Jpn. J. Pharmacol. 81, 194–199 (1999)

Two Different P2Y Receptors Linked to Steroidogenesis in Bovine Adrenocortical Cells

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Received May 6, 1999 Accepted July 7, 1999

ABSTRACT—Both extracellular adenosine 5′-triphosphate (ATP) and uridine 5′-triphosphate (UTP) induced corticoid production (steroidogenesis) concentration-dependently in bovine adrenocortical cells (BA cells). Pertussis toxin (PTX, approx. 2 μg/ml) partially inhibited (approx. 55% inhibition) extracellular ATP (100 μM)-induced steroidogenesis in BA cells. However, PTX did not inhibit extracellular UTP (100 μM)-induced steroidogenesis. Both ATP- and UTP-induced steroidogeneses were significantly inhibited by suramin (50–200 μM). These effects were inhibited significantly by reactive blue-2 (more than 100 μM) and pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (more than 100 μM). Both nucleotides (1–100 μM) induced inositol phosphates accumulation and intracellular Ca^{2+} mobilization, but PTX did not inhibit them. The RT-PCR procedure identified only $P2Y_2$-receptor mRNA in BA cells. These results suggest that extracellular ATP induces steroidogenesis via a unique P2 receptor linked to PTX-sensitive guanine nucleotide-binding protein (G-protein), while extracellular UTP induces steroidogenesis via P2 receptor linked to PTX-insensitive G-protein. Thus, it was concluded that at least two different P2Y-like receptors linking to steroidogenesis exist in BA cells.

Keywords: Steroidogenesis, P2 receptor, Bovine adrenocortical cell, ATP, UTP

Extracellular adenosine 5′-triphosphate (ATP) has been well-known as a chemical mediator. Its receptor is called the P_2-purinoceptor (P2 receptor) (1), and these receptors are divided into two types pharmacologically (2, 3); one is intrinsic ion channel P2X and the other is G-protein coupled P2Y. Each receptor type has several subtypes. Recently, in the case of P2Y, several laboratories have reported the co-existence of two different subtypes in the same organ or cell with respect to rat vascular smooth muscle (4, 5), bovine aortic endothelial cells (6), bovine adrenomedullary endothelial cells (7) and rat mesenteric cells (8).

In bovine adrenocortical cells (BA cells), both extracellular ATP (9) and uridine 5′-triphosphate (UTP) (10) induced potent steroidogenesis. These observations suggest that P2Y receptors exist and link to steroidogenesis in BA cells. However, the steroidogenic potency order of nucleotides in BA cells did not conform to the traditional ways of identifying the subtypes. Therefore, it is still not clear whether two subtypes of the P2 receptors exist or a novel type of the P2 receptors exists in BA cells. To determine the co-existence of different types of P2Y receptors, I investigated the effects of so-called P2-antagonists on ATP- and UTP-induced steroidogenesis in the cells. Furthermore, I also investigated the effects of pertussis toxin (PTX) that was reported to inhibit some actions via P2 receptors (11–13).

In this paper, I will discuss the effects of the inhibitors on the P2 receptors in BA cells and the identification of PTX-sensitive and PTX-insensitive G-protein(s) coupled P2 receptors linked to steroidogenesis in BA cells. I propose that at least two subtypes of P2Y receptor linked to steroidogenesis exist in BA cells: one is PTX-sensitive and the other is PTX-insensitive.

MATERIALS AND METHODS

Cell culture

BA cells were isolated aseptically from freshly obtained bovine adrenal cortex as described by Matsui (14). Briefly, adrenal cortical tissue was minced and treated with 0.1% collagenase (type I) and 0.005% deoxyribonuclease I in Krebs Ringer bicarbonate buffer (125 mM NaCl, 6 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 0.01 mM EGTA, 25.3 mM $NaHCO_3$, 0.2% glucose and 0.3% bovine serum albumin, pH 7.4)

(KRBGA). The treatment was carried out for 1 h at 37℃ under a gas phase of 95% O_2 – 5% CO_2 mixture. The isolated cells were cultured in Ham's F-10 medium supplemented with 5% fetal calf serum, 10% newborn calf serum, 2.5% horse serum and antibiotics. Three-day cultured cells were used for the experiments.

Assay of steroidogenesis

The 3-day primary cultured cells were incubated at 37℃ for 1 h in KRBGA with/without nucleotides and antagonists. In the case of PTX treatment, the cells were pretreated with PTX before the incubation. The total incubation medium was 1 ml. The amount of steroid in the incubation medium was determined fluorometrically with cortisol as a standard (15).

Measurement of intracellular Ca^{2+} mobilization

The BA cells, which had been cultured for 3 days on cover slips coated with cell matrix, were used for intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) measurements as previously described by Matsui (14). The cells were loaded with 5 μM fura-2 acetoxymethyl ester (fura-2/AM). The fluorescence was monitored at an emission wavelength of 510 nm. The $[Ca^{2+}]_i$ was shown as the ratio between the 510 nm fluorescence intensity excited at 340 nm (I_{340}) and that at 380 nm (I_{380}).

Assay of inositol phosphates production

Myo-[^{3}H]-inositol (74 kBq/10^5 cells) was added to the culture medium of 1-day cultured BA cells and then the cells were cultured for another 48 h. Incubation studies were carried out with KRBGA buffer containing 10 mM LiCl with or without P2-agonists for 20 min at 37℃. The reactions were terminated by adding 4% $HClO_4$. The $HClO_4$ extracts were neutralized, the total inositol phosphates (IPs) were collected by using trimethylaminopropyl SAX columns (PRN 1908), and then the radioactivity was counted.

RNA isolation and RT-PCR

Messenger RNA (mRNA) was isolated from BA cells using the FastTrack 2.0 Kit. Isolated mRNA was reverse-transcripted to cDNA using the ReadyToGo kit with random primers. PCR conditions were identical for all primers (primers: $P2Y_1$ forward 5′-TGTGGTGTTACCC CCCTCAAGTCCC-3′, reverse 5′-ATCCGTAACAGCC CAGAATCAGCA-3', expected product size 237 base pairs; $P2Y_2$ forward 5′-CCAGGCCCCCGTGCTTCTAC TTTG-3′, reverse 5′-CATGTTGATGGCGTTGAGGGT GTG-3′, expected product size 344 base pairs; $P2Y_4$ forward 5′-CGTCTTCTCGCCTCCGCTCTCT-3′, reverse 5′-GCCCTGCACTCATCCCCTTTTCT-3′, expected product size 411 base pairs): 1 μl of cDNA was added to a solution of 1 μM each of forward and reverse primer and PCR buffer, 0.2 mM dNTPs, 2.5 units of AmpliTaq DNA Polymerase and distilled H_2O in a total volume of 50 μl. Temperature cycling proceeded as follows: 1 cycle at 95℃ for 10 min to activate the enzyme, 95℃ for 60 s, 55℃ for 90 s, and 72℃ for 120 min, for 40 cycles, followed by 72℃ for 10 min. PCR products were then subjected to gel electrophoresis on a 2% agarose gel. The bands were extracted using the Geneclean gel extraction kit. Purified fragments were sequenced (Perkin Elmer DNA sequencer, model 373A; Perkin Elmer, Norwalk, CT, USA) using the same forward primers that were used to generate the PCR fragments.

Materials

The materials were purchased from the following sources: collagenase, from Worthington Biochemical (Lakewood, NJ, USA); fetal calf serum, from Cytosystems (Castle Hill, Australia); newborn calf serum, from CSL (Melbourne, Australia); horse serum, from Nacalai Tesque (Kyoto); FastTrack 2.0 Kit, from Invitrogen (Leek, Netherlands); agarose gel, from Bio-Rad (Hercules, CA, USA); Geneclean gel extraction kit, from Bio 101 Inc. (Carlsbad, CA, USA); deoxyribonuclease I, ATP and suramin, from Sigma (St. Louis, MO, USA); UTP from Boehringer (Mannheim, Germany); reactive blue-2 (RB-2), from Fluka (Milwaukee, WI, USA); pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), from RBI (Natick, MA, USA); PTX, from Seikagaku Co. (Tokyo); fura-2/AM, from Dojindo (Kumamoto); *Myo*-[^{3}H]-inositol (specific activity: 2.96–4.44 TBq/mmol) and SAX columns, from Amersham (Buckinghamshire, England); AmpliTaq DNA Polymerase, from Perkin Elmer; ReadyToGo kit with random primers, from Pharmacia Biotech (Uppsala, Sweden). All the other chemicals were of reagent grade.

Data analysis

The results were expressed as the mean±S.E.M. The statistical analysis of data was carried out by using analysis of variance (ANOVA); $P<0.05$ was considered statistically significant.

RESULTS

Effects of ATP and UTP on steroidogenesis

Both ATP (more than 1 μM) and UTP (more than 0.01 μM) stimulated steroidogenesis in concentration-dependent manners in BA cells (Fig. 1). Although UTP was more potent than ATP (approximate EC_{50} values: ATP, 1.5 μM; UTP, 0.2 μM), the efficacies of the two nucleotides were the same. Steroidogenic activity reached the maximum at 100 μM ATP or 100 μM UTP. Thus 100 μM

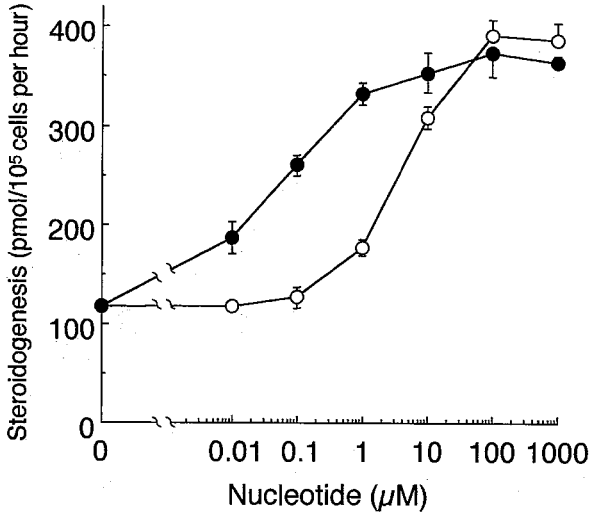

Fig. 1. Effects of ATP and UTP on steroidogenesis in primary cultured BA cells. The 3-day primary cultured monolayer cells were incubated in the presence or absence of ATP (○) and UTP (●) at 37℃ for 1 h. Cortisol in the incubation medium was determined by the methods of Slavinski et al. (15). Each point represents the mean±S.E.M. from 3 separate experiments (n=9–12).

ATP and 100 μM UTP were used in the following experiments.

Effects of P2-antagonists on ATP- and UTP-induced steroidogenesis

Figure 2 shows the effects of so-called P2-antagonists on 100 μM ATP- and 100 μM UTP-induced steroidogenesis in BA cells. Suramin inhibited both ATP- and UTP-induced steroidogenesis significantly, although the effect was remarkable for ATP-induced steroidogenesis (Fig. 2A). RB-2 slightly inhibited the steroidogenesis induced by either nucleotide (Fig. 2B). PPADS inhibited both ATP- and UTP-induced steroidogenesis slightly, and the inhibition of the ATP effect was more effective than that on the UTP effects (Fig. 2C). Both the RB-2 and PPADS treatments significantly inhibited steroidogenesis by the nucleotides in concentrations at least up to 200 μM.

Effects of PTX on ATP- and UTP-induced steroidogenesis

PTX partially (approximately 55% inhibition) inhibited ATP-induced steroidogenesis. The obvious inhibitory effect of PTX (2 μg/ml) on ATP-induced steroidogenesis was observed 2 h after PTX-pretreatment and the maximum effect of PTX was obtained by 4- to 6-h pretreatment (data not shown). Therefore, 6-h pretreatment of PTX was employed in the following experiments. Figure 3 shows the effects of PTX on steroidogenesis by ATP and UTP. PTX showed dose-dependent inhibition of 100 μM ATP-induced steroidogenesis. Even at a high concentration like 2–4 μg/ml, the inhibition by PTX was not complete. PTX did not inhibit UTP (100 μM)-induced steroidogenesis in BA cells.

Elevation of $[Ca^{2+}]_i$ caused by ATP and UTP

Effects of ATP and UTP on $[Ca^{2+}]_i$ in the presence of extracellular Ca^{2+} (1.2 mM) were examined. As shown in Fig. 4, either 100 μM ATP or 100 μM UTP caused a rapid elevation and a gradually decrease of $[Ca^{2+}]_i$ in BA cells. Thereafter, $[Ca^{2+}]_i$ declined to a steady state level (Fig. 4). The 1st phase was induced by Ca^{2+} release from intra-

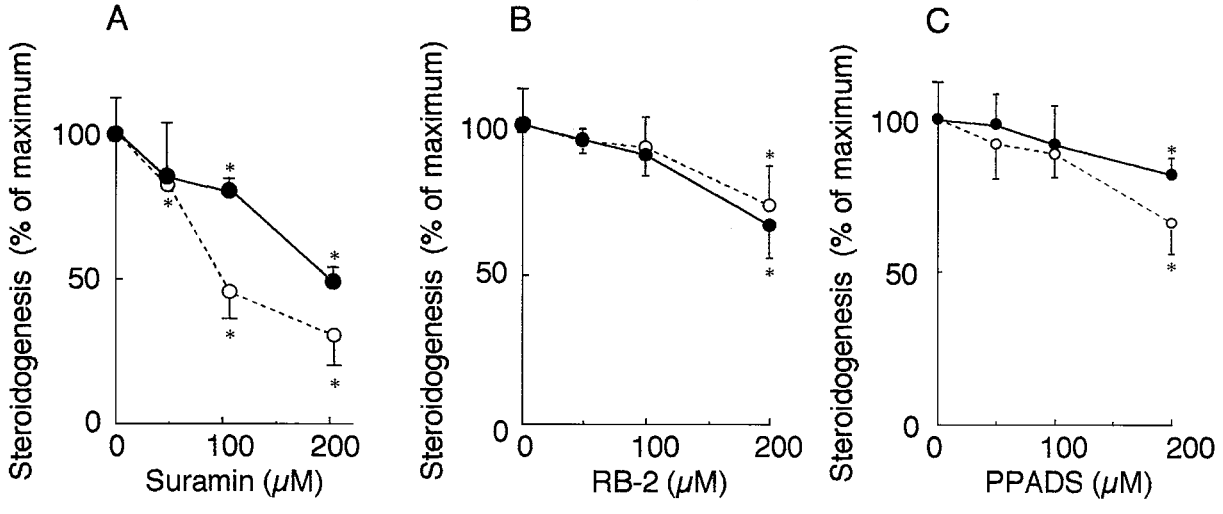

Fig. 2. Effects of suramin (A), RB-2 (B) and PPADS (C) on steroidogenesis by ATP (○) and UTP (●) in BA cells. The responses were converted to percentage of the maximum response, 100 μM ATP- and 100 μM UTP-induced steroidogenesis, without any antagonists. Data are expressed as the mean±S.E.M. (n=3–4) from a single representative experiment or 3 separate ones. *P<0.05, compared the corresponding controls.

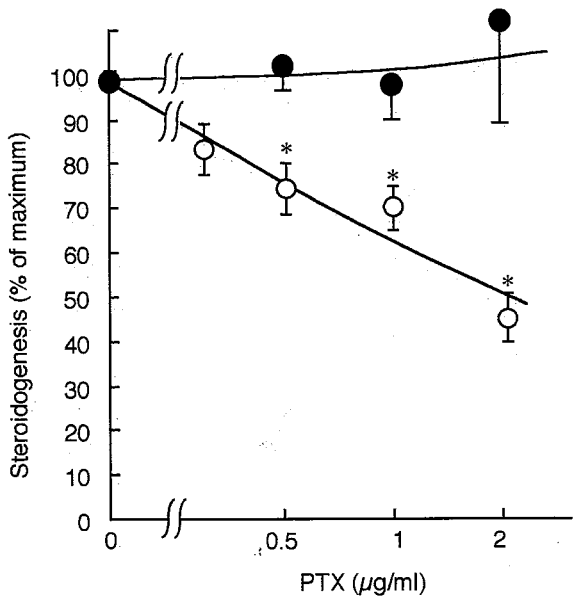

Fig. 3. Effects of PTX on steroidogenesis by ATP (○) and UTP (●) in BA cells. The responses were converted to percentage of the maximum response, 100 μM ATP- and 100 μM UTP-induced steroidogenesis, without PTX. Data are expressed as the mean±S.E.M. of 2 or 3 separate experiments (n=4–6). *P<0.05, compared to the corresponding controls.

cellular calcium stores, and the 2nd phase was induced by Ca^{2+} influx from the extracellular pool (14). PTX (4 μg/ml) pretreatment did not affect either phase induced by 100 μM ATP.

Effects of ATP and UTP on IPs production

It was reported that ATP and UTP stimulated membrane phosphoinositide turnover in BA cells (10). Thus I examined the effects of ATP and UTP on IPs production and the effect of PTX pretreatment on ATP-induced IPs production. Both ATP and UTP induced IPs production dose-dependently in BA cells (Fig. 5). The results supported the effects of nucleotides on intracellular mobilization as described above. The reactions were not saturable for 20 min. The inset shows that 100 μM ATP also induced IPs production in the PTX-treated cells (4 μg/ml, 18 h). There was no significance difference (P>0.05) between the IPs production in the PTX-treated cells and that from the non-treated cells.

RT-PCR analysis of P2Y receptor subtypes

UTP has potent steroidogenic activity and only ATP-induced steroidogenesis was abolished by PTX, so that I expected the existence of the $P2Y_2$ and/or $P2Y_4$ subtype and another subtype possessing low affinity for UTP in BA cells. However, the only P2Y-receptor mRNA that I could identify in the BA cells was that of the $P2Y_2$ subtype (Fig. 6).

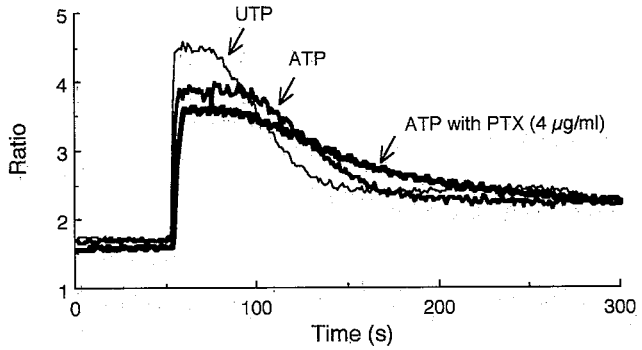

Fig. 4. Effects of 100 μM ATP and 100 μM UTP on $[Ca^{2+}]_i$ mobilization in BA cells. Typical tracings of $[Ca^{2+}]_i$, as indicated by the ratio of I_{340} to I_{380}, are shown. $[Ca^{2+}]_i$ mobilization by ATP in BA cells treated with PTX (4 μg/ml) is shown on the same tracings in a thicker line. These responses were observed in the presence of 1.2 mM $CaCl_2$.

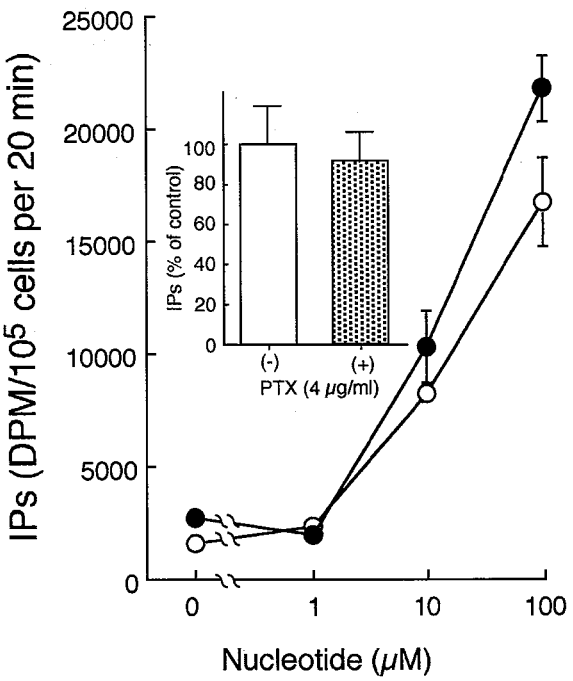

Fig. 5. Effects of ATP (○) and UTP (●) on IPs production in BA cells. The inset shows ATP-induced IPs production in PTX-treated BA cells (4 μg/ml, 18 h). Data are expressed as the mean±S.E.M. of 2 or 3 separate experiments (n=4–6).

DISCUSSION

In BA cells, both ATP and UTP stimulated steroidogenesis concentration-dependently (Fig. 1). In my preliminary work, other nucleotides and nucleosides (GTP, CTP, TTP, ITP, UDP, UMP, AMP, uridine and adenosine) had no potent steroidogenic activity, except for ADP in BA cells (data not shown). There are no available

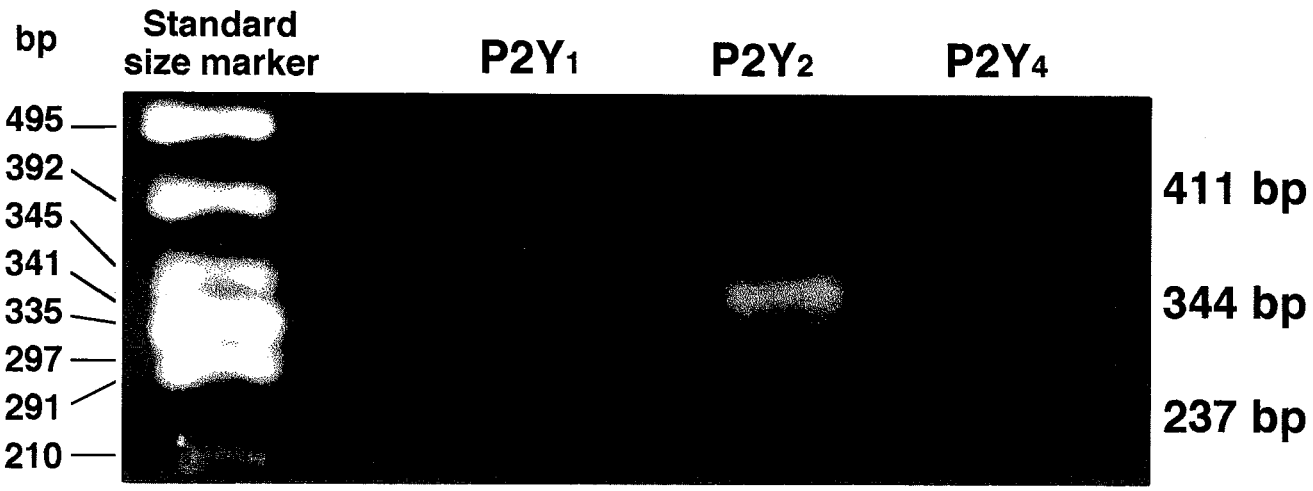

Fig. 6. Detection of the P2Y receptor subtypes from mRNA extracts using RT-PCR. If BA cells have $P2Y_1$, $P2Y_2$ or $P2Y_4$ mRNA, there could be an amplified 237 bp, 344 bp or 411 bp band, respectively. Only $P2Y_2$ mRNA but not $P2Y_1$ and $P2Y_4$ mRNAs could be identified from the cDNA of the BA cells.

specific P2-antagonists, so that the potency order of agonists for P2Y receptors is the base for subclassifying them. Accordingly, there is still some confusion about the classification of the P2Y receptors. Then, identifying the subtype of the P2 receptors, the DNA information of which is not obvious, is not an easy task. In the present study, so-called P2-antagonists were used to identify the existence of some types of P2 receptors. Suramin inhibited ATP- and UTP-induced steroidogenesis (Fig. 2A). RB-2 and PPADS indicated a slight effect on both ATP- and UTP-induced steroidogenesis (Fig. 2: B and C). However a small difference was observed: suramin and PPADS tended to inhibit ATP-induced steroidogenesis more markedly than UTP-induced steroidogenesis (Fig. 2: A and C). PPADS, known as a P2X antagonist, has been reported as a P2Y antagonist in turkey erythrocytes (16), guinea pig taenia-coli and rat duodenum (17). In bovine aorta, PPADS was also reported as an antagonist for endothelial P_{2Y}-purinoceptors ($P2Y_1$), but not as an antagonist for P_{2U}-purinoceptors ($P2Y_2$) (5, 18). According to these observations, PPADS might be a non-selective P2-antagonist. There are several reports that the 10–200 μM suramin (19, 20), RB-2 (21, 22) or PPADS (7, 17) effectively inhibited the effect of ATP or UTP. In BA cells, the inhibitory effects of these antagonists were different from those reported inhibition values of the antagonists.

There are several reports that the action of ATP via P2Y receptors is abolished by PTX, but PTX had a slight partial effect in most cases (23, 24). In BA cells, PTX also partially inhibited ATP-induced, but not UTP-induced, steroidogenesis. However, more than 0.25 μg/ml PTX was needed to inhibit steroidogenesis in BA cells (Fig. 3) and even under a higher concentration (4 μg/ml) of PTX, the inhibition was not complete (data not shown). The partial inhibition of PTX in BA cells might be based on the existence of the dual P2 receptors in BA cells. Although UTP stimulates the PTX-insensitive G-protein coupled P2 receptors, ATP probably stimulates both PTX-sensitive and PTX-insensitive G-protein coupled P2 receptors in BA cells. PTX did not abolish inositol phosphates production and the increase in $[Ca^{2+}]_i$ by ATP. Thus PTX-sensitive G-protein, which couples to the ATP receptor, may link to adenylyl cyclase, whereas PTX-insensitive G-protein which couples to UTP receptor, may link to phospholipase C.

A similar precedent was reported that both PTX-sensitive and PTX-insensitive P2 receptors existed in several organs (24–26). It was suggested that there are Gq-coupled ATP receptors (27) and Gs-coupled ATP receptors (10) in BA cells. The results suggest that at least two different types of P2Y-like receptors exist and act on steroidogenesis in BA cells.

Recently, the functional P2Y receptors were subdivided into six subtypes: $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ (*The IUPHR Compendium of Receptor Characterization and Classification, 1998*). Both $P2Y_2$ and $P2Y_4$ have potent sensitivity to UTP; thus PCR-primers were used for $P2Y_2$ and $P2Y_4$ receptors. In addition, ADP and 2-methylthio-ATP had very potent steroidogenic activity in BA cells (data not shown), so $P2Y_1$ primers were also selected for the P2Y mRNA analysis in these cells. Only the $P2Y_2$ receptor mRNA could be identified from the BA cells' cDNA in the present study (Fig. 6) However, it is difficult to explain the properties of the BA cells by a single subtype of P2 receptors, such as differences in the

inhibitory effect of suramin (Fig. 2A) or PTX (Fig. 3) on steroidogenesis. It is suggested that other subtypes (including novel types) of P2 receptors exist and link to steroidogenesis in BA cells. I am now proceeding to clone (all of) the P2 receptors in BA cells to define the P2 receptors linked to steroidogenesis in BA cells.

In conclusion, there are at least two types of P2Y-like receptors linked to steroidogenesis in BA cells, and it is suggested that the sensitivity to PTX of the P2 receptors is useful for subclassifying the G-protein-coupled P2 receptors.

Acknowledgments

The author thanks Prof. Masahiro Kawamura for his valuable criticisms and suggestions for improving this study.

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