Effect of Adrenaline on Steroidogenesis in Primary Cultured Bovine Adrenocortical Cells

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Abstract—In primary 2-day cultured bovine adrenocortical cells, adrenaline stimulated the steroidogenesis, while the effect of adrenaline did not appear in the freshly isolated cells. Thus the primary 2-day cultured cells were used to study the effect of adrenaline on steroidogenesis. Adrenaline showed the steroidogenesis-stimulating effect at concentrations higher than $10^{-9}$ M, and the maximum effect was obtained between $10^{-6}$ M and $10^{-5}$ M in the primary 2-day cultured cells. The maximum effect of adrenaline was 50–70% of that of adrenocorticotropic hormone (ACTH). Noradrenaline, isoproterenol and phenylephrine also stimulated the steroidogenesis. However, the order of the potency was isoproterenol $=$ adrenaline $=$ noradrenaline $>$ phenylephrine. Propranolol and alprenolol inhibited the effect of adrenaline, but phenoxybenzamine and phentolamine did not inhibit the effect. Moreover, adrenaline stimulated the cyclic AMP production dose-dependently at concentrations higher than $10^{-8}$ M. These results suggest that there are steroidogenesis-linked adrenergic receptors in primary 2-day cultured bovine adrenocortical cell membrane and that the steroidogenesis-stimulating effect of adrenaline occurs through the $\beta$-adrenergic receptor.

Under stressful conditions, not only ACTH but also adrenaline, which secretes from the adrenal medulla, increase in the blood flow. From this point of view, the participation of adrenaline in adrenocortical function has been proposed. As shown by Naumenko (1) and Inaba et al. (2) in vivo, there is a possibility that adrenocortical function is affected by systemically circulating adrenaline via the hypothalamo-pituitary-adrenocortical axis. The stimulative effect of adrenaline on ACTH secretion was also suggested by Giguère and Labrie (3) using cultured rat anterior pituitary cells. On the other hand, the possibility of the direct steroidogenesis stimulative effect of adrenaline on adrenocortical cells was reported using hypophysectomized dogs (4) and rats (5). However, some workers have reported the lack of steroidogenic activity of adrenaline in vivo (6, 7) and in vitro (8). Therefore, it has not yet been established whether or not adrenaline has some direct stimulative effect on adrenocortical steroidogenesis. To clarify this situation, we have been conducting experiments on the steroidogenic effect of adrenaline in primary cultured bovine adrenocortical cells, and in this report we propose that there are steroidogenesis-linked adrenergic receptors in primary cultured bovine adrenocortical cells.

Materials and Methods

Materials
Synthetic ACTH$_{1-24}$ was donated by Daiichi Seiyaku Co. Ltd.; l-adrenaline bitartrate, dl-isoproterenol hydrochloride and ethylene glycol bis (l-aminooxylylether)-N,N,N',N'-tetraacetic acid (EGTA) were obtained from Nakarai Chemicals Ltd.; phenoxybenzamine hydrochloride from Tokyo Chemical Industry Co. Ltd.; phentolamine mesylate (Regitin) from CIBA; dl-propranolol hydrochloride, l-alprenolol-d-tartrate, (l)-noradrenaline bitartrate and l-
phenylephrine hydrochloride from Sigma Chemical Co.; trypsin from Difco Laboratories; and bovine serum albumin (fraction V) (BSA) from Armour Pharmaceutical. Ham’s F-10 was obtained from Gibco Laboratories, and sera were obtained from Flow Laboratory. The cyclic AMP $^{[125I]}$ RIA kit was purchased from New England Nuclear.

Methods

1. Cell culture: The isolated bovine adrenocortical cells were prepared by the trypsin digestion method (9) under aseptic conditions. The isolated cells were washed once with the Ham’s F-10 medium containing 5% fetal calf serum, 10% newborn calf serum, 2.5% horse serum, 100 IU/ml penicillin, 100 $\mu$g/ml streptomycin, and 50 $\mu$g/ml gentamicin. The washed cells were suspended to a cell density of about 4x$10^5$ cells/ml in the above culture medium. The cells were seeded in 2 cm$^2$ multiwells at a cell density of 8-9x$10^4$ cells/ml and were cultured at 37°C in a humidified atmosphere of 5% CO$_2$ in air. Three hours after the cell-seeding, the medium was changed to the Ham’s F-10 medium without gentamicin. The culture medium was renewed every 2 days during primary culture when the cells were cultured more than 2 days.

2. Assay of steroidogenesis: The adherent cultured bovine adrenocortical cells were washed twice with Ca$^{++}$-free, phosphate-buffered saline containing 0.5 mM EGTA, followed by washing once with Krebs-Ringer-bicarbonate buffer, pH 7.4, which contained 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$^{++}$-free KRBGA). The washed cells were incubated at 37°C for 1 hr in the 1.2 mM CaCl$_2$-containing, Ca$^{++}$-free KRBGA (pH 7.4) in a total incubation volume of 1 ml in a humidified atmosphere of 5% CO$_2$ in air. At the end of the incubation, 0.5 ml of each incubation medium was removed for the corticosteroid assay. Then the cells were detached for a 10 min incubation period at 37°C in 0.1% trypsin containing Ca$^{++}$-free KRBGA to count the cell number by a hemocytometer. Assay of corticosteroid was carried out fluorometrically by the method of Silber et al. (10) using cortisol as a standard.

3. Assay of cyclic AMP: The washed cells were incubated for 30 min at 37°C in 1.2 mM CaCl$_2$-containing, Ca$^{++}$-free KRBGA in a total incubation volume of 0.5 ml. The total cyclic AMP was determined by radio-immunoassay (11) using a commercially obtained RIA kit.

Results

1. Effect of adrenaline and ACTH on the steroidogenesis: The steroidogenic response to adrenaline and ACTH on primary cultured bovine adrenocortical cells was examined every 24 hr after the cell-seeding up to day 5 (Fig. 1). In the freshly isolated cells, $10^{-5}$ M adrenaline did not stimulate cortisol production after 1 hr exposure of the cells, while $10^{-9}$ M ACTH showed a potent stimulating effect on the steroidogenesis. However, during primary culture, the cells began to have a steroidogenic response to adrenaline, and the effect of adrenaline reached its

![Fig. 1. Effect of adrenaline and ACTH on the steroidogenesis in primary cultured bovine adrenocortical cells. The incubations were carried out every 24 hr after the cell-seeding in the absence (O-O) and presence of $10^{-5}$ M adrenaline ( ) and $10^{-9}$ M ACTH ( ) for 1 hr at 37°C in a humidified atmosphere of 5% CO$_2$ in air. Each point represents the mean±S.E. of the values from three wells.](image-url)
maximum on day 2, as it did for ACTH. The steroidogenic effect of 10^{-6} M adrenaline in the primary 2-day cultured cells was about 50-70% that of ACTH. From the results, the primary 2-day cultured cells were used in the following experiments.

In the primary 2-day cultured cells, 10^{-6} M adrenaline and 10^{-9} M ACTH stimulated cortisol production time-dependently, at least up to 1 hr incubation (data not shown). Thus a 1 hr incubation was chosen as a suitable incubation period. Under these conditions, adrenaline (10^{-7} M) and ACTH (10^{-12} M and 10^{-9} M) did not show an additive or a synergistic effect on the steroidogenesis (Fig. 2).

2. Effect of adrenergic drugs on the steroidogenesis: The steroidogenic effect of adrenaline and other adrenergic drugs was examined in primary 2-day cultured bovine adrenocortical cells. As shown in Fig. 3, adrenaline, noradrenaline and isoproterenol had a dose-dependent stimulating effect on cortisol production. Though noradrenaline had a steroidogenic effect as potent as that of adrenaline, the effect of isoproterenol was more potent than that of adrenaline. The ED50 values of adrenaline, noradrenaline and isoproterenol were approximately 4 \times 10^{-8} M, 10^{-7} M and 10^{-11} M, respectively.

On the other hand, as shown in Table 1, phenylephrine also had a weak but obvious steroidogenic effect in the primary 2-day cultured cells.

3. Effect of adrenergic blocking agents on adrenaline-induced steroidogenesis: Effects of propranolol, phenoxybenzamine and phentolamine on 10^{-5} M adrenaline-induced cortisol production in the primary 2-day cultured cells were examined (Fig. 4). Phentolamine or phenoxybenzamine at 10^{-8}-10^{-6} M did not inhibit 10^{-5} M adrenaline-induced cortisol production, while the same dose of propranolol could inhibit the effect of adrenaline, and 10^{-6} M propranolol

Fig. 2. Effect of adrenaline on ACTH-induced steroidogenesis in primary 2-day cultured bovine adrenocortical cells. The incubations were carried out for 1 hr at 37°C in a humidified atmosphere of 5% CO₂ in air in the presence and absence of 10^{-7} M adrenaline and 10^{-12} M or 10^{-9} M ACTH. Hatched columns show the presence of both agonists in the incubation medium. The number of the incubated cells was 10X10^4 cells/well. Each value represents the mean±S.E. of the values from three wells.

Fig. 3. Effect of adrenaline, noradrenaline and isoproterenol on steroidogenesis in primary 2-day cultured bovine adrenocortical cells. The incubations were carried out for 1 hr at 37°C in a humidified atmosphere of 5% CO₂ in air in the presence of several concentrations of adrenaline (O ----O), noradrenaline (●—●) and isoproterenol (△—△). The number of the incubated cells was 17.4X10^4 cells/well. Each point represents the mean of the values from three wells.
arrested the effect of 10^{-5} M adrenaline completely. Propranolol at 10^{-6} M did not have an effect on 10^{-9} M ACTH-induced cortisol production (Fig. 5). Propranol at 5 \times 10^{-8} M shifted the dose-response curve to adrenaline on cortisol production to the right. However, the maximum cortisol production rate by adrenaline did not change even in the presence of the blocking agent (Fig. 6). These suggest that there are steroidogenesis-linked \(\alpha\)- and \(\beta\)-adrenergic receptors in

### Table 1. Effect of adrenaline and phenylephrine on steroidogenesis in primary 2-day cultured bovine adrenocortical cells

| Agonist (M) | Cortisol production* (pmol/10^4 cells/hr) |
|-------------|-----------------------------------------|
| ---         | ---                                     |
| 10^{-8}     | 40.6 ± 2.2                              |
| 10^{-7}     | 379.0 ± 14.0                            |
| 10^{-6}     | 620.7 ± 11.0                            |
| ---         | 809.9 ± 10.9                            |
| 10^{-4}     | 142.0 ± 12.6                            |
| 10^{-3}     | 150.8 ± 12.6                            |
| ---         | 217.0 ± 6.7                             |

The cultured cells were incubated for 1 hr at 37°C in the presence and absence of adrenaline and phenylephrine as indicated in the Table. *Values represent the mean±S.E. of the values from three wells.

**Fig. 4.** Effect of adrenergic blocking agents on adrenaline-induced steroidogenesis in primary 2-day cultured bovine adrenocortical cells. The incubations were carried out in the presence of several concentrations of propranolol (○ --- ○), phenoxybenzamine (● --- ●) and phentolamine (△ --- △) with 10^{-5} M adrenaline for 1 hr at 37°C in a humidified atmosphere of 5% CO\(_2\) in air. [--- ---]: In the absence of adrenaline. The number of the incubated cells was 16.9 \times 10^4 cells/well. Each point represents the mean of the values from two wells.

**Fig. 5.** Effect of propranolol on adrenaline- and ACTH-induced steroidogenesis in primary 2-day cultured bovine adrenocortical cells. The incubations were carried out in the absence (open columns) and presence (hatched columns) of 10^{-6} M propranolol with or without (A) 10^{-6} M adrenaline (B) and 10^{-9} M ACTH (C) for 1 hr at 37°C in a humidified atmosphere of 5% CO\(_2\) in air. The number of the incubated cells was 10\times 10^4 cells/well. Each value represents the mean±S.E. of the values from four wells.
primary 2-day cultured bovine adrenocortical cells and that the effect of adrenaline on steroidogenesis is revealed mainly via the β-receptor.

Furthermore as shown in Fig. 7, propranolol and alprenolol had an inhibitory effect on 10⁻⁶ M adrenaline-induced steroidogenesis, and the effect of alprenolol was as potent as that of propranolol. This indicates the possibility that the subtype of the adrenocortical β-receptor involved in the effect is β₁.

4. Effect of adrenaline on the cyclic AMP production: As shown in Table 2, adrenaline

![Fig. 6](image1.png)

**Fig. 6.** Effect of propranolol on adrenaline-induced steroidogenesis in primary 2-day cultured bovine adrenocortical cells. The incubations were carried out in the absence (○—○) and presence (●—●) of 5X10⁻⁸ M propranolol with several concentrations of adrenaline for 1 hr at 37°C in a humidified atmosphere of 5% CO₂ in air. The number of the incubated cells was 15x10⁴ cells/well. Each point represents the mean±S.E. of the values from three wells.

![Fig. 7](image2.png)

**Fig. 7.** Effect of propranolol and alprenolol on adrenaline-induced steroidogenesis in primary 2-day cultured bovine adrenocortical cells. The incubations were carried out in the presence of various concentrations of propranolol (○—○) and alprenolol (●—●) with 10⁻⁶ M adrenaline for 1 hr at 37°C in a humidified atmosphere of 5% CO₂ in air. Δ: Control value. The number of the incubated cells was 17.4X10⁴ cells/well. Each point represents the mean±S.E. of the values from three wells.

| Adrenaline (M) | Cortisol production* (pmol/10⁶ cells/hr) | Cyclic AMP production* (pmol/10⁶ cells/hr) |
|----------------|------------------------------------------|-------------------------------------------|
|                |                                          |                                           |
| —              | 330.8±48.6                               | 7.5±0.2                                   |
| 10⁻⁸           | 424.8±65.2                               | 10.8±1.8                                  |
| 10⁻⁷           | 1032.0±59.7                               | 11.5±0.6                                  |
| 10⁻⁶           | 1143.8±51.5                               | 26.4±2.1                                  |

The cultured cells were incubated for 30 min at 37°C in the presence of adrenaline as indicated in the Table, and cortisol was determined fluorometrically (10) and total cyclic AMP was determined by radioimmunoassay (11). *Values represent the mean±S.E. of the values from three wells. a): P<0.5, b): P<0.01.
(10^{-8}–10^{-6} \text{ M}) enhanced the total cyclic AMP production dose-dependently as in the case of cortisol production in the primary 2-day cultured cells. It is well known that the \( \beta \)-receptor in various organs is linked to adenylate cyclase to increase intracellular cyclic AMP (12). Thus the result may also indicate that the steroidogenic effect of adrenaline is revealed via the \( \beta \)-receptor in primary cultured bovine adrenocortical cells.

**Discussion**

The effect of adrenaline on the steroidogenesis in primary cultured bovine adrenocortical cells was studied. In the primary 2-day cultured cells, adrenaline accelerated cortisol production dose-dependently, while the maximum effect of adrenaline was less than that of ACTH. Our results suggest the existence of steroidogenesis-linked \( \alpha \)- and \( \beta \)-adrenergic receptors, which differ from those of ACTH, in primary cultured bovine adrenocortical cell membrane. However, the steroidogenic effect of adrenaline might be shown via the \( \beta \)-adrenergic receptor, and the effect occurs through the enhancement of intracellular cyclic AMP.

In freshly isolated bovine adrenocortical cells, adrenaline did not have any effect on steroidogenesis, while ACTH showed a potent steroidogenesis stimulating effect. However, the cells began to have a good response to adrenaline and ACTH during primary culture, and the response to adrenaline and ACTH of the cells reached the maximum on day 2. The result also confirms the existence of different receptors from those of ACTH in primary cultured bovine adrenocortical cell membrane. Although the change of the steroidogenic response to ACTH during primary culture will be discussed elsewhere (manuscript in preparation), it is very interesting to note that adrenaline had no effect on the steroidogenesis in the freshly isolated cells.

The lack of steroidogenic activity of adrenaline in the freshly isolated cells might be caused by the trypsin digestion method which we used to isolate the cells. Although, in the case of ACTH, Malamed et al. (13) reported that the isolation procedure of rat adrenal cells by the trypsin digestion method did not show any significant effect on the electronmicroscopic morphology and the steroidogenic effect of ACTH in the cells. However, Stiles and Lefkowits (14) indicated that trypsin had an effect on some components of the hormone sensitive adenylate cyclase system in human platelet membrane. On the other hand, there is a possibility that there are no adrenergic receptors in adrenocortical cell membrane in vivo, and the receptors are synthesized during primary culture as are those in rat hepatocytes (15). The latter was suggested by the report which showed the lack of steroidogenic activity of adrenaline on perfused, isolated calf adrenal gland (8). However, as it was indicated by the author, the negative result probably resulted from the low concentration of adrenaline in the perfusate. It was reported that exogenous adrenaline could increase plasma corticosterone level in hypophysectomized rats (5); and quite recently, Shima et al. (16) suggested the existence of \( \beta \)-adrenergic receptors in rat adrenal tissue. Therefore, it would seem that the former possibility is more likely.

There appear to be two possibilities for interpreting our observations: 1) Trypsin destroys the adrenaline receptor on the bovine adrenocortical cell membrane surface, and 2) Trypsin inhibits \( \beta \)-adrenergic receptor-linked guanine nucleotide regulatory units and/or the catalytic units of the adenylate cyclase. Since \( 10^{-9} \) M ACTH shows enough steroidogenic activity in freshly isolated bovine adrenocortical cells, the possibility for the effect of trypsin on the catalytic unit of adenylate cyclase may be ruled out. More investigations are necessary to clarify the phenomenon.

Although the existence of a steroidogenesis-linked \( \beta \)-adrenergic receptor in primary 2-day cultured bovine adrenocortical cells was indicated, further investigation is necessary to know whether or not adrenaline has a physiological role in the regulation of adrenocortical function; this is because the effective dose of adrenaline was higher than the physiological concentration in the blood flow, and adrenaline did not show an additive or a synergistic effect on the ACTH action. However, as it was reported by Inaba and...
Kamata (5), there is some possibility that adrenaline, under stressful conditions, has some compensatory effect on the steroidogenesis in the adrenal cortex if ACTH secretion is damaged.

Finally, primary cultured bovine adrenocortical cells will be an interesting tool to study the β-adrenergic receptor and β-adrenegic response because the end-product of the reaction is easily assayable corticosteroid.

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