STUDIES ON CYCLIC NUCLEOTIDES IN THE ADRENAL GLAND
VII. CYCLIC AMP SYSTEMS IN THE ADRENAL MEDULLA
IN VIVO

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Abstract—3',5'-cyclic adenosine monophosphate (cyclic AMP) systems in the anesthetized rat adrenal medulla were studied using the technique of a direct infusion of various agents into the adrenal gland, and such is referred to as 'the retrograde-adrenal vein infusion' and 'the intra-adrenal infusion'. A direct administration of acetylcholine into the adrenal gland by the retrograde infusion produced a rapid stimulation of the catecholamine-release, followed by a delayed activation of the adenylate cyclase in the adrenal medulla. The acetylcholine-induced increase in hormonal secretion and cyclic AMP levels was completely blocked by hexamethonium, indicating the mechanism of nicotinic trans-synaptic stimulation. Intra-adrenal infusion of epinephrine also prevented an increase in cyclic AMP accumulation induced by acetylcholine. This suggests an inhibitory mechanism on the cyclic AMP systems by catecholamines within the medullary cells. The infusion of propranolol or phentolamine into the gland, in some cases denervated, increased cyclic AMP amounts in the medulla, suggesting a decrease in catecholamine-induced inhibition by α or β-antagonists. These results favor the assumption that the mechanism of catecholamine inhibition of the adenylate cyclase system is mediated by adrenergic interaction. The present studies suggest a mechanism of regulation by catecholamines of the cyclic AMP systems at the early stage of trans-synaptic stimulation, such leading to the delayed increase in the adenylate cyclase activation of the adrenal medulla in vivo.

Recent reports (1–6) have stated that trans-synaptic activation in vivo induced an increase in cyclic AMP accumulation, followed by subsequent events including activation of protein kinase and induction of tyrosine 3-monooxygenase in the rat adrenal medulla. Previous studies (7–9) in vitro showed that incubation of slices from bovine adrenal medulla with acetylcholine increased tissue levels of cyclic AMP some time after stimulation of the catecholamine-release. It was of interest that a renewal of the incubation medium produced a greater increase in cyclic AMP levels after the acetylcholine-stimulation (9). These results suggested the possibility that an increase in cyclic AMP levels of the adrenal medulla would in part be regulated by catecholamine released and/or accumulated during the incubation. Since the experiment using adrenal medulla slices is not an ideal system (8, 10) the method referred to 'the retrograde-adrenal vein infusion' (11–12) and 'the intra-adrenal infusion' (13–14), in which an unilateral adrenal of the rat is exposed directly to the drugs and the release of hormones and cyclic nucleotides in the adrenal venous effluent is estimated in situ, was employed in the present studies. Experiments in vivo reported hitherto demonstrated the difficulty in eliminating the systemic factors on the gland which had been indirectly influenced by the method of systemic administration of the drugs (1–2). Although many
surgical factors including anesthesia and bleeding cannot be ruled out, the infusion method reported here is considered to be advantageous when attempting to determine the direct action of the drugs on the adrenal gland. The present experiments were also designed to investigate in detail the relationship between hormonal secretion and mechanisms regulating the adenylate cyclase-cyclic AMP generating systems in the adrenal medulla in vivo.

MATERIALS AND METHODS

The following compounds were obtained for these studies; acetylcholine chloride, hexamethonium chloride (Wako, Co. Ltd., Tokyo.). Adenosine 3',5'-monophosphate (cyclic AMP) (Daiichi Seiyaku, Co. Ltd., Tokyo.) and epinephrine (Merck, Darmstadt.). Propranolol and phentolamine were generously donated by Sumitomo Chemical, Osaka and CIBA-Geigy, Basle.

Normal male Donryu strain rats weighing 200 to 300 g were anesthetized with sodium pentobarbital (25 mg/kg i.p.). In the first experiment, acetylcholine 10^{-6} mole/kg or hexamethonium 3.2 \times 10^{-6} moles/kg dissolved in 0.9\% saline was infused for 5 sec in a `retrograde' fashion (Fig. 1) (11-12) against the blood stream up the left adrenal vein cannulated with a polyethylene catheter, ligations of renal and other blood vessels being made to prevent the dispersion of the drugs to sites other than the adrenal. Immediately after infusion, the adrenal vein was allowed to bleed through the polyethylene catheter. Adrenal venous blood was collected for 2 to 3 min at a time every 5 min for the determination of plasma cyclic AMP and catecholamines. In the second experiment, epinephrine (740 n moles/min), propranolol (210 n moles/min) or phentolamine (20 n moles/min) dissolved in 0.9\% saline was infused directly into the left adrenal through a hypodermic needle of 25-gauge which has been inserted in the central portion of the gland, referred to 'intra-adrenal infusion' (Fig. 1) (13-14). Intra-adrenal infusion was made at a constant rate (7.4 \mu l/min) for 10 to 30 min by a infusion pump. During infusion, the adrenal vein was allowed to bleed through the polyethylene catheter cannulated into the vein similar to the case of retrograde infusion (Fig. 1). In some experiments rats were denervated by cutting the nerve fibers leading from the main splanchnic trunk to the left adrenal, in which 'intra-adrenal infusion' was performed. At various intervals indicated in the text, after or during infusion, an aliquot of collected blood plasma was submitted for determination of catecholamines, as the sum of adrenaline and

![Diagram](image-url)
noradrenaline, according to the method of Anton and Sayre (15). The remaining plasma and the medullary portion of adrenal glands were used for determination of cyclic AMP levels based on the method of Gilman (16). Estimation of the adenylate cyclase activity was performed as previously described (8). Medullary portions were homogenized in 10 vol of ice-cold 10 mM Tris-HCl buffer (pH 7.4) containing 0.25 M sucrose, 2 mM glycylglycine, 6 mM mercaptoethanol, 1 mM EDTA, and 2 mM MgCl₂ in a Potter-Elvehjem homogenizer with a close fitting teflon pestle. The homogenate was centrifuged at 43,500 x g for 20 min. Subsequent pellets washed twice with the homogenization medium were recentrifuged.

The assay mixture contained 235 μg of tissue protein, 2 mM MgCl₂, 10 mM theophylline, 5 mM ATP and an ATP regenerating system consisting of the 100 μg/mg pyruvate kinase (Sigma Chemical Co., St. Louis Mo.) and 5 mM phosphoenol pyruvate in a total volume of 0.6 ml 40 mM Tris-HCl buffer (pH 7.4). The incubation was carried out at 30°C for 15 min, and terminated by boiling. Cyclic AMP levels were determined according to the method of Gilman (16). The protein concentration was measured by the method of Lowry et al. (17).

RESULTS

Acetylcholine (10⁻⁶ moles/kg) infused retrogradely produced a rapid increase in catecholamine-secretion, while an increase in cyclic AMP concentrations of the adrenal
medulla was observed 20 to 30 min after acetylcholine-infusion when stimulation of hormonal secretion no longer occurred (Fig. 2). Basal concentrations of cyclic AMP in adrenal venous plasma (55±2.9 p moles/ml plasma) were similar to those in peripheral venous plasma (53±3.5 p moles/ml plasma). In contrast with an increase in intra-cellular cyclic AMP levels, secretion by the adrenal gland of cyclic AMP was not altered (Fig. 2). Hexamethonium (3.2×10^{-6} moles/kg) infused with acetylcholine completely blocked catecholamine-release and cyclic AMP increase elicited by acetylcholine (Fig. 2). Adenylate cyclase activities of the medullary tissue 30 min after acetylcholine increased about 4-fold as compared with controls (saline infused). This increase in the enzyme activities was prevented by hexamethonium (Table 1).

As shown in Fig. 3, intra-adrenal infusion of saline (7.4 μl/min) for 30 min increased cyclic AMP concentrations in the adrenal medulla. In contrast, 30 min-infusion of epinephrine decreased cyclic AMP concentration as compared to that of 30 min-infusion of saline. This inhibitory effect of epinephrine on cyclic AMP accumulation became more apparent in the adrenal medulla which had been stimulated by acetylcholine (Fig. 4). Cyclic AMP accumulation inhibited by epinephrine, however, was reversed when propranolol or phentolamine was infused with epinephrine (Fig. 3). The reversal effects on cyclic AMP

### Table 1. Adenylate cyclase activities in adrenal medulla 30 min after infusion of acetylcholine

| Treatment                        | Adenylate cyclase activity (mean±SE) | Cyclic AMP formed p moles/mg protein/min |
|----------------------------------|--------------------------------------|------------------------------------------|
| Control                          |                                      | 8.46±1.77                                |
| Acetylcholine                    |                                      | 34.12±2.88                               |
| Acetylcholine + Hexamethonium    |                                      | 8.04±0.87                                |

Acetylcholine (10^{-6} moles/kg) was infused continuously for 5 sec in a "retrograde fashion". Each value is the mean and S.E. from 4 incubations.

### Table 2. Effects of various chemical agents infused in an "intra-adrenal fashion", on cyclic AMP levels of denervated adrenal medulla

| Intra-adrenal infusion for 10 min | Cyclic AMP p moles/mg protein (mean±S.E.) |
|----------------------------------|------------------------------------------|
| Saline                           | 17.50±1.50                               |
| Epinephrine                      | 6.97±1.44*                               |
| Epinephrine + Propranolol        | 62.15±2.26*                              |
| Epinephrine + Phentolamine       | 30.56±4.92                               |
| Propranolol                      | 31.80±0.92*                              |
| Phentolamine                     | 34.17±2.64*                              |

Methods were the same as in Fig. 3. Each value is the mean and S.E. from 4 experiments. *P<0.05 compared with the values obtained with saline infusion.
FIG. 3. Cyclic AMP levels of adrenal medulla infused with various chemical agents. Saline (7.4 µl/min), epinephrine (740 n moles/7.4 µl/min), propranolol (210 n moles/7.4 µl/min) or phentolamine (20 n moles/7.4 µl/min) was infused continuously for 10 to 30 min in an “intra-adrenal fashion”. Acetylcholine (10⁻⁶ moles/kg) was infused for 5 sec in a “retrograde fashion”. Each bar represents the mean ± S.E. of values in 4 experiments. *P<0.05 compared with intact controls.

FIG. 4. Effects of epinephrine and propranolol infusion on cyclic AMP levels of acetylcholine-stimulated adrenal medulla. Acetylcholine (10⁻⁶ moles/kg) was infused for 5 sec in a retrograde fashion at the beginning of intra-adrenal infusion. Epinephrine (740 n moles/7.4 µl/min) and propranolol (210 n moles/7.4 µl/min) were infused continuously for 10 to 30 min in an intra-adrenal fashion.

TABLE 3. Adenylate cyclase activities in adrenal medulla infused with various chemical agents

| Intra-adrenal infusion for 10 min | Adenylate cyclase activity | Cyclic AMP formed p moles/mg protein/min (mean±SE) |
|----------------------------------|-----------------------------|-----------------------------------------------|
| Saline                           | 4.28±0.07                   |                                               |
| Epinephrine                      | 3.40±0.21                   |                                               |
| Propranolol                      | 6.42±0.11                   |                                               |
| Phentolamine                     | 7.28±0.12                   |                                               |

Saline (7.4 µl/min), epinephrine (740 n moles/7.4 µl/min), propranolol (210 n moles/7.4 µl/min) or phentolamine (20 n moles/7.4 µl/min) was infused continuously for 10 min in an “intra-adrenal fashion”. Each value is the mean and S.E. from 4 experiments. *P<0.05 compared with the values obtained with saline infusion.
accumulation were also observed in the denervated adrenal medulla (Table 2). Infusion of propranolol or phentolamine alone was also effective in increasing cyclic AMP levels (Fig. 3). It is also interesting that intra-adrenal infusion of propranolol produced cyclic AMP accumulation at no time-dependent trends in the adrenal medulla which had been stimulated by acetylcholine (Fig. 4). Meanwhile, a delayed increase in cyclic AMP levels was observed when the gland was stimulated by acetylcholine alone (Fig. 2). Adenylate cyclase activities of the medullary tissue infused with propranolol or phentolamine increased when compared with the epinephrine-infused (Table 3).

DISCUSSION

Retrograde infusion of acetylcholine into the adrenal vein of the rat produced a rapid stimulation of the catecholamine-release, followed by a delayed increase in cyclic AMP accumulation in the adrenal medulla (Fig. 2). This supports the results obtained in previous experiments in vitro on the bovine adrenal medulla (7-9). There was no increase in cyclic AMP levels in the adrenal venous effluent, suggesting that this increase in the adrenal medulla is confined within the tissue. Contrarily, ACTH stimulation to the adrenal cortex caused a rapid increase in adrenal cyclic AMP output with a positive correlation between tissue levels of cyclic AMP and steroid secretion (18-20). Blockade of acetylcholine-induced catecholamine release by hexamethonium was accompanied by inhibition of cyclic AMP elevation (Fig. 2); this suggests that these responses are interrelated and are elicited by activation of nicotinic transmission. Kurosawa et al (6) reported that cyclic AMP accumulation, cytosol kinase activation and tyrosine 3-monooxygenase induction in the rat adrenal medulla elicited by carbamylcholine were not abated by atropine, but were abated by hexamethonium. The delayed increase in medullary cyclic AMP content after acetylcholine-stimulation appeared to be due to activation of the adenylate cyclase (Table 1). This is the first finding for activation of the adenylate cyclase of the adrenal medulla in vivo by the acetylcholine-stimulation. Previous experiments (9) have shown that accumulation of medullary cyclic AMP is enhanced by exchanging the incubation medium. This raises the possibility that the cyclic AMP generating system would be regulated by catecholamine release/and or cyclic AMP antagonist accumulated during incubation. A hormone antagonist (21), or feedback regulator (22) has been found in the incubation medium of adipocytes following stimulation by a hormone that raises cyclic AMP levels (23-25).

Thirty min-intra-adrenal infusion of saline resulted in elevation of cyclic AMP (Table 2). Thirty min-infusion of epinephrine into the adrenal showed cyclic AMP levels to be lower than those of the saline-infused. An increase in cyclic AMP contents was also abated by epinephrine infused into the adrenal which had been stimulated by acetylcholine (Fig. 4). This indicates that the excess of epinephrine infused or catecholamines released exerts an inhibitory action on cyclic AMP systems in the adrenal medulla. Guidotti and Costa (2) have shown inhibitory effects on the adenylate cyclase activity by addition of epinephrine to the incubation of the assay. An elevation of cyclic AMP levels after a long-term (30 min) infusion of saline into the adrenal gland may be due to dilution of catecholamines spon-
taneously released, and such in turn induces a decrease in the amines inhibitory to cyclic AMP systems. The observations that infusion of epinephrine produced an inhibitory effect on cyclic AMP generating systems support a putative mechanism inhibiting cyclic AMP systems by catecholamine. The increase in cyclic AMP levels by adrenergic antagonists, propranolol or phentolamine, would be thus interpreted in part as the blockade of the inhibitory action by catecholamine, resulting in freeing cyclic AMP systems from end-product inhibition. Nikodijevic et al (26) postulated the inhibitory control of adenylate cyclase by \( \beta \)-adrenergic agents in secretory vesicle membranes from the bovine adrenal medulla. Meanwhile, Gutman and Boonyaviroj reported \( \alpha \)-adrenergic stimulation which inhibits catecholamine-release (27-28) and \( \beta \)-adrenergic activation which stimulates the catecholamine release as well as the adenylate cyclase in the adrenal medulla (29). These conflicting results may partly be attributed to differences in experimental conditions between studies \textit{in vivo} and \textit{in vitro} and further investigations are required. Moreover, either epinephrine or isoproterenol given \textit{in vitro} (unpublished) failed to reproduce the results of Boonyaviroj and Gutman (29) who observed increased cyclic AMP levels and activated adenylate cyclase of the adrenal medulla by \( \beta \) agonists. An increase in cyclic AMP accumulation produced by propranolol or phentolamine alone (Fig. 3) could also be interpreted as the result of these agents preventing the inhibitory effect of the endogenous catecholamine released spontaneously. It is of further interest that the experiments of propranolol or phentolamine infused with epinephrine and of propranolol infused into the adrenal which had been stimulated by acetylcholine were shown to increase cyclic AMP contents at rapid intervals (Figs. 3 to 4). It is tempting to suggest however, that the mechanism in chromaffine cells of catecholamines, inhibiting cyclic AMP generation in a way of negative feedback, is mediated by the site(s) for \( \alpha \) or \( \beta \)-adrenergic interaction.

Large doses of reserpine reportedly elicit an increase in sympathetic nerve activity with cyclic AMP levels increased in the adrenal medulla (1, 3). Reserpine, phenoxybenzamine or 6-hydroxydopamine which interfere with the sympathetic nervous system may result in a reflex activation of the sympatho-adrenal system accompanying tyrosine 3-monoxygenase induction (30-32). In the present experiments, propranolol or phentolamine infused into the adrenal gland may be considered to act on trans-synaptic activation through a sympathetic reflex. As shown in Table 2, however, increases in cyclic AMP levels by infusion of these agents were also demonstrated in the rat whose splanchnic nerves had been denervated, indicating that effects of these agents are not involved in sympathetic activation. The results obtained in the present experiment do not exclude the possibility that propranolol or phentolamine exerts some effect upon direct stimulation of the cyclic AMP generating system of the adrenal medulla. A transient but substantial increase in cyclic AMP in the rat adrenal medulla has been reported in the case where a single dose of propranolol was administered intraperitoneally (3). The results from the present studies support the hypothesis that stimulation of the adenylate cyclase activity induced trans-synaptically is inhibited by catecholamines in the medullary cells at the early stage of hormonal secretion, thus leading to delayed activation of the adenylate cyclase after the end of the catecholamine release (8-9).
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