Action of Partially Purified ACTH-Potentiating Substance from Rat Serum on Isolated Rat Adrenal Cells

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Abstract—A serum extract possessing ACTH-potentiating activity was partially purified by Sephadex G-100 gel filtration. Fractions from 9,000 to 40,000 in molecular weight (APS-Fr) potentiated the corticosterone production induced by synthetic adrenocorticotropic hormone (ACTH1-24). Approximately 5 μg (as protein) of APS-Fr in 0.5 ml medium showed the maximum activity to potentiate the response of isolated rat adrenal cells to ACTH1-24. Puromycin, cycloheximide and actinomycin D did not abolish the potentiation by APS-Fr of ACTH1-24-induced steroidogenesis. The log-dose response curve for ACTH1-24 was shifted toward the side representing lower doses of ACTH1-24 by APS-Fr, but the maximum response was not changed. In a typical experiment, the median effective dose (ED50) for ACTH1-24 was decreased from $5.2 \times 10^{-11}$ M to $3.0 \times 10^{-12}$ M. ACTH1-24-induced production of cyclic AMP was also increased by APS-Fr. Time courses of both corticosterone and cyclic AMP production induced by ACTH1-24 did not differ qualitatively in either the presence or absence of APS-Fr, although they showed a marked difference quantitatively. When dibutyryl cyclic AMP was used in place of ACTH1-24, APS-Fr did not potentiate steroidogenesis. These results suggest that APS-Fr acts at a step between the binding of ACTH to its receptor and the formation of cyclic AMP. ACTH1-24-induced steroidogenesis which was increased by increasing concentration of calcium in the medium, was further increased by addition of APS-Fr. The calcium requirement for the same degree of response was decreased by addition of APS-Fr, in a similar way to when a higher concentration of ACTH1-24 was used.

The trypsin digest of 16K fragment, the amino-terminal region of proopiomelanocortin, has a potentiating effect on the action of ACTH1-24 in isolated adrenal cells (1). Synthetic $\gamma_3$-melanotropin (MSH), representing a portion of the 16K fragment, potentiates ACTH-induced corticosterone and aldosterone biosynthesis in vivo in the hypophysectomized rat (2). These peptides have been shown to stimulate the activity of cholesterol ester hydrolase in the adrenal cortex (1, 2). A naturally occurring human pro-$\gamma$-MSH glycopeptide potentiates the ACTH-induced steroidogenic response of isolated perfused rat and human adrenocortical cells (3). This potentiation has been suggested to be due to stimulation of mRNA synthesis (4).

The extract obtained from ACTH-free rat serum by the QUSO G-32 adsorption method also potentiates ACTH1-24-induced steroidogenesis in isolated rat adrenal cells (5). Most of the ACTH-potentiating activity in the serum extract is found in the fractions between 9,000 and 40,000 in molecular weight (APS-Fr) on Sephadex G-100 gel filtration, and a small portion of the activity is found in the range of lower molecular weights. The potentiating activity in APS-Fr is markedly decreased by hypophysectomy, accompanying a quantitative decrease of some of the peptides which are revealed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of the fractions (5).
The mechanism of action of these ACTH-potentiating substances still remains to be elucidated, although various mechanisms have been proposed as described above. In the present study, possible sites of the action of APS-Fr on rat adrenal cells were examined.

Materials and Methods

Male adult rats of the Donryu strain were used. The animals were decapitated, and trunk blood was collected at room temperature. The adrenals were used for the preparation of isolated adrenal cells. The blood was centrifuged at 4°C to obtain the serum, which was then stored in a freezer at −12°C until use. Remaining ACTH activity in the serum was eliminated by thawing the serum and leaving it to stand at room temperature before use.

Siliconized glassware, and plastic tubes and pipettes were used throughout the experiments.

Drugs: ACTH1-24 (Cortrosyn) was purchased from Daiichi Pharmaceutical Co., cyclic AMP-Na and dibutyryl cyclic AMP-Na were purchased from Yamasa Shoyu Co., and puromycin dihydrochloride, cycloheximide and actinomycin D were purchased from Sigma Chemical Co.

Assay of ACTH-potentiating activity: Isolated rat adrenal cells were prepared by the method described in the previous paper (5), except that before preparation of the final cell suspension, cell purification was carried out by the method of Goverde et al. (6), and usual Krebs-Ringer bicarbonate glucose solution (2.54 × 10^{-3} M calcium) was used for the final cell suspension. Preparation of the serum extract and gel filtration on Sephadex G-100 were carried out by the methods described previously (5). Since most of the ACTH-potentiating activity in the serum extract was found in the fractions from 9,000 to 40,000 in molecular weight on Sephadex G-100 gel filtration (5), these fractions were combined and used for the experiments. This combined fraction containing ACTH-potentiating substance is referred to as 'APS-Fr' in this paper. Aliquots (50 μl each, corresponding to approx. 5 μg of protein) of APS-Fr were evaporated to dryness in plastic tubes by aeration at 50°C.

The same volumes of the elution buffer (0.5 M ammonium acetate buffer, pH 4.5) were evaporated to dryness as a control. Fifty microliters of ACTH1-24 or dibutyryl cyclic AMP solution and 0.45 ml of the cell suspension were added to the tube containing dried APS-Fr. The mixture was incubated at 38°C for a definite time. The diluent for ACTH1-24 was a solution of 0.5% bovine serum albumin (Fraction V, Armour Pharmaceutical Co.) in 0.9% NaCl, and its pH was adjusted to 3.5 with 0.1 N HCl (7). The diluent for dibutyryl cyclic AMP was 0.9% NaCl. APS-Fr or ACTH1-24 alone was incubated with the cell suspension to confirm the absence of ACTH-like activity in APS-Fr or to estimate the activity of ACTH1-24 (control activity), respectively. Corticosterone produced in the incubate (cells and medium) was determined by a modification (5) of the method of Silber et al. (8).

Inhibitors of potentiation by APS-Fr: Actinomycin D was dissolved in 0.9% NaCl adjusted to pH 3.5 with 0.1 N HCl. Puromycin dihydrochloride was dissolved in 0.9% NaCl and neutralized with 0.154 M NaHCO3. Cycloheximide was dissolved in 0.9% NaCl. Each of these inhibitors in a volume of 40 μl was added to 0.6 ml of the adrenal cell suspension and preincubated for 30 min at 38°C. After the preincubation, 0.48 ml of the incubate was transferred into a tube containing ACTH1-24 in a volume of 20 μl and dried APS-Fr. The incubation was then carried out for 90 min at 38°C.

Assay of cyclic AMP: For determination of cyclic AMP, 50 μl of 1.1 N HCl and 5 μl of 0.5 M ethylenediaminetetraacetic acid (EDTA) were added to the incubate. The mixture was heated for 3 min in a boiling water bath and centrifuged at 3,000 rpm for 15 min at 4°C (9). Cyclic AMP in the supernatant was determined using a YAMASA cyclic AMP kit (Yamasa Shoyu Co.).

Measurement of protein: Protein content in the APS-Fr was measured by the method of Lowry et al. (10). The amount of APS-Fr was expressed in terms of protein content.

Results

The response of rat adrenal cells to ACTH1-24 was different more or less from
one experiment to another. This seems to have been due to seasonal variation, age of rats, unexpected stress to rats and so on. However, potentiation by APS-Fr of the response was observed in all the experiments.

The maximum potentiation of ACTH$_{1-24}$-induced steroidogenesis was found in the presence of 5 μg (as protein) of APS-Fr in 0.5 ml of medium (Fig. 1). On the basis of this result, approx. 5 μg of APS-Fr was used in the subsequent experiments. APS-Fr did not show any ACTH-like activity in the amounts used in this series of experiments.

ACTH$_{1-24}$-induced steroidogenesis was slightly inhibited by $10^{-6}$–$10^{-5}$ M actinomycin D, but the potentiation by APS-Fr of the steroidogenesis was not inhibited at all. Although the steroidogenesis was markedly inhibited by $10^{-4}$ M actinomycin D, the potentiation was not abolished (Fig. 2). Actinomycin D interfered with the corticosterone determination at $10^{-4}$ M as a result of its yellowish color. However, the interference was not enough to distort the result described above.

Puromycin and cycloheximide inhibited ACTH$_{1-24}$-induced steroidogenesis in a concentration-dependent manner. However, the potentiation by APS-Fr was observed as long as the action of ACTH$_{1-24}$ remained; consequently, when the action of ACTH$_{1-24}$ was abolished completely by these inhibitors, no potentiation was observed (Fig. 3).

The log dose response curve for ACTH$_{1-24}$ was shifted toward the side representing lower doses of ACTH$_{1-24}$ by the addition of APS-Fr, but the maximum response was not changed. The result of a typical experiment is shown in Fig. 4. The ED50 for ACTH$_{1-24}$ was approx. $5.2 \times 10^{-11}$ M, but it was decreased to $3.0 \times 10^{-12}$ M by APS-Fr. The affinity of the adrenal cells to ACTH$_{1-24}$ was apparently increased about 17 times by APS-Fr. In the same experiment, production of cyclic AMP was also increased by APS-Fr (Fig. 5).

Figure 6 shows the time courses of production of corticosterone and cyclic AMP induced by two concentrations of ACTH$_{1-24}$. The production of these substances was markedly increased by the addition of APS-Fr. Corticosterone production still continued at 180 min, but cyclic AMP formation reached a maximum within 30 min. These time courses did not differ qualitatively in the presence or absence of APS-Fr, although they showed a marked difference quantitatively.

When dibutyl cyclic AMP was used in place of ACTH$_{1-24}$, the potentiation by APS-Fr of the steroidogenesis was not observed at any concentration of dibutyl cyclic AMP (Fig. 7), and a similar result was obtained with cyclic AMP. It was clear that APS-Fr did not have any activity of potentiation of cyclic AMP-induced steroidogenesis.

Figure 8 shows the relationship between concentrations of calcium in the medium and the response of adrenal cells to ACTH$_{1-24}$ in the presence or absence of APS-Fr. ACTH$_{1-24}$-induced steroidogenesis was increased by increasing the concentration of
calcium in the medium. The addition of APS-Fr further increased the response of the cells. The calcium requirement for the same degree of response was decreased when APS-Fr was added as well as when a higher concentration of ACTH_1-24 was used.

**Discussion**

The finding that pro-α-MSH peptides potentiate ACTH-induced steroidogenesis in adrenal cells has been confirmed by several investigators (1-4). APS-Fr also potentiates such steroidogenesis as shown in the previous paper (5), although it is not known whether APS-Fr is a pro-α-MSH peptide.

Immunoreactive pro-α-MSH peptides or immunoreactive α-MSH is present in the circulation of rats (11-13) and man (14, 15). It has been reported that highly specific antiserum to synthetic rat α_3-MSH peptide eliminates the ACTH-induced rise of cortisol, 18-hydroxycorticosterone and aldosterone in chronically cannulated rats, while it has no effect on basal corticosteroid levels (16). APS-Fr also exists endogenously in the circulation of rats, as it can be obtained from rat serum by extraction. The maximum potentiation of ACTH_1-24-induced corticosterone production was observed with 5 μg of APS-Fr (Fig. 1). This amount of APS-Fr was obtained from about 110 μl of rat serum. As previously reported (5), the serum extract corresponding to about 500 μl of rat serum, showed the maximum potentiation. This difference in the volumes of serum corresponding to the amounts of APS-Fr and the serum extract for producing the maximum potentiation may be mostly due to the elimination of the inhibiting substance by the gel filtration of the serum extract (5). APS-Fr was about five times more purified than the serum extract.

It has been reported that human α-MSH precursor potentiates ACTH-induced steroidogenesis by stimulating mRNA synthesis, the potentiation being completely inhibited by
4 × 10^{-6} M actinomycin D (4). However, in the present experiment, the potentiating action of APS-Fr was not abolished by 10^{-6} to 10^{-4} M actinomycin D (Fig. 2). Puromycin and cycloheximide inhibited both ACTH_1-24-induced steroidogenesis and its potentiation by APS-Fr, but the degree of inhibition was almost always the same in the presence or absence of APS-Fr, no abolition of potentiation being observed (Fig. 3). Therefore, it appeared that APS-Fr potentiation of ACTH_1-24-induced steroidogenesis did not occur by stimulation of either RNA synthesis or protein synthesis.

Synthetic bovine Lys-γ3-MSH has been found to potentiate the steroidogenic action of ACTH during incubation of rat adrenal cells in vitro (17). On the other hand, Lys-γ3-MSH did not increase ACTH-induced production of cyclic AMP or phosphatidyl-inositol (17). This finding with regard to Lys-γ3-MSH differs from the results obtained for APS-Fr. APS-Fr potentiated not only ACTH_1-24-induced steroidogenesis but also ACTH_1-24-induced production of cyclic AMP (Figs. 4, 5 and 6). As shown in Fig. 4, as a result of the presence of APS-Fr, the log-dose response curve for ACTH_1-24 was shifted toward the side representing lower doses of ACTH_1-24. Comparison of ED50 values for ACTH_1-24 in the presence or absence of APS-Fr revealed that the affinity of the cells to ACTH_1-24 was increased about 17 times by the addition of APS-Fr. However, the maximum response of the cells to ACTH_1-24 was not changed by the addition of APS-Fr. This result indicates that when a saturated concentration of ACTH_1-24 was used for the cells, APS-Fr was no longer effective. These findings suggest that APS-Fr potentiates the response of the cells to ACTH_1-24 by increasing the affinity of the cells to ACTH_1-24.

In this experiment, a remarkable increase of cyclic AMP was also observed in the presence
Fig. 4. Effect of APS-Fr on corticosterone production induced by various concentrations of ACTH$_{1-24}$ in isolated rat adrenal cells. Fifty microliters (5 μg as protein) of APS-Fr or 50 μl of the elution buffer was evaporated to dryness. Each concentration of ACTH$_{1-24}$ and 9.5×10⁴ cells were added to the dried sample to make a total volume of 0.5 ml. The mixture was incubated for 180 min at 38°C. Each point represents the mean of values obtained from duplicate incubations. Basal value (0.035 μg/0.5 ml) obtained from the incubation of the cells alone was subtracted from all the values in the figure. The value obtained from incubation of the cells with APS-Fr was almost the same as the basal value. ED50 was estimated from the figure.

Fig. 5. Effect of APS-Fr on production of cyclic AMP induced by various concentrations of ACTH$_{1-24}$ in isolated rat adrenal cells. All the experimental conditions were the same as those of the experiment shown in Fig. 4. Each point represents the mean of values obtained from duplicate incubations. Basal value (0.2 pmol/0.5 ml) was subtracted from all the values in the figure. The value obtained from the incubation of the cells with APS-Fr was almost the same as the basal value.
of APS-Fr (Fig. 5). A similar increase by APS-Fr of ACTH$_{1-24}$-induced production of corticosterone and cyclic AMP was also observed in the experiment shown in Fig. 6. Time courses of the production of these substances induced by ACTH$_{1-24}$ did not differ qualitatively in the presence or absence of APS-Fr, although they showed a marked difference quantitatively. It therefore seemed likely that when APS-Fr was added to ACTH$_{1-24}$, the response of the cells was similar to that when a higher concentration of ACTH$_{1-24}$ was used.

When dibutyryl cyclic AMP was used in place of ACTH$_{1-24}$, APS-Fr did not potentiate the steroidogenesis induced by dibutyryl cyclic AMP (Fig. 7). This result suggests that APS-Fr does not affect the pathway of steroidogenesis beyond cyclic AMP formation, i.e., APS-Fr potentiates ACTH$_{1-24}$-induced steroidogenesis at a step between the binding of ACTH to its receptor and the formation of cyclic AMP.

Since the first observation that calcium is required for stimulation of adrenal steroidogenesis by ACTH (18), the calcium requirement for the effects of ACTH has been widely accepted. When calcium is omitted from the incubation medium, a parallel reduction in both corticosterone and cyclic AMP formation by rat adrenal cells in response to ACTH occurs, and when calcium concentration in the medium is increased (from zero to $7.65 \times 10^{-3}$ M), production of corticosterone and accumulation of cyclic AMP are increased (19). It has also been reported that the requirement of calcium diminishes when higher concentrations of ACTH are used (19, 20). In order to define the role of calcium in the hormonal control of adenylate cyclase activity, ACTH binding, enzyme activation and the catalytic formation of cyclic AMP have all been examined independently. On the basis of the resulting findings, it has been...
Fig. 7. Effect of APS-Fr on dibutyryl cyclic AMP-induced steroidogenesis in isolated rat adrenal cells. Fifty microliters (5 μg as protein) of APS-Fr or 50 μl of the elution buffer was evaporated to dryness. Each concentration of dibutyryl cyclic AMP and 1 × 10⁵ cells were added to the dried sample to make a total volume of 0.5 ml. The mixture was incubated for 90 min at 38°C. Each column represents the mean of values obtained from duplicate incubations. Each point represents each value in duplicate incubations.

Fig. 8. Effect of APS-Fr on ACTH₁-24-induced steroidogenesis in isolated rat adrenal cells, incubated in medium containing various concentrations of calcium. Fifty microliters (5 μg as protein) of APS-Fr or 50 μl of the elution buffer was evaporated to dryness. ACTH₁-24 and 1.1 × 10⁵ cells in medium containing each concentration of calcium were added to the dried sample to make a total volume of 0.5 ml. The mixture was incubated for 90 min at 38°C. Each point represents the mean of values obtained from duplicate incubations.
concluded that the stimulatory effect of calcium on ACTH action is exerted at a step involving guanidine nucleotide interaction with the enzyme complex (21). Calcium, required for adenylate cyclase activation by ACTH, might come from the extracellular space, since ACTH stimulates the calcium influx (22). Also in this study, ACTH and calcium, but not APS-Fr, were found to be essential for steroidogenesis. The action of APS-Fr was found only in the simultaneous presence of ACTH and calcium. As shown in Fig. 8, the corticosterone production by adrenal cells in response to a fixed concentration of ACTH1-24 was increased by increasing concentration of calcium in the medium. The response of the cells was further increased by the addition of APS-Fr. The concentration of calcium required to produce the same degree of response as that induced by ACTH1-24 alone was decreased by the addition of APS-Fr. A similar decrease of calcium requirement was found when a higher concentration of ACTH1-24 was used without APS-Fr. This result suggests that APS-Fr increases the amount of ACTH1-24 binding to the cells. It may be that APS-Fr potentiates the action of calcium, but it cannot be determined whether APS-Fr affects the action of calcium either directly or indirectly by increasing the affinity of ACTH1-24 to the adrenal cells.

The results obtained in the present study indicate that APS-Fr increases production of both corticosterone and cyclic AMP by ACTH1-24 as though a higher concentration of ACTH1-24 were being used without APS-Fr. It has been shown that partially purified plasma membrane preparations from rat adrenal cortex exhibit a specific and high affinity for a Lys-r3-MSH, which has ACTH-potentiating activity. The affinity of the peptide for the binding site is 100-fold higher than that of ACTH1-24, but unlike ACTH1-24, concentrations of Lys-r3-MSH up to 10^-6 M fail to stimulate membrane-associated adenylate cyclase and guanylate cyclase activities (23). The molecular weights of several peptides isolated from APS-Fr were 16,000-41,000, and these peptides resembled proopiomelanocortin-related peptides in some of their characteristics (24). Because ACTH can stimulate steroidogenesis without entering its target cells (25), APS-Fr which has a much larger molecular weight than ACTH, may act on the surface of the adrenal cell membrane. Based on these facts, it is assumed that the binding sites for APS-Fr exist on the surface of the adrenal cells; APS-Fr may increase the binding of ACTH molecules to their specific receptors by occupying the APS-Fr binding sites, or APS-Fr may modulate the conformation of ACTH receptors to bind ACTH much more easily by becoming attached to its own binding sites.

In conclusion, APS-Fr was shown to potentiate ACTH-induced steroidogenesis by isolated rat adrenal cells at a step between the binding of ACTH to its receptor and the formation of cyclic AMP; notably, the step of ACTH binding seemed to be the site of action of APS-Fr. The exact site and mechanism of action of ACTH-potentiating substance remain to be studied.

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