Extracellular ATP Potentiates Steroidogenic Effect of Adrenocorticotropic Hormone in Bovine Adrenocortical Fasciculata Cells

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ABSTRACT—We examined the effect of extracellular adenosine 5'-triphosphate (ATP) on adrenocorticotropic hormone (ACTH)- and angiotensin II-induced steroidogenesis in bovine adrenocortical fasciculata cells. The low concentration of ATP (5 μM) potentiated ACTH-induced steroidogenesis synergistically. However, the purine derivative did not affect angiotensin II-induced steroidogenesis. Although adenosine (100 μM) (a metabolite of ATP) showed a weak steroidogenic effect, it did not potentiate ACTH-induced steroidogenesis. ATP also enhanced the steroidogenesis by NaF synergistically in bovine adrenocortical cells, but did not potentiate forskolin- and dibutyryl cyclic AMP-induced steroidogenesis. The stimulating effect of ACTH on cyclic AMP production was synergistically accelerated by ATP (5 μM), which has no effect by itself on cyclic AMP formation. These results suggest that extracellular ATP affected the ACTH receptor-adenylyl cyclase coupling processes, and potentiation of steroidogenesis by ACTH ensued in bovine adrenocortical fasciculata cells.

Keywords: Adrenal cortex, Adrenocorticotropic hormone, Steroidogenesis, ATP, P2Y receptor

Extracellular adenosine 5'-triphosphate (ATP) plays many biological roles in diverse cell functions via its cell membrane receptors (1 – 4). Receptors for ATP are classified into two groups: a ligand-gated ion channel P2X receptor family and a G protein-coupled P2Y receptor family (5). We previously reported that extracellular ATP stimulated glucocorticoid production (steroidogenesis) in bovine adrenocortical fasciculata cells (BAFC) via P2Y receptors at micromolar concentrations (6).

Recently, the importance of cross-talk between two biological active substances on the functions in several kinds of cells and organs has been mentioned. In the case of extracellular ATP, the nucleotide potentiates the insulin secretion stimulated by acetylcholine in isolated perfused rat pancrease (7), and the cytosolic Ca²⁺ response to parathyroid hormone in rat osteoblastic cells (8). In steroidogenic organs, the cross-talk between serotonin and angiotensin II (9), interleukin-6 and adrenocorticotropic hormone (ACTH) (10) in rat adrenal steroidogenesis and angiotensin II and ACTH on cyclic AMP formation in bovine adrenal glomerulosa cells (11, 12) had been reported. From these observations, there might be possible cross-talk between extracellular ATP and ACTH (and/or another steroidogenic substances) on glucocorticoid production in BAFC. Therefore, we investigated the effect of ATP on ACTH- and angiotensin II-induced steroidogenesis in BAFC.

MATERIALS AND METHODS

Primary culture of BAFC

Isolated bovine adrenocortical fasciculata cells were prepared aseptically using collagenase and deoxyribonuclease I as previously described (13). 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 in a 24-well-type dish (10 – 15 × 10⁵ cells/well) in a CO₂ incubator (humidified atmosphere of 5% CO₂ in air) at 37°C as reported (14). The 3-day primary cultured monolayer cells were used for the experiments.

Determination of steroidogenic activity

The cultured monolayer cells were washed by phosphate-buffered saline (pH 7.4). The washed cells were incubated in Krebs-Ringer bicarbonate buffer (pH 7.4) containing 125 mM NaCl, 5.9 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1.2 mM CaCl₂, 25.3 mM NaHCO₃, 2 mg

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/ml glucose and 3 mg/ml bovine serum albumin in a total incubation medium of 1 ml for 1 h at 37°C in a CO₂ incubator (humidified atmosphere of 5% CO₂ in air) as previously described (14). Steroid in the incubation medium was fluorometrically determined using cortisol as a standard (15).

Determination of cyclic AMP production
The cultured monolayer cells were incubated in the above buffer (Krebs-Ringer bicarbonate glucose albumin buffer (pH 7.4)) in the presence of 3-isobutyl-methylxanthine (IBMX) (200 μM) in a total incubation medium of 0.5 ml for 15 min at 37°C in a humidified atmosphere of 5% CO₂ in air. In the Ca²⁺-free buffer, CaCl₂ was substituted by 0.5 mM EGTA. The reactions were terminated by adding ice-cold ethanol (final concentration, 70%) and the extracted cyclic AMP was determined by a commercially available radioimmunoassay kit.

Statistical analyses
The statistical analysis was carried out by ANOVA and Student’s t-test. The significance was assumed at P<0.05.

Materials
ATP, adenosine, angiotensin II, dibutyryl cyclic AMP (db-cyclic AMP), forskolin and IBMX were purchased from Sigma Chemical Co. (St. Louis, MO, USA). ACTH₁₋₂₄ (Cortrosine) was obtained from Daiichi Seiyaku Co. (Osaka) and Ham’s F-10 medium was obtained from Gibco Laboratories Inc. (Grand Island, NY, USA). The cyclic AMP assay kit was purchased from Yamasa Co. (Tokyo). All the other chemicals were reagent grade.

RESULTS
Effects of ATP on ACTH- and angiotensin II-induced steroidogenesis
Table 1 shows the effect of ATP (1 and 10 μM) on steroidogenesis induced by 10 pM ACTH in BAFC. In this preliminary result, ATP potentiated ACTH-induced steroidogenesis synergistically. Although 1 μM of ATP might be the threshold concentration on steroidogenesis, 5 μM ATP was used in the following experiments. As shown in Fig. 1, 5 μM ATP potentiated the steroidogenic effect of ACTH in a synergistic manner.

Effect of adenosine on ACTH-induced steroidogenesis
Because extracellular ATP might be metabolized to adenosine by ecto-nucleotidases in the incubation period (16), the potentiating effect of ATP on ACTH-induced steroidogenesis could be achieved by adenosine. Accordingly the effect of adenosine on ACTH-induced steroidogenesis in BAFC was examined. Although adenosine (100 μM) stimulated cortisol production slightly, it did not potentiate ACTH-induced steroidogenesis (Fig. 2). The other tested concentrations of adenosine (1 and 10 μM) also did not enhance the ACTH effect (data not shown).

Effect of ATP on angiotensin II-induced steroidogenesis
Angiotensin II is another physiological steroidogenic substance. As shown in Fig. 3, angiotensin II enhanced steroidogenesis, and this enhancement was concentration-dependent in BAFC. ATP at 5 μM showed only an additive effect on angiotensin II-induced steroidogenesis.

Table 1. Effect of ATP on ACTH-induced steroidogenesis in bovine adrenocortical cells

| ATP (μM) | Cortisol production (pmol/10⁵ cells per hour) | ACTH (−) | ACTH (10 pM) |
|---------|---------------------------------------------|----------|--------------|
| 0       | 0                                           | 605.6 ± 85.3 |              |
| 1 μM    | 42.3 ± 19.8                                 | 1276.3 ± 61.9* |             |
| 10 μM   | 491.7 ± 36.7                                | 1500.9 ± 71.4* |             |

The incubations were carried out for 1 h at 37°C in a humidified atmosphere of 5% CO₂ in air in the presence or absence of ACTH and ATP as indicated in the Table. Total incubation medium was 1 ml. Each value represents the mean ± S.E.M. from triplicate determinations. *Significantly different from ACTH-induced steroidogenesis (P<0.01).

Fig. 1. Effect of ATP on ACTH-induced steroidogenesis. Incubations were carried out for 1 h at 37°C in a humidified atmosphere of 5% CO₂ in air in the presence or absence of ACTH and 5 μM ATP. ATP (−), open circles; ATP (5 μM), closed circles. Each point expresses the mean ± S.E.M. from 3 separate experiments. *Significantly different from ACTH-induced steroidogenesis in the absence of ATP, respectively, at P<0.05.
Effects of ATP on NaF- and forskolin-induced steroidogenesis

We examined the effect of 5 μM ATP on steroidogenesis elicited by NaF, a stimulant of Gs to enhance adenylyl cyclase activity (17, 18), and forskolin, an adenylyl cyclase activating agent (19). As shown in Fig. 4, ATP highly potentiated the effect of NaF. However, the nucleotide did not augment the steroidogenic effect of forskolin (Fig. 5). Steroidogenesis by db-cyclic AMP was also not affected by ATP (data not shown).
Fig. 6. Effect of ATP on ACTH-induced cyclic AMP production. Incubations were carried out for 15 min at 37°C with 200 μM IBMX in a humidified atmosphere of 5% CO₂ in air in the presence or absence of 5 μM ATP and ACTH. Each point expresses the mean ± S.E.M. from 3 separate experiments. ATP (―), open circles; ATP (5 μM), closed circles. *Significantly different from in the absence of ATP, at P<0.05.

We also investigated the effect of indomethacin, a prostaglandins (PGs) synthesis inhibitor (20), on the potentiation effect of ATP on NaF-induced steroidogenesis. However, indomethacin did not inhibit the effect of ATP (data not shown).

Effect of ATP on ACTH-induced cyclic AMP production

The effect of 5 μM ATP on cyclic AMP formation elicited by ACTH in the presence of IBMX, a potent nonspecific phosphodiesterase inhibitor, was examined. As shown in Fig. 6, although ATP itself did not enhance cyclic AMP production, the nucleotide potentiated ACTH-induced cyclic AMP production synergistically. The absence of extracellular Ca²⁺ significantly attenuated cyclic AMP formation by 1 nM ACTH. The amounts of cyclic AMP production (pmol/10⁶ cells per 15 min) were as follows: 1.2 mM Ca²⁺, 0.5 ± 0.05; ACTH + Ca²⁺, 47.5 ± 1.08; ACTH, 2.3 ± 0.15 (mean ± S.E.M., n = 3).

DISCUSSION

During the last decade, the role of extracellular ATP in several cellular functions via its receptors has been studied extensively, and the interactions between extracellular ATP and other physiological substances in several cell systems were examined. Bertrand et al. (7) reported the potentiating effect of ATP on acetylcholine-induced insulin secretion in a rat pancreas, and Kaplan et al. (8) indicated the potentiation effect of ATP on parathyroid hormone-evoked intracellular Ca²⁺ mobilization in rat osteoblastoma cells. These reports stress the modificational effect of extracellular ATP on biological active substance-induced cellular functions rather than its direct effect on cell functions via ATP receptors. We investigated the effect of ATP on steroidogenic action of ACTH and angiotensin II in bovine adrenocortical fasciculata cells. Extracellular ATP synergistically potentiated both cortisol and cyclic AMP production elicited by ACTH. However, the nucleotide did not affect the steroidogenic effect of angiotensin II. Because the effect of angiotensin II is revealed by a phospholipase C – inositol phosphates – Ca²⁺ system (21) and that of ACTH is shown via an adenylyl cyclase-cyclic AMP system (22), the results suggest that ATP may enhance the effect of a steroidogenic secretagogue that links to the adenylyl cyclase-cyclic AMP system.

However, the mechanism of the potentiating effect of ATP on ACTH-induced steroidogenesis is obscure. It was reported that ATP stimulates PGs formation (23) and PGs receptors exist in BAFC membrane (24). Although these reports suggest the involvement of PGs in the potentiation effect in an autocrine fashion, lack of the inhibitory effect by indomethacin on the potentiation effect of ATP indicates that the above possibility might be unlikely.

Ecto-nucleotidases metabolize ATP to adenosine (16, 25). Adenosine combines with A₁ receptors that are linked to Gₛ protein (26) and may cross-talk with Gᵢ protein-coupled ACTH receptor. However, this line of thinking seems improbable because adenosine does not augment the steroidogenic effect of ACTH. Also, Hoey et al. showed that the only 50% of ATP was metabolized during 1 h incubation to ADP and AMP (not to adenosine) in bovine adrenocortical cells (25).

There are ATP receptors linked to steroidogenesis in BAFC (6, 25). ATP receptors are classified into ligand-gated channel (P2X) and G protein-coupled (P2Y) receptors (5). It has been proposed that the receptor in bovine adrenocortical cells was P2Y from the potency order of the tested ATP analogues on steroidogenesis and its ability to activate phospholipase C (6, 25). In BAFC, ATP increases the intracellular concentration of Ca²⁺ ([Ca²⁺]) in a biphasic manner; the first rise in [Ca²⁺] is due to Ca²⁺ release from the intracellular Ca²⁺ stores by inositol 1,4,5-trisphosphate and subsequent rise in [Ca²⁺] due to Ca²⁺ influx from an extracellular pool (13). Hoey et al. (25) indicated that UTP also stimulates steroidogenesis in the potency order of UTP > ATP in BAFC. Therefore, the potentiating effect of ATP on ACTH-induced steroidogenesis might be brought about via the Gₛ-protein coupled P2Y₂ or P2Y₄ receptor (27).

ACTH interacts with its receptor to activate adenylyl cyclase through Gₛ protein and increases intracellular cyclic
AMP level. As a consequence, steroidogenesis is stimulated in adrenocortical cells (22). Thus the possible sites of the ATP action are a) ACTH-receptor interaction, b) ACTH receptor-Gs protein coupling, c) Gs protein-adenylyl cyclase interaction, and d) the steroidogenic pathway after cyclic AMP production.

ATP could not potentiate cortisol production induced by db-cyclic AMP, a membrane permeable cyclic AMP analogue, and ATP augmented ACTH-induced cyclic AMP production. These results suggest that the effect of ATP is not achieved at a point downstream of cyclic AMP production, but on the cyclic AMP producing process.

It was reported that ATP increased the sensitivity of acetylcholine to a nicotinic receptor in bullfrog sympathetic ganglion cells in a noncompetitive manner via P2 receptor (28). ATP potentiated NaF-induced steroidogenesis synergistically. Because NaF activates Gs protein to stimulate adenylyl cyclase activity (17, 18), ATP might not affect the interaction between ACTH and its receptors; and the absence of the effect of ATP on steroidogenesis elicited by forskolin, an adenylyl cyclase activator (19), predicts that the site of ATP effect on ACTH-induced steroidogenesis is Gs protein-adenylyl cyclase coupling process in BAFC.

The precise mechanism of cross-talk of intracellular events between ATP and ACTH receptors is still uncertain. However, it has been reported that angiotensin II, a Ca2+ mobilizing hormone via phospholipase C activation (21), potentiates ACTH-induced cyclic AMP formation in bovine adrenal glomerulosa cells (11, 12). Although, both reports emphasized an involvement of the increase in intracellular Ca2+ by angiotensin II in the cross-talk between angiotensin II and ACTH on cyclic AMP production, Baulak et al. (11) suggested the participation of calcineurin, a Ca2+/calmodulin-dependent protein phosphatase, in this “cross-talk.” Nine adenylyl cyclase subfamilies (type I–IX) have been cloned, and the three types of Ca2+-activated cyclases are types I, III and VIII (29). Burnay et al. (12) showed the expression of type III adenylyl cyclase in bovine adrenal glomerulosa cells and surmised that Ca2+ influx elicited by angiotensin II activates pre-stimulated adenylyl cyclase by Gs protein. In our experimental condition, extracellular Ca2+ is obligatory upon adenylyl cyclase activation by ACTH in BAFC, suggesting adenylyl cyclase subfamilies in BAFC might be one of the types I, III and VIII. These isoforms are stimulated by calcium-calmodulin (Ca/CaM) (29). Ca/CaM has been reported to participate in the effect of ATP on lysis of monocyte leukemia cells (30) and Cl− conductance in epididymal cells (31). Therefore, ATP stimulates Ca2+ influx via P2Y receptor and Ca/CaM might activate synergistically pre-stimulated adenylyl cyclase by ACTH or NaF in BAFC. Further detailed studies are necessary to elucidate the precise mechanism of the cross-talk between extracellular ATP and ACTH in BAFC.

ATP is co-secreted with catecholamines from adrenal medullary chromaffin cells into the blood stream, and it was reported that a mass of chromaffin cells was present in the adrenal cortex (32). Under stressful conditions, ATP may act in a paracrine fashion to modulate the steroidogenic effect of ACTH and/or adrenocortical cells might be exposed by ATP in blood stream to potentiate the effect of ACTH. Therefore, the sensitizing effect of extracellular ATP on adrenocortical fasciculata cells to ACTH is rational to protect us from stress.

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