British Journal of Pharmacology (2002) 137, 177–184

www.nature.com/bjp

ADP-sensitive purinoceptors induce steroidogenesis *via* adenylyl cyclase activation in bovine adrenocortical fasciculata cells

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1 The role of P2Y receptors in the production of cAMP and the activation of protein kinase A (PKA) was studied with respect to the regulation of the steroidogenesis in primary cultures of bovine adrenocortical fasciculata cells (BAFCs).

2 ADP and ATP stimulated cAMP production with EC_{50} values of 23.7 ± 6.8 μM and 40.1 ± 5.5 μM, respectively. In contrast, the EC_{50} of BzATP for cAMP production was 153.0 ± 37.4 μM. Adenosine and AMP (0.1–1000 μM) were much less effective than ADP and ATP. 2MeSADP and UTP did not exert detectable effects. ADP (10 and 100 μM) significantly stimulated steroidogenesis; the process was blocked by an adenylyl cyclase inhibitor SQ22536 (100 μM) but not by the $P2Y_1$ receptor antagonist MRS2179 (100 μM).

3 Real-time imaging of the PKA activity with the dye ARII, which became less fluorescent upon phosphorylation, revealed that ADP (100 μM) immediately activated PKA. These effects could be mimicked by forskolin (100 μM) and were blocked by the PKA inhibitor H89 (50 μM). UTP (100 μM) did not activate PKA.

4 The cytoplasm harvested from morphologically and electrophysiologically identified single BAFCs contained mRNA for $P2Y_2$ but not for $P2Y_1$, $P2Y_4$, $P2Y_{11}$ or $P2Y_{12}$ receptors, as confirmed by single-cell RT–PCR amplification (50 cycles).

5 These results suggest an expression of an ADP-sensitive G_s-coupled purinoceptor in BAFCs. We propose that this not yet described type of P2Y receptor might mediate the extracellular purine-activated steroidogenesis *via* cAMP/PKA-mediated pathways, independently from the pathways involving $InsP_3$ production and consequent intracellular Ca^{2+} increase.
British Journal of Pharmacology (2002) **137,** 177–184. doi:10.1038/sj.bjp.0704847

Keywords: P2Y receptor; cyclic AMP dependent protein kinase; cortisol; steroidogenesis

Abbreviations: BzATP, 2′- and 3′-*O*-(4-benzoylbenzoyl)-ATP; cAMP, cyclic AMP; H89, ethyl-5-isoquinolinesulfonamide; IBMX, 3-isobutyl-1-methylxanthine; $InsP_3$, inositol 1,4,5-triphosphate; 2MeSADP, 2-methylthio-ADP; 2MeSATP, 2-methylthio-ATP; MRS2179, N^6-methyl-2′-deoxyadenosine-3′,5′-bisphosphate; PKA, cyclic AMP dependent protein kinase; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid; RB2, reactive blue 2

Introduction

ATP and UTP stimulate cortisol production through activation of P2Y receptors in bovine adrenocortical fasciculata cells (BAFCs) (Kawamura *et al*., 1991; Nishi, 1999). Steroidogenesis with ATP and UTP is mediated by an increase in intracellular concentrations of $InsP_3$ and Ca^{2+} resulting from activation of $P2Y_2$ receptors (Nishi, 1999). However, the effects of ATP and UTP in BAFCs are differently mediated as shown by the following observations: (1) ATP increases intracellular cAMP concentration but UTP does not (Niitsu, 1992; Hoey *et al*., 1994; Nishi *et al*., 1998). (2) Cortisol production stimulated by ATP is sensitive to pertussis toxin but such production stimulated by UTP is not (Nishi, 1999). (3) Cortisol production with 100 μM ATP was decreased by suramin (100 μM) to 46%, whereas that with 100 μM UTP was decreased to only 80% of the control (Nishi, 1999).

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These observations led us to speculate that the effect of ATP on steroidogenesis involves a pathway other than the one linked to $InsP_3$ production (Burnstock, 2001), presumably a pathway which involves activation of adenylyl cyclase. The objective of the present study was to identify the receptor and the mechanisms underlying ATP-induced steroidogenesis involving cAMP production in BAFCs. Here we demonstrate that ADP and ATP stimulate steroidogenesis through activation of a not yet known type of P2Y receptor which activates adenylyl cyclase in BAFCs, while UTP does not stimulate such steroidogenesis. In addition to pharmacological analyses, we used fluorometric methods which allow real-time visualization of protein kinase A (PKA) activity (Higashi *et al*., 1997) in order to observe directly the consequences of the activation of this receptor. Our results indicate that BAFCs express a not-yet-identified P2Y receptor, the specific activation of which by ADP or ATP results in the immediate stimulation of adenylyl cyclase and PKA, leading to the enhanced production of cortisol.

Methods

Cell culture

BAFCs were isolated under aseptic conditions from freshly obtained bovine adrenal cortex and were cultured in Ham's F-10 medium supplemented with 5% foetal calf serum, 10% newborn calf serum, 2.5% horse serum and antibiotics. The cells were cultured in 24-well dishes or on cover slips (diameter of 12 mm) as previously described (Matsui, 1991). On the third day of culture, the BAFCs were washed with phosphate buffered saline solution containing (in mM): NaCl 136.8, KCl 2.7, KH_2PO_4 1.4, Na_2HPO_4 7.8 and EGTA 0.5 (pH = 7.4). The washed cells were exposed to KRHGA buffer solution composed of: (in mM) NaCl 123.2, KCl 6, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.2, EGTA 0.1, and HEPES 10; with 0.2% (w/v) glucose and 0.1% bovine serum albumin (pH = 7.4).

Measurement of cAMP production

The three-day primary cultured cells were incubated at 37°C for 20 min in KRHGA containing 200 μM IBMX with or without nucleotides and antagonists. Cyclic AMP was extracted by ethanol and measured by use of a commercially available radioimmunoassay kit (Yamasa, Chiba) as previously described (Niitsu, 1992).

Cortisol production assay

The BAFCs were incubated at 37°C for 1 h in KRHGA in the presence and absence of nucleotide agonists. The amount of total incubation medium was 1 ml. The amount of steroid in the incubation medium was determined fluorometrically with cortisol as a standard (Slavinski *et al*., 1976; Niitsu, 1992).

Real-time measurement of protein kinase A (PKA) activity

Fluorometric demonstration of PKA activity was performed according to the method described by Higashi *et al*. (1997) with slight modifications. BAFCs on cover slips were incubated with KRHGA containing 100 μg ml^{-1} ARII, a PKA-specific probe, for 90 min at room temperature. After loading, the coverslips with BAFCs were transferred to a recording chamber continuously perfused (1–2 ml min^{-1}) with fresh KRHGA containing 200 μM IBMX and 1 μM cyclosporin-A. The cells were observed with an inverted epifluorescent microscope (TE300, Nikon, Tokyo) with an objective lens of 100× magnification. The fluorescence images monitored at an excitation wavelength of 366 nm and an emission wavelength of 525 nm were captured with a cooled CCD camera (C-6790, Hamamatsu Photonics, Hamamatsu, Japan) and analysed with a fluorescence image analysis program (Aquacosmos, Hamamatsu Photonics). The region of interest was selected so that the fluorescence signal was obtained from a single BAFC. The fluorescent intensity was shown as the ratio of fluorescence at time t (F_t) to the initial intensity (F_o). The agonists were dissolved in KRHGA perfusate at the final concentration and substituted for control perfusate. The temperature of the perfusate in the recording chamber was kept at 35°C.

Single cell RT–PCR

In order to analyse P2Y receptor proteins expressed in identified BAFCs, RT–PCR analysis of the cytoplasmic mRNAs was performed using tight-seal patch-clamp pipettes to avoid contamination by any types of cells in the culture other than BAFCs. The cytoplasm was harvested with a patch-clamp glass electrode to collect mRNAs from visually and electrophysiologically identified single BAFCs. The electrodes were filled with intracellular solution (8 μl) composed of (in mM) potassium gluconate 102, KCl 45, $MgCl_2$ 3, HEPES 10, EGTA 5 (pH = 7.2). The seal resistance at the cell-attached configuration was higher than 5 GΩ. After establishment of the whole-cell configuration by rupturing the sealed membrane with a small negative pressure and a short voltage pulse, the cytoplasm was slowly aspirated into the glass electrode under visual control. The harvested cytoplasm was expelled into a microtube containing 10U RNAsin, an RNAse inhibitor, and 10 mM dithiothreitol. After reverse transcription, PCR procedures were carried out using a commercially available PCR kit (Takara, Otsu, Japan) with specific primers for $P2Y_1$, $P2Y_2$ (Chen & Lin, 1999), $P2Y_4$ (Webb *et al*., 1996), $P2Y_{11}$ (Communi *et al*., 1997) or $P2Y_{12}$ (Hollopeter *et al*., 2001). The gel images were loaded by use of CCD digital image stock system, FAS-III (Toyobo, Osaka, Japan).

Statistics

Except for the data shown in Figure 2, the measurement of cAMP and cortisol production was made in duplicate or triplicate for the cultured BAFCs. The mean and the standard error of the mean (s.e.) were calculated from the measurements for BAFCs from different animals. In these cases, the mean ± s.e.mean are shown in the text and figures with n indicating the number of animals. For the data shown in Figure 2, BAFCs from a single bovine cultured in 40 wells were subjected to cAMP measurement under distinct conditions. In this series of experiments, the mean and the standard deviation (s.d.) for the values obtained from BAFCs in four wells (i.e., $n = 4$) with the same pharmacological treatment are shown in the text and figure legends.

Concentration-response curves (Hill equation) were fitted onto the data by non-linear least-square analysis. The EC_{50} and s.d. of the estimate obtained by chi-square method implanted in IgorPro (Wavemetrics, OR, U.S.A.) are shown in the text and the Summary. Data were compared for differences by Student's t-test, Mann–Whitney's U-test or one-way ANOVA. A P-value of less than 0.05 was considered to be statistically significant (*).

Drugs used

Most drugs were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.); exceptions were 2-MeSATP from Funakoshi (Tokyo, Japan), ARII from Dojindo Laboratories (Tokyo, Japan), UTP from Boehringer Mannheim (Mannheim, Germany), H-89 from Seikagaku Co (Tokyo, Japan), cAMP assay kit including [^{125}I]-cAMP (59.2 kBq) from

Yamasa Shoyu (Chiba, Japan), foetal calf serum from Cytosystems (Castle Hill, N.S.W., Australia), newborn calf serum from CSL (Victoria, Australia) and horse serum from Nacalai Chemicals (Kyoto, Japan). All chemicals were of reagent grade.

Results

ADP stimulates steroidogenesis through pathways involving adenylyl cyclase

ATP and UTP stimulate cortisol production through activation of $P2Y_2$-$InsP_3$ pathways (Nishi, 1999). However, it has also been reported that ATP markedly increases the production of cAMP (Niitsu, 1992; Hoey *et al*., 1994). This is well known to be the essential mediator of the effect of ACTH in stimulating steroidogenesis in adrenal cortex. We first analysed the effect of various nucleotides on the cAMP concentration (Figure 1). ATP and ADP markedly stimulated cAMP production. The potency of ADP (EC_{50}, 23.7 ± 6.8 μM; Hill constant, 1.2 ± 0.4) was slightly higher than that of ATP (EC_{50}, 40.1 ± 5.5 μM; Hill constant, 1.0 ± 0.1). The absolute values of the accumulated cAMP were 8.87 ± 0.64 pmol 10^5 cells^{-1} and 9.12 ± 0.47 pmol 10^5 cells^{-1} ($n=4$) in the presence of 100 μM ATP and 100 μM ADP, respectively. These values were almost half that of the cAMP production stimulated by ACTH at 10 pM, a concentration slightly higher than the EC_{50} of ACTH in this system (Kawamura *et al*., 2001). Such high values indicate that ATP and ADP are potent stimulants of cAMP production, though they are weaker than ACTH. 2MeSADP and BzATP are potent $P2Y_1$ and $P2Y_{11}$ agonists, respectively, but here they were less effective than ATP and ADP. The EC_{50} of BzATP was 153.0 ± 37.4 μM and that for 2MeSADP could not be estimated (>10 mM). These results suggest that $P2Y_1$ and $P2Y_{11}$ receptors are less likely to be involved in the ATP- and ADP-activated cAMP production (Communi *et al*., 1999). UTP, a potent $P2Y_2$ agonist, did not stimulate cAMP production. AMP and adenosine were much less effective in stimulating cAMP production than ADP, excluding the possibility of the activation of adenosine receptors (Figure 1A). UDP, α,β-methylene-ATP and β,γ-methylene-ATP did not exert detectable effects (data not shown). The increases in cAMP accumulation due to ADP and ATP were significantly attenuated by a membrane-permeable adenylyl cyclase inhibitor SQ22536 (reduced to 47.8 ± 4.80% for ADP and to 47.9 ± 7.31% for ATP of the cAMP accumulation in the absence of 100 μM SQ22536; $n=4$). In addition, both ADP and ATP significantly stimulated cortisol production, which was significantly inhibited by 100 μM SQ22536 (Figure 1B). These data further suggest that activation of purinoceptors with ADP as well as ATP stimulates steroidogenesis via activation of cAMP–PKA mediated pathways. The steroidogenesis by UTP, which has been reported to be mediated by $InsP_3$–Ca^{2+} pathways (Nishi, 1999), was not affected by SQ22536. Cortisol production in the absence of SQ22536 with 10 μM and 100 μM UTP was 749.6 ± 52.3 pmol 10^5 cells^{-1} and 652.4 ± 51.2 pmol 10^5 cells^{-1} respectively, and that in the presence of SQ22536 was 702.1 ± 3.8 pmol 10^5 cells^{-1} and 640.2 ± 60.3 pmol 10^5 cells^{-1}, respectively ($n=5$). Such results suggest that the steroidogenesis activated by ADP and UTP involves distinct intracellular messenger pathways.

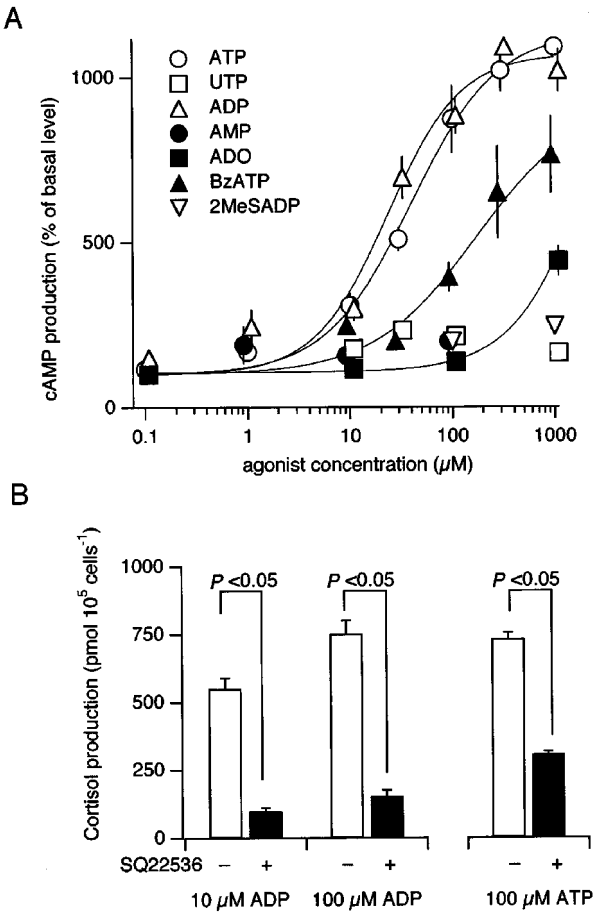

Figure 1 (A) Effects of purines and pyrimidines on cAMP production. The ordinate shows the amount of cAMP produced after 20-min of incubation with agonists as expressed as the percentage of the basal level measured in the absence of agonists. The four curves are estimated Hill plots for the dose-response relation of ATP, ADP, BzATP and adenosine. Results from incubations with AMP and 2MeSADP were not fitted to Hill plots. Values shown represent mean (±s.e.) of six or three (BzATP, adenosine and 2MeSADP) measurements. The basal level of cAMP in the absence of purines and pyrimidines was 0.75 ± 0.272 pmol/10^{-5} cells ($n=18$; range, $0.431-1.221$ pmol 10^{-5} cells). (B) Effect of ADP on cortisol production in the absence and presence of SQ22536 (100 μM). Values shown are mean (±s.e.) of five measurements. *Significant difference between means; $P<0.05$.

Effect of inhibitors of extracellular breakdown of ATP and ADP

In rat glioma cells, ATP-induced production of cAMP is mediated by activation of adenosine receptors following the extracellular conversion of ATP to adenosine (Ohkubo *et al*., 2000). To confirm that the breakdown of ATP and/or ADP was not essential for the stimulation of cAMP production in BAFCs, we examined the effects of inhibitors of the extracellular breakdown of ATP (Zimmermann, 2000) on cAMP production. Even in the presence of α,β-methylene ADP (1 mM), an inhibitor of the ecto 5-nucleotidase (CD73), β-glycerophosphate (10 mM) or levamisole (1 mM), inhibitors

of ecto-alkaline phosphatase, or PPADS (1 mM), an ecto-nucleotide pyrophosphatase (PDNP1) inhibitor (Ohkubo *et al*., 2000) at concentrations reported to inhibit these enzymes in rat glioma cells (Ohkubo *et al*., 2000), both ATP and ADP elicited cAMP production to similar levels (Figure 2). These results mean that extracellular catabolism of ATP to ADP or to adenosine is not necessary, but rather that both ATP and ADP stimulate cAMP production themselves. In support of our findings, Hoey *et al*. (1994) reported that, after 60 min of incubation at 37°C, only 30% of ATP is converted to ADP and only 20% to AMP and that adenosine was not detected as measured in BAFCs. It is therefore not likely either that a significant amount of adenosine was produced from ATP or ADP or that ATP is mostly broken down to ADP during the present incubation for only 20 min at 37°C.

ADP-induced PKA activation

To demonstrate that the production of cAMP and consequent activation of PKA occur immediately following activation of ADP-sensitive purinoceptors, we performed real-time imaging of PKA phosphorylation. The real-time imaging of PKA activity was made possible by introducing ARII (Higashi *et al*., 1997), a PKA-specific fluorescent substrate, whose fluorescence signal is decreased when it is phosphorylated by activated PKA. In BAFCs pre-loaded with ARII, extracellular ADP (100 μM) strongly and promptly reduced the fluorescence intensity (Figure 3). The decrease in the fluorescent signal, i.e., the phosphorylation of ARII, started just after the onset of ADP application (Figure 3). This decrease in fluorescent signal due to ADP was mimicked by forskolin (Figure 3) and was completely blocked by a PKA inhibitor H89 (Figure 3, dashed curve). UTP (100 μM) did not exert any detectable effect on the PKA activity in BAFCs (data not shown). The rate of change in fluorescence signal (-dF/dt), which reflects the rate of phosphorylation (i.e., kinase activity), was dependent on the concentration of ADP in the range from 0 to 100 μM (Figure 4).

Effect of receptor antagonists and enzyme inhibitor on the response to ADP in BAFC

Effect of non-selective P_2 receptor antagonists In order to compare the effect of ADP on cAMP production with previously described P2Y receptors, we examined the effects of three non-selective P_2-antagonists on the ADP-induced cAMP production (Figure 5). The cAMP production with ADP in the presence of suramin and RB2 was significantly reduced to 77.6±5.9% and 81.5±4.3%, respectively, of the production in the absence of these antagonists (Figure 5). PPADS did not significantly affect cAMP production with ADP (reduced only to 91.7±3.9%).

Effect of cyclo-oxygenase inhibitor P_2-agonists enhance cAMP production *via* an autocrine/paracrine mechanism mediated by prostaglandin secretion in Madin–Darby canine kidney epithelial (MDCK) cells (Post *et al*., 1996). To examine the possibility that the increase of cAMP by ADP involves this pathway, we examined the effect of indomethacin on the ADP-induced cAMP production. The cAMP production by 100 μM ADP in BAFC in the presence of indomethacin (10 μM) was 93.9±2.9% of the value in the absence of indomethacin (Figure 6). From this finding and the rapid onset of the PKA activation upon ADP application

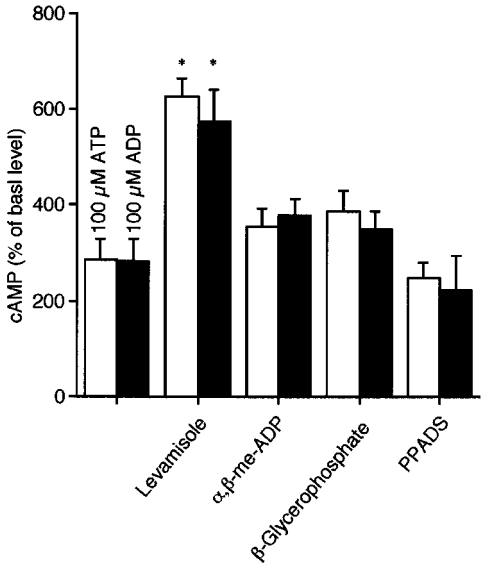

Figure 2 Effects of inhibitors on the stimulation of cAMP producton by ADP and ATP. Levels of cAMP are expressed as the percentage of the basal (non-stimulated) amounts of cAMP. The first set of bars represent cAMP production after incubation of the cells with 100 μM ADP or ATP under control conditions, i.e. in the absence of antagonists. Values shown are means±s.d. of four assays. The inhibitors were applied 5 min before the incubation with ADP or ATP. *Significantly different from control values; $P<0.05$.

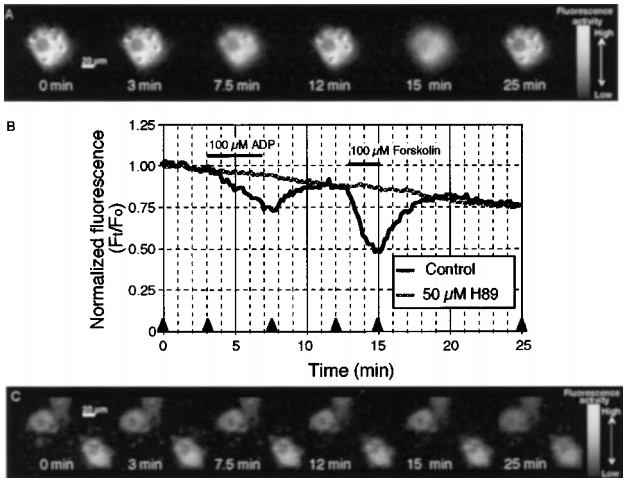

Figure 3 Effect of ADP on the phosphorylation of the fluorescent dye ARII. (A) Original fluorescence images of an ARII-loaded BAFC. ADP (100 μM) and forskolin (100 μM) were applied at 3 min and 13 min after the start of recording, respectively). Note the decrease in the fluorescent signal in the images at 7.5 min and 15 min. (B) Time-course of the fluorescent signal of a single ARII-loaded cell. The ordinate gives the fluorescence intensity normalized to the initial value. The solid curve represents the data from the experiment shown in A. Stimuli were applied in the perfusate to yield the concentrations shown over the periods indicated by the horizontal bars (see Methods). Note that the rate of decrease in the fluorescence following application of ADP and forskolin is clearly larger than the spontaneous trend. The gray curve represents the effects of ADP and forskolin in the presence of 50 μM H89. The original images of the latter experiments are shown in C.

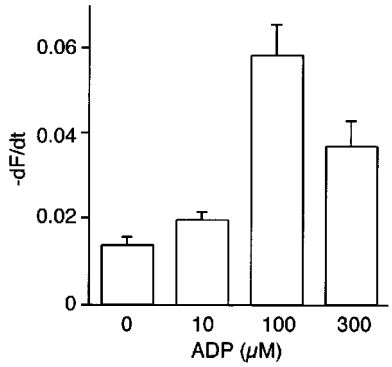

Figure 4 Effect of ADP on ARII phosphorylation as measured by fluorescence imaging. The ordinate shows the rate of the decrease of the fluorescence intensity, expressed as reduction of the intensity per second. The abscissa shows the concentrations of ADP (μM) used in these experiments; 0 represents the spontaneous rate of decrease, i.e. without ADP. Values shown are means$\pm$s.e. of six cells.

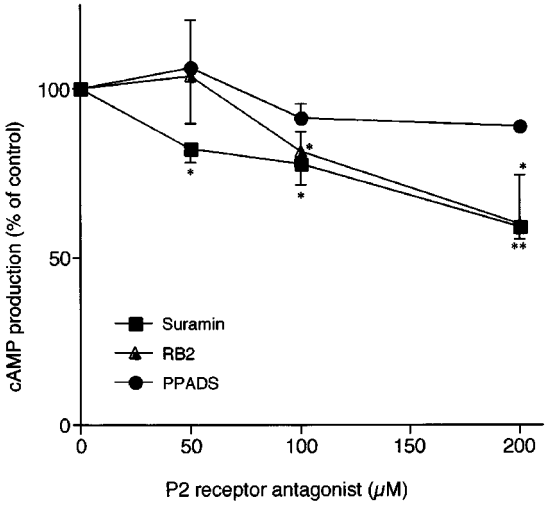

Figure 5 Effects of P_2 receptor-antagonists on stimulation of cAMP production by ADP. Values are expressed as the percentage of the amount of cAMP determined after the incubation of the cells with 100 μM ADP under control conditions, i.e. in the absence of antagonists. Values shown are means$\pm$s.e. of three separate experiments, each performed in duplicate or triplicate. The antagonists were applied 5 min before the incubation with ADP. *,$P<0.05$; **,$P<0.01$ significantly different from control.

(Figure 3), it is unlikely that the cAMP increase is mediated by autocrine and/or paracrine secondary extracellular messengers.

Effect of selective blockade of $P2Y_1$ receptors $P2Y_1$ receptors are activated more potently by ADP than by ATP, and these receptors are linked to G_q, the activation of which does not result in cAMP production (Communi *et al*., 1999). In order to examine the possibility that ADP activates $P2Y_1$ receptors and thereby induces cAMP accumulation, the effect of selective blockade of $P2Y_1$ receptors was tested on the ADP-induced cAMP production. As can be seen from Figure 6B, the increase in cAMP concentration induced by ADP was not affected by MRS2179 (100 μM; Figure 6).

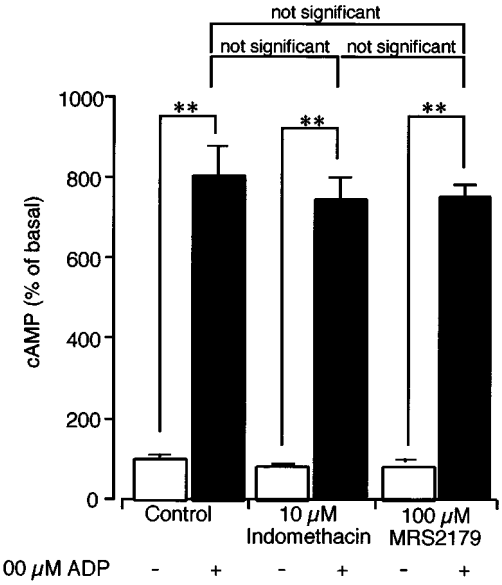

Figure 6 Effects of indomethacin and MRS2179 on the ADP-activated production of cAMP. Open columns give the levels of cAMP after 20-min of incubation of cells without addition of ADP as expressed as the percentage of the basal initial levels; filled columns represent the increase in cAMP stimulated by 100 μM ADP in the absence of antagonists (control), in the presence of 10 μM indomethacin and in the presence of 100 μM MRS2179. The increase in cAMP levels by ADP was neither significantly affected by indomethacin nor by MRS2179. Values shown are means ($\pm$s.e.) from four separate experiments. **$P<0.01$ significantly different from the value obtained in the absence of ADP.

Expression of P2Y mRNAs in BAFCs

The characteristics demonstrated above, i.e. stimulation of cAMP production by ADP, cannot be attributed to any of the previously identified P2Y receptors (Burnstock, 2001). $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors are all coupled to G_q and do not affect cAMP levels. $P2Y_{11}$ receptors are the only P2Y receptors known to be coupled with G_s and to cause PKA activation (Burnstock, 1997). These receptors, however, are activated more potently by BzATP than by ATP (van der Weyden *et al*., 2000). $P2Y_1$ and $P2Y_{12}$ receptors are readily activated by ADP but are not linked to G_s. Indeed, an activation of $P2Y_{12}$ receptors in platelets results in a decrease in cAMP concentration (Hollopeter *et al*., 2001).

In order to confirm the absence of mRNAs for $P2Y_1$, $P2Y_4$, $P2Y_{11}$ and $P2Y_{12}$ in the BAFCs, we analysed mRNA harvested from morphologically and electrophysiologically identified BAFCs ($n=20$; Figure 7A). The single cell RT–PCR method was chosen because, in the primary cultures of BAFCs there is a possible contamination from blood cells, fibroblasts and vessel cells. These contaminating cell types which do not take part in steroidogenesis may express P2Y receptor subtypes that do not exist in BAFCs. We selected healthy BAFCs with visible intracellular vesicles typical for steroid secreting cells with a stable resting membrane potential. Despite known expression of $P2Y_2$ mRNA in primary cultured BAFCs (Nishi, 1999), mRNAs for $P2Y_1$, $P2Y_4$, $P2Y_{11}$ and $P2Y_{12}$ receptors were never detected even after 50 PCR cycles amplification in all 20 cell samples analysed (Figure 7B). As the $P2Y_{11}$ receptor

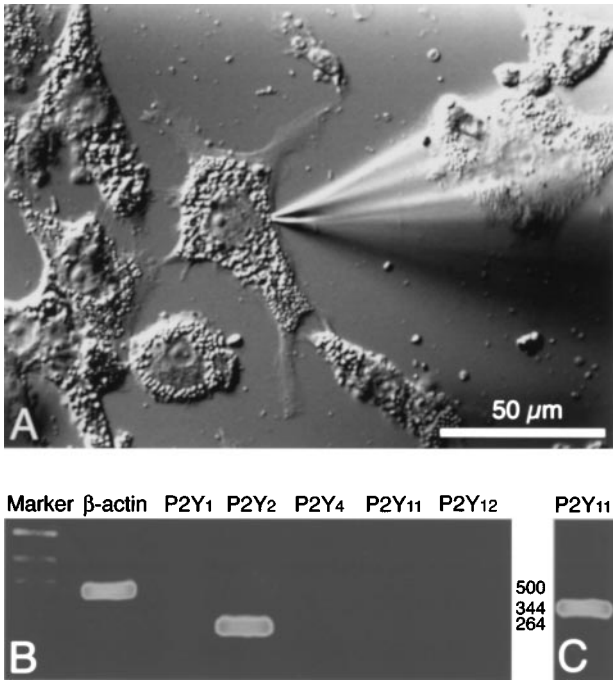

Figure 7 RT–PCR amplification of P2Y receptor mRNAs from BAFC cytoplasm derived from a single cell. (A) Whole-cell tight-seal patch-clamp of a BAFC imaged with differential interference contrast video microscopy. A morphologically identified BAFC with a patch-clamp glass pipette to collect cytoplasm for mRNA analysis is shown on the right. (B) PCR fragments for P2Y receptor mRNAs harvested from a single BAFC analysed on a 2% agarose gel. Contrast was intensified with PhotoShop software (Adobe Systems Inc, San Jose, CA, USA) in order to show clearly the fluorescent band. (C) PCR product amplified with bovine $P2Y_{11}$ receptor-based primer for the tissue extract from bovine placenta.

shares some common pharmacological features with the ADP-activated receptor analysed in the present study (e.g., production of cAMP, inhibition profiles by P_2-antagonists), we examined the efficacy of detection of $P2Y_{11}$ mRNA in bovine placenta using the same primer sequence as used here based on the $P2Y_{11}$ sequence cloned from human placenta cDNA. A clear band for the $P2Y_{11}$ mRNA was readily observed after 50 PCR cycle amplification for the samples taken from bovine placenta, indicating efficiency of detection and supporting the absence of $P2Y_{11}$ in BAFCs. Identical results, i.e., presence of $P2Y_2$ mRNA and absence of those for $P2Y_1$, $P2Y_4$, $P2Y_{11}$ and $P2Y_{12}$, were obtained with the same RT–PCR amplification procedures applied to samples from the whole BAFC population (data not presented), suggesting that the BAFC populations used for cAMP and steroid measurements in this study were close to homogeneous.

Discussion

Our results argue for the expression of a not yet known type of purinoceptor that is activated by ADP and is linked to cAMP production in BAFCs. First, we discuss the possibility of involvement of previously identified P2Y receptors and then we mention other possible mechanisms which may or may not explain the present observations.

Among P2Y receptors identified to date, $P2Y_1$ and $P2Y_{12}$ are the only receptors at which ADP can be a potent agonist (Burnstock, 2001). The following lines of evidence argue against the possibility that the responses described in the present study are mediated by $P2Y_1$ or $P2Y_{12}$ receptors. First, 2MeSADP, a potent $P2Y_1$ agonist, failed to stimulate cAMP production in BAFCs. Second, steroidogenesis and cAMP production with ADP were not affected by MRS2179, a selective $P2Y_1$ receptor antagonist. Third, cAMP production was increased with ADP in BAFCs, whereas stimulation of $P2Y_{12}$ receptors results in reduction of cAMP (Hollopeter *et al*., 2001). In addition, we failed to detect mRNAs for $P2Y_1$ and $P2Y_{12}$ from the identical cytoplasmic samples in which $P2Y_2$ mRNA was clearly detected. These lines of evidence indicate that activation of $P2Y_1$ and $P2Y_{12}$ receptors does not mediate the increase in cAMP production, PKA activation and cortisol production by ADP in BAFCs. At present, $P2Y_{11}$ receptor is the only purinoceptor shown to be coupled to G_s protein, leading to adenylyl cyclase activation (Burnstock, 2001). However, cAMP production in the present study is not likely to be mediated by $P2Y_{11}$ receptors because (i) BzATP, a potent stimulant of cAMP production in cells heterologously expressing $P2Y_{11}$, was much less effective in BAFCs; (ii) ADP, which presents only weak effects in $P2Y_{11}$-expressing cells (Communi *et al*., 1997), was the most potent agonist in the present study; (iii) suramin, which reduces cAMP production by ATP to less than $\sim$15% at a concentration of 100 μM in CHO cells heterologously expressing $P2Y_{11}$ receptors (Chen & Lin, 1999), decreased cAMP production only to $\sim$78% in BAFC (Figure 5); and (iv) mRNA for $P2Y_{11}$ was not detected in BAFCs. All other P2Y receptors identified to date, i.e., $P2Y_2$, $P2Y_4$, $P2Y_6$ are linked to $G_{q/11}$ protein (Burnstock, 2001) and therefore are not likely to mediate cAMP production by ADP in BAFCs. Indeed, despite a clear expression of $P2Y_2$ in BAFCs (Nishi, 1999), UTP, a potent $P2Y_2$ agonist which increases $InsP_3$ and Ca^{2+} concentration in BAFCs, did not induce cAMP production (Figure 1A). We therefore conclude that ADP-stimulated cAMP production and PKA activation in BAFCs were not mediated by any of the previously identified P2Y receptors.

Post *et al*. (1996) demonstrated in MDCK cells that activation of purinoceptors with ATP and UTP activates PGE_2 synthesis, which in turn results in a rise in cAMP concentration. However this is not a likely pathway on our experiments, because indomethacin did not affect ADP-induced cAMP production (Figure 6), at a concentration 10 times higher than that which completely inhibits prostaglandin synthesis and steroidogenesis in BAFCs (Rolland *et al*., 1981). Therefore, prostaglandin synthesis, at least that inhibited by indomethacin, does not mediate cAMP production by ADP in BAFCs. The rapid onset of the PKA activation upon ADP application, as revealed with real-time phosphorylation imaging (Figure 3), also supports the notion that autocrine/paracrine processes are not involved in the activation of adenylyl cyclase by ADP.

The agonist activity of ADP and the resulting increased cAMP production are the most prominent features of the responses observed in the present study. Although expression of P2X receptors in BAFCs is not yet demonstrated, heterologously expressed $P2X_1$ and $P2X_{1/5}$ receptors show significant sensitivity to ADP with EC_{50} values below 30 μM

(North & Surprenant, 2000). These P2X receptors form non-specific cation channels, through which Ca^{2+} may enter and activate various intracellular processes. The involvement of $P2X_1$ and $P2X_{1/5}$ receptors, however, is unlikely in our system because α,β-methyleneATP and β,γ-methyleneATP did not cause any detectable increase in cAMP concentration in BAFCs in the presence of extracellular Ca^{2+} (1.2 mM). In the same context, an intracellular Ca^{2+} increase caused by activation of $P2Y_2$ receptors expressed in BAFCs (Nishi, 1999) does not directly mediate cAMP production by ADP. This is true because UTP, which markedly increases intracellular Ca^{2+} concentration (Nishi, 1999), did not stimulate cAMP production but did stimulate steroidogenesis in an adenylyl cyclase-independent fashion. However, it is possible that a rise in intracellular Ca^{2+} may play a synergistic role in stimulating cAMP production upon ADP application through enhancing Ca-dependent adenylyl cyclase in BAFCs (Kawamura *et al*., 2001) because ADP also increases intracellular Ca^{2+} (our unpublished observations). Finally, it is unlikely that adenosine or AMP are produced from ATP and ADP by extracellular enzymes, despite the expression of ectonucleotidase in BAFCs (Hoey *et al*., 1994).

Another interesting possibility is that the ADP-activated cAMP production is mediated by a heterodimer complex of different G-protein-coupled receptors (Yoshioka *et al*., 2001), one of which is activated by ADP (e.g., $P2Y_{12}$) and the other of which is linked to G_s (e.g., $P2Y_{11}$). The evidence arguing against this possibility is that mRNAs for ADP-sensitive receptors ($P2Y_1$ and $P2Y_{12}$) were not detected in BAFCs. The possibility that such a heterodimer is indeed formed in BAFC membranes remains to be examined in future studies.

In conclusion, the most plausible explanation for our present results is that the cAMP production, PKA activation and the resulting cortisol production by ADP in BAFCs are mediated by some novel type of ADP-sensitive G_s protein-coupled P2Y receptor. It will be a challenging issue in future studies to identify this receptor and compare its amino acid sequence to the known P2Y receptors. This receptor might be a completely novel P2Y receptor, adding a new G_s protein-coupled member to the metabotropic purine receptor family (Burnstock, 2001), or it could be an unidentified variant of human placental $P2Y_{11}$ receptor specifically expressed in the bovine adrenal cortex with distinct sensitivities to nucleotides and P_2 receptor antagonists.

A very recent study reported an ADP-activated receptor with a high homology to human $P2Y_{12}$ (Communi *et al*., 2001) and referred to as '$P2Y_{13}$'. This ADP receptor, however, differs from that mediating cAMP synthesis observed in the present study, in the following three aspects. First, the '$P2Y_{13}$' receptor was coupled to G_i, not to G_s. Second, the '$P2Y_{13}$' receptor was not activated by ATP, which was almost equipotent to ADP in our system (Figure 1A); Third, 2MeSADP was a potent agonist at the '$P2Y_{13}$' receptor but was only weakly active in stimulating cAMP production in the BAFCs.

The present study, together with our previous report (Nishi, 1999), clearly indicates that there are two distinct pathways linking a rise in extracellular nucleotides to an increase in steroidogenesis in the adrenal cortex: one is mediated by $InsP_3-Ca^{2+}$ systems and the other by cAMP–PKA systems. The former system, activated not only by ATP and UTP but also by other ligands including angiotensin II (Bird *et al*., 1989), and the latter system, activated not only by ATP and ADP but also by ACTH (Schimmer, 1980) and other chemical transmitters, probably function together for the optimal regulation of cortisol production in response to hormonal and neural stimulations under various conditions. In particular extracellular purine concentration may change under pathophysiological situations such as stress responses and hypoxia (Gordon, 1986) in which higher levels of cortisol would be beneficial. It has been suggested that BAFCs express functional enzyme chains that convert ATP to ADP (Hoey *et al*., 1994). The rate of this conversion, i.e., the balance between ATP and ADP in the extracellular milieu, might determine the extent to which either of the $InsP_3-Ca^{2+}$ and the cAMP–PKA pathways is activated. The elucidation of the physiological difference between the two modes of purine-activated steroidogenesis mediated by Ca^{2+} or by cAMP, including the differences in biochemical processes downstream to these messengers, and confirmation of synergistic modulation between these pathways should help our understanding of the mechanisms of protection and recovery from pathophysiological situations by means of cortisol secretion (Kawamura *et al*., 2001).

The authors thank Dr Manfred Thiel for critical reading of this manuscript and for his recommendation. We thank Drs Noboru Nakamichi and Seiji Hori for their valuable criticisms and suggestions for improving this study. We appreciate the assistance received from Professor Frank Scott Howell of Sophia University in polishing the English of this paper. This work was supported in part by Grants-in Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology of Japan to E. Masaki (No. 11671525) and F Kato (No. 13680902).

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(Received December 3, 2001
Revised May 15, 2002
Accepted June 19, 2002)