Calcium Is Essential for ATP-Induced Steroidogenesis in Bovine Adrenocortical Fasciculata Cells

Akiyoshi Niitsu

Department of Internal Medicine (III), The Jikei University School of Medicine, Nishi-shinbashi, Minato-ku, Tokyo 105, Japan

Received June 18, 1992 Accepted August 24, 1992

ABSTRACT—The effect of extracellular ATP on steroidogenesis in primary cultured bovine adrenocortical fasciculata cells was investigated. I observed that in the presence of extracellular Ca²⁺, ATP caused a dose-dependent elevation of intracellular Ca²⁺ ([Ca²⁺]i) and induced steroidogenesis concentration- and time-dependently. However, in the absence of extracellular Ca²⁺, ATP had no effect on steroidogenesis. In the presence of extracellular Ca²⁺, calmodulin inhibitors inhibited the ATP-induced steroidogenesis, but dihydropyridine calcium channel blockers did not. Furthermore, ATP did not cause an elevation of cyclic AMP in bovine adrenocortical fasciculata cells even if extracellular Ca²⁺ existed. These results suggest that extracellular ATP might have an influence on bovine adrenocortical cells via the purinoceptor (P2Y) in connection with calcium mobilization, open the non-selective calcium channel and induce steroidogenesis by means of an elevation of [Ca²⁺], via the calcium-calmodulin system.

Keywords: ATP, Purinoceptor, Steroidogenesis, Calcium, Adrenocortical fasciculata cell

Extracellular purine analogues have been known to induce various responses via purinergic receptors (purinoceptors) in a number of cell types. Purinoceptors have been divided into P1- and P2-subtypes on the bases of agonist potency order and functional response (1). Furthermore, according to the actions, the rank order of agonist potency and the effects of antagonists, P2-purinoceptors have been subdivided into at least 2 subtypes: P2X and P2Y (2–5).

We have recently suggested that extracellular ATP stimulated steroidogenesis via P2Y-purinoceptors in bovine adrenocortical fasciculata cells (6). It is reported that the modulation of intracellular calcium concentration ([Ca²⁺]i) could play an important role in the ATP-induced physiological processes via P2Y-purinoceptors (7–10). Thus it is considered that ATP-induced steroidogenesis might have a close connection to intracellular calcium mobilization.

In the present study, I investigated whether calcium could be essential for the ATP-induced steroidogenesis in primary cultured bovine adrenocortical fasciculata cells.

MATERIALS AND METHODS

Chemicals

Collagenase (type I) was purchased from Cooper Biomedical; deoxyribonuclease (DNase), ATP, ADP, AMP and nicardipine, from Sigma Chemical Co.; adenosine, from Kohjin Co.; ACTH₁₋₂₄ (Cortrosin), from Dai-ichi Seiyaku Co., (+)PN200-110, from Sandoz; N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide-HCl (W-7), from Seikagaku Kogyo Co.; and trifluoperazine malate, from Yoshitomi Seiyaku Co. Ham’s F-10 medium was obtained from GIBCO Laboratories; fura 2/acetoxymethyl ester (fura 2/AM), from Dojin Laboratories; and bovine serum albumin (BSA, fraction V), from Armour Pharmaceutical Co. The cyclic AMP [¹²⁵I] assay system was obtained from Amersham Co.

Cell culture

The isolated bovine adrenocortical fasciculata cells were prepared aseptically by the collagenase-DNase digestion method (11). The isolated cells were cultured in Ham’s F-10 medium containing 5% fetal calf serum, 10% newborn calf serum, 2.5% horse serum and antibiotics (100 IU/ml penicillin G, 100 μg/ml streptomycin and 50 μg/ml gentamicin) in 24-well dish at a cell den-
sity of approximately $15 \times 10^4$ cells/well, except for the measurement of $[Ca^{2+}]_i$, at $37^\circ C$ in a humidified atmosphere of 5% CO$_2$ in air. The culture medium was changed in 2 days after cell seeding, and the 3-day monolayer cultured cells were used for the experiments.

**Assay of steroidogenesis**

The 3-day cultured cells were washed twice with Ca$^{2+}$-free phosphate-buffered saline containing 0.5 mM EGTA (pH 7.4), followed by washing once with Ca$^{2+}$-free Krebs-Ringer bicarbonate buffer, pH 7.4, containing 125 mM NaCl, 6 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgSO$_4$, 25.3 mM NaHCO$_3$, 0.1 mM EGTA, 2 mg/ml glucose and 3 mg/ml BSA (Ca$^{2+}$-free KRBGA). The cells were incubated at 37°C for 1 hr in Ca$^{2+}$-free KRBGA with or without various reagents (CaCl$_2$, purine analogues, etc.). Glucocorticoid in the incubation medium was determined fluorometrically with cortisol as a standard (12).

After the incubation, the cells were dispersed by a 15-min incubation at 37°C in Ca$^{2+}$-free KRBGA containing 0.1% trypsin. The number of cells were counted by a hemocytometer. The results were expressed as picomoles cortisol production per $10^5$ cells per one hr (pmol/10$^5$ cells/hr).

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

For the measurement of $[Ca^{2+}]_i$, the isolated cells were cultured in 60-mm culture dishes at a cell density of approximately $8-9 \times 10^4$ cells/cm$^2$ under the above conditions. The culture medium was changed at 2 days after cell seeding. The 3-day cultured cells were suspended by collageanase. The suspended cells were loaded with fura 2/AM in Krebs-Ringer HEPES buffer (10 mM HEPES, pH 7.4) supplemented with 0.2% glucose and 0.1% BSA. The fura 2-loaded cells ($100 \times 10^4$ cells/ml) were placed in a quartz cuvette. The fluorescence was monitored by a HITACHI F-2000 spectrofluorometer (Hitachi Industry, Japan) with an incubation system (37°C) at an emission wavelength of 510 nm, while the excitation wavelength alternated every 0.5 sec between 340 and 380 nm. Fluorescence intensity at 340 and 380 nm was corrected for autofluorescence of the unloaded cells. $[Ca^{2+}]_i$ in the suspended cells was calculated by the equation: $[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)](S_{CA}/S_{BO})$ using $K_d = 224$ nM according to Grynkiewicz et al. (13).

**Measurement of cyclic AMP**

The 3-day cultured cells were incubated at 37°C for 1 hr in KRBGA with or without various reagents. Cyclic AMP was extracted by ethanol and measured by a commercially available radioimmunoassay system. The results were expressed as picomoles cyclic AMP production per $10^5$ cells per 1 hr (pmol/10$^5$ cells/hr).

**Statistical analysis**

Results are expressed as means ± S.E. Student’s $t$-test was used for statistical analyses. P values of less 0.05 were considered to indicate a significant difference.

**RESULTS**

**Effect of extracellular ATP on steroidogenesis**

The effect of incubation time on ATP-induced steroidogenesis was examined. As shown in Fig. 1, $10^{-4}$ M ATP induced steroidogenesis time-dependently at least until 60 min in the presence of extracellular Ca$^{2+}$ (1.2 mM). Because of this, we used a 60-min incubation in the following experiments.

The effect of extracellular Ca$^{2+}$ on ATP-induced steroidogenesis was examined. The ATP-induced steroidogenesis did not appear in the absence of extracellular Ca$^{2+}$, and the ATP-induced steroidogenesis was dependent on the extracellular calcium concentration. Because the maximum response was obtained above 1.2 mM CaCl$_2$ (Fig. 2), our experiments were performed in the presence of 1.2 mM extracellular Ca$^{2+}$.

**Effect of calmodulin inhibitors on ATP-induced steroidogenesis**

The effect of trifluoperazine and W-7 (calmodulin in-
hibitors) on ATP-induced steroidogenesis was examined. In the presence of extracellular Ca\(^{2+}\), both trifluoperazine and W-7 blocked the ATP-induced steroidogenesis dose-dependently, and the maximum inhibiting effect was obtained by 50 \(\mu\)M (Fig. 3). These effects resembled each other in inhibiting pattern.

**Effect of calcium channel blockers on ATP-induced steroidogenesis**

The effect of nicardipine and (+)PN200-110 (dihydropyridines calcium channel blockers) on ATP-induced steroidogenesis was examined. As shown in Figs. 4 and 5, both nicardipine and (+)PN200-110 did not block...
the ATP-induced steroidogenesis in contrast to 30 mM KCl-induced steroidogenesis. High potassium concentration potassium steroidogenesis by causing a calcium influx via voltage-operated (L-type) calcium channels (14).

Effect of ATP on $[Ca^{2+}]_i$

ATP (above $10^{-6}$ M) caused a rapid elevation of $[Ca^{2+}]_i$ from the quiescent level to a peak level within 2–5 sec in the presence of extracellular Ca$^{2+}$ in the suspended bovine adrenocortical fasciculata cells; and then, $[Ca^{2+}]_i$ gradually declined to a steady state. The peak increase in $[Ca^{2+}]_i$ caused by ATP was dose-dependent (Fig. 6).

Effect of ATP on cyclic AMP

The relationship between ATP-induced steroidogenesis and cyclic AMP production was examined. The ATP-induced steroidogenesis was dose-dependent, but the cyclic AMP production induced by ATP was not (Table 1). The cyclic AMP production by less than $10^{-3}$ M ATP was not significantly different from the control group, but that by $10^{-4}$ M was significantly different. The difference between $10^{-5}$ M and $10^{-6}$ M is significant in the steroidogenesis, but not in the cyclic AMP production. This indicates that the ATP-induced steroidogenesis did not correspond to the cyclic AMP production.

DISCUSSION

It is known that ATP is released from platelets, neurons, the cells of the adrenal medulla, and so on (1); and blood plasma ATP levels are augmented in various conditions such as shock (1) and hemostasis (15). If P2-purinoceptors exist in bovine adrenocortical fasciculata cells and ATP induces steroidogenesis, it is an appropriate system on protecting the body against shock.

Purinoceptors have been divided into P1- and P2-subtypes on the bases of agonist potency order and functional response (1). In general, ATP induces various responses via P2-purinoceptors. Furthermore, according to the actions, the rank order of agonist potency and the effects of antagonists, P2-purinoceptors were first subdivided into at least two more subtypes, P2X and P2Y (2–5); and further observations provided evidence for at least four subtypes [P2X, P2Y, P2Z, P2T] (16, 17). Modulation of $[Ca^{2+}]_i$ is an important pathway for regulation of the ATP-induced physiological processes via purinoceptors (7–10).

We previously reported that P2-purinoceptor agonists

| Concentration | Cortisol production (pmol/10^6 cells/hr) | Cyclic AMP production (pmol/10^6 cells/hr) |
|---------------|----------------------------------------|------------------------------------------|
| Control       | 3.12 ± 1.13                            | 106.69 ± 16.08                           |
| ATP $10^{-7}$ M | 8.68 ± 2.78                            | 76.83 ± 3.78                             |
| ATP $10^{-6}$ M | 20.06 ± 6.72                            | 90.88 ± 10.99                            |
| ATP $10^{-5}$ M | 111.72 ± 14.75                          | 118.92 ± 10.14                           |
| ATP $10^{-4}$ M | 216.47 ± 6.29                           | 253.05 ± 21.06**                         |

The 3-day cultured cells were stimulated by a variety of extracellular ATP concentrations in the presence of 1.2 mM CaCl$_2$. The cyclic AMP production was observed. Results are shown as the mean ± S.E. of 2 separate experiments. The difference in the cyclic AMP production between $10^{-6}$ M ATP and $10^{-5}$ M ATP is significant (***P < 0.01).
stimulated steroidogenesis in the presence of extracellular Ca\(^{2+}\) in bovine adrenocortical fasciculata cells (6). It was considered that ATP-induced steroidogenesis had a close connection to ATP-induced calcium mobilization. The present studies were performed to determine if ATP-induced steroidogenesis could be caused by calcium mobilization or not.

Extracellular ATP (10\(^{-4}\) M) induced time-dependent steroidogenesis in the presence of extracellular Ca\(^{2+}\). ATP-induced steroidogenesis was dose-dependent as described previously (6). The ATP-induced steroidogenesis was detected only in the presence of extracellular Ca\(^{2+}\). The effect of extracellular Ca\(^{2+}\) was dose-dependent and the maximum response was obtained at CaCl\(_2\) concentrations above 1.2 mM. Trifluoperazine and W-7, calmodulin inhibitors, inhibited the ATP-induced steroidogenesis in the presence of extracellular Ca\(^{2+}\). However, as the incubation medium was used to measure the cortisol production in this study, it was possible that the calmodulin inhibitors could affect not only the cortisol production but also the cortisol secretion. It was reported that the steroid stimulated by ACTH might diffuse through the cytoplasm and across the cell membrane (18). In experiments using isolated adrenocortical cells, calmodulin inhibitors completely inhibited the ACTH-induced steroidogenesis (19). In the experiments using the isolated adrenocortical cells, the incubation mixture (both the incubation medium and the adrenocortical cells) was used to measure the cortisol production. It was considered that if the calmodulin inhibitors had an inhibitory effect on the cortisol secretion, they probably could not inhibit steroidogenesis completely. Thus, it is unlikely that the calcium-calmodulin system could have an effect on the cortisol secretion. My results suggested that the presence of extracellular Ca\(^{2+}\) was essential for ATP to induce steroidogenesis, and ATP might enhance Ca\(^{2+}\) influx into the cell and stimulate steroidogenesis via the calcium-calmodulin system. This possibility was supported by the experiment using the fura 2/AM loaded cells (Fig. 6). Matsui also reported that extracellular ATP caused an elevation of [Ca\(^{2+}\)], in bovine adrenocortical fasciculata cells (11). As described previously, voltage-operated calcium channels exist in bovine adrenocortical fasciculata cells, and a high concentration of potassium in the incubation mixture induced steroidogenesis by causing a calcium influx (14). Though nicardipine and (+)PN200-110, dihydropyridines calcium channel blockers, inhibited the 30 mM KCl-induced steroidogenesis, they did not abolish the ATP-induced steroidogenesis in bovine adrenocortical fasciculata cells. These results suggest that extracellular ATP causes the calcium influx via non-selective calcium channels and ATP induces the elevation of [Ca\(^{2+}\)]. Considering that the effect on ATP is connected to calcium mobilization, the present results indicate that these effects are induced via P2-purinoceptors. Since it is known that the increase of cyclic AMP induces steroidogenesis in bovine adrenocortical fasciculata cells (20), there is a possibility that extracellular ATP causes the increase of cyclic AMP in bovine adrenocortical fasciculata cells. However, cyclic AMP increased only at the high ATP concentration (10\(^{-4}\) M). Furthermore, contrasting the increase of cyclic AMP with the steroidogenesis, it is likely that ATP-induced steroidogenesis has no connection to the increase of cyclic AMP. Furthermore, adenosine (10\(^{-4}\) M) induced a remarkable increase of cyclic AMP in bovine adrenocortical fasciculata cells (data not shown). It is well-known that adenosine has the effect on cyclic AMP production via P1-purinoceptors (4, 16). Thus it is considered that ATP is metabolized to adenosine by ectonucleotidases during the incubation period. Therefore, the increase of cyclic AMP by ATP under our experimental conditions might involve the metabolite adenosine, via low-affinity P1-purinoceptors in bovine adrenocortical fasciculata cells. It is reported that P2Y-purinoceptors have a close connection with inositol phosphate metabolism (8). Therefore, further investigations on the connection between ATP and inositol phosphate metabolism should be performed in bovine adrenocortical fasciculata cells.

**Acknowledgments**

The author thanks Prof. Masahiro Kawamura (Department of Pharmacology(I), The Jikei University School of Medicine) for his encouragement during this study.

**REFERENCES**

1. Gordon, J.L.: Extracellular ATP: effects, sources and fate. Biochem. J. 233, 309 – 319 (1986)
2. Burnstock, G.: A basis distinguishing two types of purinergic receptor. In Cell Membrane Receptors for Drugs and Hormones. Edited by Straub, R.W. and Bolis, L., p. 107 – 118, Raven Press, New York (1978)
3. Burnstock, G. and Kennedy, C.: Is there a basis for distinguishing two types of P2-purinoceptor? Gen. Pharmacol. 16, 433 – 440 (1985)
4. Kennedy, C.: P1- and P2-purinoceptor subtypes – an update. Arch. Int. Pharmacodyn. Ther. 303, 30 – 50 (1990)
5. Caswell, A.M., Leong, W.S. and Russell, R.G.G.: Evidence for the presence of P2-purinoceptors at the surface of human articular chondrocytes in monolayer culture. Biochem. Biophys. Acta 1074, 151 – 158 (1991)
6. Kawamura, M., Matsu, T., Nitsu, A., Kondo, T., Ohno, Y. and Nakamichi, N.: Extracellular ATP stimulates steroidogenesis in bovine adrenocortical fasciculata cells via P1
purinoceptors. Japan. J. Pharmacol. 56, 543–545 (1991)
7 Carter, T.D., Hallam, T.J., Cusack, N.J. and Pearson, J.D.: Regulation of P_2Y-purinoceptor-mediated prostacyclin release from human endothelial cells by cytoplasmic calcium concentration. Br. J. Pharmacol. 95, 1181–1190 (1988)
8 Sasakawa, N., Nakaki, T., Yamamoto, S. and Kato, R.: Stimulation by ATP of inositol trisphosphate accumulation and calcium mobilization in cultured endothelial cells. J. Neurochem. 52, 441–447 (1989)
9 Benham, C.D.: ATP-activated channels gate calcium entry in single smooth muscle cells dissociated from rabbit ear artery. J. Physiol. (Lond.) 419, 689–701 (1989)
10 Soitoff, S.P., McMillian, M.K., Lechleiter, J.D., Cantley, L.C. and Talamo, B.R.: Elevation of [Ca^{2+}]_i and the activation of ion channels and fluxes by extracellular ATP and phospholipase C-linked agonists in rat parotid acinar cells. Ann. N.Y. Acad. Sci. 603, 76–90 (1990)
11 Matsui, T.: Biphasic rise caused by extracellular ATP in intracellular calcium concentration in bovine adrenocortical fasciculate cells. Biochem. Biophys. Res. Commun. 178, 1266–1272 (1991)
12 Silber, R.H., Busch, R.D. and Oslaps, R.: Practical procedure for estimation of corticosterone or hydrocortisone. Clin. Chim. 4, 278–285 (1958)
13 Grynkiewicz, G., Poenie, M. and Tsien, R.Y.: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. 260, 3440–3450 (1985)
14 Yanagibashi, K., Kawamura, M. and Hall, P.F.: Voltage-dependent Ca^{2+} channels are involved in regulation of steroid synthesis by bovine but not rat fasciculata cells. Endocrinology 127, 311–318 (1990)
15 Born, G.V.R. and Kratzer, M.A.A.: Source and concentration of extracellular adenosine triphosphate during haemostasis in rats, rabbits and man. J. Physiol. (Lond.) 354, 419–429 (1984)
16 Burnstock, G.: Purinergic mechanisms. Ann. N.Y. Acad. Sci. 603, 1–17 (1990)
17 Cusack, N.J. and Hourani, S.M.O.: Subtypes of P_2-purinoceptors studies using analogues of ATP. Ann. N.Y. Acad. Sci. 603, 172–181 (1990)
18 Bloodworth, J.M.B. and Powers K.L.: The ultrastructure of the normal dog adrenal. J. Anat. 102, 457–476 (1968)
19 Sekimoto, T., Nakamichi, N., Hanyu, J., Uzawa, S., Amamoto, T., Yonezawa, Y., Tsurui, M., Ishiwatari, S., Imagawa, N., Tanaka, Y., Tomita, C. and Matsuba, M.: A possible regulatory role of Ca^{2+}-calmodulin system in cellular cholesterol ester hydrolysis in the steroidogenic response to ACTH in bovine adrenocortical cells. Endocrinol. Japon. 30, 199–204 (1983)
20 Schimmer, B.P.: Cyclic nucleotides in hormonal regulation of adrenocortical function. Adv. Cyclic Nucleotide Res. 13, 181–214 (1980)