Fredholm *et al.*, 1994). In this study, however, UTP was at least 10 times more potent than ATP. Although UTP has been found to be slightly more potent than ATP in nucleotide receptors in pituitary cells (Davidson *et al.*,

1990), rat liver parenchymal cells (Van Rhee *et al.*, 1993) and cloned human P_{2U}-purinoceptors (Lazarowski *et al.*, 1995), UTP and ATP are considered to be essentially equipotent in these receptors. Recently, Communi *et al.* (1995) cloned a novel type of P_{2Y}-receptor, P_{2Y4}, in which UTP is significantly more potent than ATP. Our results suggest that the purinoceptor investigated in this study has some similarity to this P_{2Y4} subtype. Another difference in potency order obtained in this study from that generally accepted at P_{2U}-receptors is that 2-MeSATP was considerably less potent than α,β-Me ATP or ADP. Therefore, it is possible that the receptor described in this study is a novel type of nucleotide receptor.

In the present study, responses to ADP and α,β-Me ATP suddenly became prominent as the concentration of these agonists was increased from 100 μM to 1 mM. In a recent study with P_{2U}-receptors expressed in *Xenopus* oocytes, from cDNA encoding mouse P_{2U}-purinoceptors, similar phenomena with the responses to these agonists was observed (Lustig *et al.*, 1993), although the size of the responses to these agonists in this study was nearly 70% of the response to ATP at the same concentration.

UTP concentration-dependently increased the production of IP_3 in this study. These findings are in good agreement with the signal transduction pathway of P_{2U}-purinoceptors previously described (Gerwins & Fredholm, 1992). However, recently obtained data suggest that the increase in $[Ca^{2+}]_i$ can be attributed not only to Ca^{2+} release from intracellular Ca^{2+} store sites but also to the influx of Ca^{2+} from the extracellular space (Van Rhee *et al.*, 1993; Kitajima *et al.*, 1994). If this is the case, the significant depression of the changes in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} observed in this study can be attributed to the loss of the influx of Ca^{2+} from the extracellular space. The response to α,β-Me ATP, however, was not affected by removing Ca^{2+} from the extracellular space (compare Figures 2a and 4). The difference in the effect of removing Ca^{2+} on the response between ATP and α,β-Me ATP can be explained by assuming that these agents bind different receptors. Nevertheless, this assumption is not plausible, since α,β-Me ATP depressed the response to ATP but not that to CCh. Therefore, we need further experiments to clarify the role of extracellular Ca^{2+} on purinoceptor-mediated responses in swine TSMCs in primary culture.

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References

ADVENIER, C., BIDET, D., FLOCH-SAINT-AUBIN, A. & RENIER, A. (1982). Contribution of prostaglandins and thromboxanes to the adenosine and ATP-induced contraction of guinea-pig isolated trachea. *Br. J. Pharmacol.*, **77**, 39–44.

AKSOY, M.O. & KELSEN, S.G. (1994). Relaxation of rabbit tracheal smooth muscle by adenine nucleotides: mediation by P_2-purinoceptors. *Am. J. Respir. Cell. Mol. Biol.*, **10**, 230–236.

CANDENAS, M.L., DEVILLIER, P., NALINE, E. & ADVENIER, C. (1992). Contractile effect of α,β-methylene ATP on the guinea-pig isolated trachea. *Fundam. Clin. Pharmacol.*, **6**, 135–144.

COMMUNI, D., PIROTTON, S., PARMENTIER, M. & BOEYNAEMS, J. (1995). Cloning and functional expression of a human uridine nucleotide receptor. *J. Biol. Chem.*, **270**, 30849–30852.

DAVIDSON, J.S., WAKEFIELD, I.K., SOHNIUS, U., ANTON VAN DER MERWE, P. & MILLAR, R.P. (1990). A novel extracellular nucleotide receptor coupled to phosphoinositidase-C in pituitary cells. *Endocrinology*, **126**, 80–87.

DEVORE-CARTER, D., MORWAY, P.F. & WEISS, E.B. (1988). Isolation and characterization of guinea-pig tracheal smooth muscle cells that retain differentiated function in long-term subculture. *Cell Tissue Res.*, **251**, 325–331.

DOWNES, C.P., HAWKINS, P.T. & IRVINE, R.F. (1986). Inositol 1,3,4,5-tetrakisphosphate and not phosphatidylinositol 3,4-bisphosphate is the probable precursor of inositol 1,2,4-trisphosphate in agonist-stimulated parotid gland. *Biochem. J.*, **238**, 501–506.

FREDHOLM, B.B., BRODIN, K. & STRANDBERG, K. (1979). On the mechanism of relaxation of tracheal muscle by theophylline and other cyclic nucleotide phosphodiesterase inhibitors. *Acta Pharmacol. Toxicol.*, **45**, 336–344.

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

GERWINS, P. & FREDHOLM, B.B. (1992). ATP and its metabolite adenosine act synergistically to mobilize intracellular calcium via the formation of inositol 1,4,5-triphosphate in a smooth muscle cell line. *J. Biol. Chem.*, **267**, 16081–16087.

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.

KITAJIMA, S., OZAKI, H. & KARAKI, H. (1994). Role of different subtypes of P_2 purinoceptor on cytosolic Ca^{2+} levels in rat aortic smooth muscle. *Eur. J. Pharmacol.*, **266**, 263–267.

LAZAROWSKI, E.R., WATT, W.C., JACKSON STUTTS, M., BOUCHER, R.C. & KENDALL HARDEN, T. (1995). Pharmacological selectivity of the cloned human P_{2U}-purinoceptor: potent activation by diadenosine tetraphosphate. *Br. J. Pharmacol.*, **116**, 1619–1627.

LUSTIG, K.D., SHIAU, A.K., BRAKE, A.J. & JULIUS, D. (1993). Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. USA*, **90**, 5113–5117.

O'CONNOR, S.E. (1992). Recent developments in the classification and functional significance of receptors for ATP and UTP, evidence for nucleotide receptors. *Life Sci.*, **50**, 1657–1664.

O'CONNOR, S.E., WOOD, B.E. & LEFF, P. (1990). Characterization of P_{2X}-receptors in rabbit isolated ear artery. *Br. J. Pharmacol.*, **101**, 640–644.

PANETTIERI, R.A., MURRAY, R.K., DEPALO, L.R., YADVISH, P.A. & KOTLIKOFF, M.I. (1989). A human airway smooth muscle cell line that retains physiological responsiveness. *Am. J. Physiol.*, **256**, C329–C335.

SPARROW, M.P. & MITCHELL, H.W. (1990). Contraction of smooth muscle of pig airway tissues from before birth to maturity. *J. Appl. Physiol.*, **68**, 468–477.

VAN RHEE, A.M., VAN WINDEN, E.C.A., NAGELKERKE, J.F., DE BONT, H.J.G.M., IJZERMAN, A.P. & SOUDIJN, W. (1993). Binding of the radioligand [^{35}S]adenosine 5'-o-(2-thiodiphosphate) and intracellular calcium response in rat liver parenchymal cells. *Biochem. Pharmacol.*, **45**, 801–807.

WELFORD, L.A. & ANDERSON, W.H. (1988). Purine receptors and guinea-pig trachea: evidence for a direct action of ATP. *Br. J. Pharmacol.*, **95**, 689–694.

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