STUDIES ON CYCLIC NUCLEOTIDES IN THE ADRENAL GLAND V. ADENYLATE CYCLASE IN THE ADRENAL MEDULLA

Sumio SHIMA, Yoshiko KAWASHIMA, Masanao HIRAI
and Hiroshi KOUYAMA*

Department of Pharmacology and *Radioisotope Institute for Basic Medicine,
St. Marianna University School of Medicine, Kawasaki, Kanagawa 213, Japan

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Abstract—Effects of various chemical agents eliciting the catecholamine-release on the adenylate cyclase-cyclic AMP generating system have been studied in the secretory process of the bovine adrenal medulla slices. Cyclic AMP levels were not affected at the interval of the maximal increase of the catecholamine-release by acetylcholine, but increased gradually some time after the end of the release/or at the beginning of the restoration of catecholamine in the medulla tissue. This delayed increase in the medullary cyclic AMP is not attributed to a direct involvement in 'stimulus-secretion coupling process' of the medullary secretion, but rather may be caused by release of intracellular catecholamine.

In previous studies, (1-2) the rat adrenal was divided into the fasciculata-reticularis (the cortex), the glomerulosa (the cortex), and the medulla and immobilization stress produced a great increase in cyclic AMP levels to a similar extent in the two cortex zones, but with no changes in the medulla. Moreover, the higher activity of cyclic AMP-dependent protein kinase from the cortex than that of the medullary enzyme was attributed to difference in the cyclic AMP-binding activity of the regulatory proteins associated with the enzymes (3-4). These findings suggest that the medullary cyclic AMP systems could be different from those in the cortex in the adrenal gland. Guidotti and Costa (5) reported that an increase in the medullary cyclic AMP by carbamylcholine was involved in the drug-induced increase of tyrosine hydroxylase activity in the rat adrenal medulla. Contrarily, the adenylate cyclase activity in cell-free preparations from the bovine adrenal medulla was unaffected by cholinergic agents (6-7). The present work was designed to examine the effects of various chemical agents, at doses which elicit the release of catecholamine, on the adenylate cyclase-cyclic AMP generating system in the medulla of the bovine adrenal gland.

MATERIALS AND METHODS

The following compounds were obtained for these studies: acetylcholine chloride, hexamethonium chloride, histamine free base, and serotonin (Wako, Co. Ltd., Tokyo.); angiotensin II (Ciba-Geigy, Basle.); epinephrine (Merck, Darmstadt.) and adenosine 3',5'-monophosphate (cyclic AMP) (Daiichi Seiyaku, Co. Ltd., Tokyo.)

Portions of the medulla from the bovine adrenal obtained fresh at Teikokuzoki, Co. Ltd., Tokyo, were sliced in a room the temp of which was approx. 4°C. Thirty-min pre-
incubation of 100 to 200 mg of slices in 3 ml of Locke's solution at 37°C in the gas phase of 95% O₂-CO₂ was followed by a further incubation with acetylcholine, nicotine, histamine, serotonin, angiotensin II, atropine, and hexamethonium, in the presence of 10 mM theophylline. At various intervals after incubation, catecholamines were determined in an aliquot of the medium as the sum of adrenaline and noradrenaline, according to the method of Anton and Sayre (8). The remaining medium and the tissue were used for determination of cyclic AMP levels as based on the method of Gilman (9).

Effects of acetylcholine as well as other secretagogues on the adenylate cyclase activity in cell-free preparations were also examined as follows: sliced medulla was weighed and homogenized in 10 vol of ice-cold 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 for 1.5 min. The homogenate was filtered through 2 layers of gauze to remove the debris. The first preparations obtained by centrifugation at 1,100 × g for 15 min were referred to as 1,100 × g particles and the second at 43,500 × g for 20 min as 43,500 × g particles. Subsequent pellets washed twice with the homogenization medium were recentrifuged. The assay mixture contained 955 µg of tissue protein, 2 mM MgCl₂, 10 mM theophylline, 5 mM ATP and ATP regenerating system consisting of the 100 µg/ml pyruvate kinase (Sigma Chemical, 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 10 min, and terminated by boiling. Cyclic AMP levels were determined according to the method of Gilman (9). The protein concentration was measured by the method of Lowry et al (10), using bovine serum albumin as the standard protein.

RESULTS

Acetylcholine stimulated the catecholamine-release from the medulla slices for 10 min incubation, while no evident effect on cyclic AMP levels was observed (Fig. 1). Hexamethonium prevented the hormone-release induced by acetylcholine. Nicotine which produced the stimulatory catecholamine-release had no effect on the cyclic AMP accumulation (Fig. 2). No changes in the cyclic AMP levels were observed during stimulation of catecholamine-release by histamine (Fig. 3).

Serotonin and angiotensin II were also revealed to be catecholamine-releasers (Fig. 3). Levels of medullary cyclic AMP were unaffected during stimulation of the catecholamine-release.

Medullary slices were homogenized and centrifuged to give 1,100 × g and 43,500 × g particles, presumably rich in membrane or vesicle fractions.

These two fractions incubated for 10 min with acetylcholine, serotonin and angiotensin II showed no stimulatory effect on the enzyme activity (Table 1).

The relationship between catecholamine-release and cyclic AMP accumulation as a function of time following addition of various secretagogues is shown in Fig. 4 and Table 2. Cyclic AMP levels did not change during stimulation of the catecholamine-release for the
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### FIG. 1. Effects of acetylcholine on catecholamine release and cyclic AMP levels in adrenal medulla slices with a 10-min incubation.

| Cyclic AMP Levels | Additions | CA Release |
|-------------------|-----------|------------|
|                   | NONE      |            |
|                   | CONTROL   |            |
|                   | ACH       |            |
|                   | ACH + ATP |            |
|                   | ACH + ATROPINE |            |
|                   | ACH + CG |            |
|                   | ATROPINE |            |
|                   | NO        |            |
|                   | MEDIUM    |            |

CA: catecholamine, NONE: incubated without theophylline, CONTROL: incubated with theophylline, ACH: acetylcholine, 5 x 10^-5 M, C6: hexamethonium, 10^-6 M, atropine, 10^-6 M. Number of incubations is shown in parentheses. Each value is the mean and standard error.

### FIG. 2. Effects of nicotine on catecholamine release and cyclic AMP levels in adrenal medulla slices with a 10-min incubation.

| Cyclic AMP Levels | Additions | CA Release |
|-------------------|-----------|------------|
|                   | CONTROL   |            |
|                   | NICOTINE  |            |
|                   | NICOTINE + C6 |        |
|                   | NICOTINE + CG |        |
|                   | NO        |            |
|                   | MEDIUM    |            |

CA: catecholamine, C6: hexamethonium, 10^-6 M, nicotine, 10^-4 M, ND: not determined. Number of incubations is shown in parentheses. Each value is the mean and standard error.

### FIG. 3. Effects of various chemical agents on catecholamine release and cyclic AMP levels in adrenal medulla slices with a 10-min incubation.

| Cyclic AMP Levels | Additions | CA Release |
|-------------------|-----------|------------|
|                   | CONTROL   |            |
|                   | HISTAMINE |            |
|                   | 5HT       |            |
|                   | NO        |            |
|                   | MEDIUM    |            |

CA: catecholamine, histamine, 10^-3 M, 5HT: serotonin, 10^-4 M, Ang II: angiotensin II, 10^-5 M ND: not determined. Number of incubations is shown in parentheses. Each value is the mean and standard error.
TABLE 1. Adenylate cyclase activities in cell-free preparations from adrenal medulla

| Additions          | Cyclic AMP p moles/mg protein/min | 1,100 x g particles | 43,500 x g particles |
|--------------------|-----------------------------------|---------------------|----------------------|
| Control            | 15.30 ± 2.42                     | 24.82 ± 4.16        |
| Acetylcholine, 5 x 10^{-5} M | 17.36 ± 2.81                  | 19.58 ± 4.56        |
| Nicotine, 10^{-4} M | 17.16 ± 2.28                     | 20.48 ± 1.79        |
| Histamine, 10^{-3} M | 17.96 ± 3.02                   | 23.92 ± 2.60        |
| Serotonin, 10^{-1} M | 14.76 ± 1.93                    | 24.95 ± 2.08        |
| Angiotensin II, 10^{-9} M | 18.06 ± 2.58                  | 28.35 ± 2.87        |

Each value is the mean and standard error of three incubations.

TABLE 2. Effects of various chemical agents on cyclic AMP levels and catecholamine release of adrenal medulla slices as a function of time

| Additions          | Cyclic AMP p moles/g tissue | 10 min | 60 min | Catecholamine µg/g tissue | 10 min | 60 min |
|--------------------|----------------------------|--------|--------|---------------------------|--------|--------|
| Control            |                            | (4)    |        |                           | (4)    |        |
| Acetylcholine, 5 x 10^{-5} M | 344 ± 10                | 542 ± 56         | 91 ± 5  | 148 ± 5                   | 542 ± 56         |
| Histamine, 10^{-4} M | 423 ± 55                 | 1210 ± 60       | 161 ± 8 | 175 ± 9                   | 1210 ± 60       |
| Serotonin, 10^{-4} M | 280 ± 53                 | 585 ± 73        | 145 ± 7 | 234 ± 14                  | 585 ± 73        |
| Angiotensin II, 10^{-9} M | 316 ± 22                | 476 ± 67        | 186 ± 15| 275 ± 25                  | 476 ± 67        |
| Epinephrine, 2 x 10^{-3} M | 432 ± 45               | 361 ± 32        |        |                           | 432 ± 45       |

Number of incubations is shown in parentheses.

Fig. 4. Effects of acetylcholine on catecholamine release and adenyl cyclase activities as a function of time. Results are expressed as percentages of control values per g tissue from 1, 10, 30 and 60 min incubation and were 170 ± 10 p moles, 330 ± 28 p moles, 552 ± 61 p moles and 610 ± 56 p moles for cyclic AMP levels and 68 ± 2 μg, 71 ± 2 μg, 108 ± 1 μg, 138 ± 7 μg for catecholamine respectively. ACH: acetylcholine, 5 x 10^{-5} M, C6: hexamethonium, 10^{-6} M, CA: catecholamine.
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first 10 min after addition of acetylcholine, but increased considerably 30 to 60 min later, when the stimulatory secretion was no longer observed. In spite of inhibition of the acetylcholine-stimulation by the 10 min-incubation with hexamethonium, a further incubation for 30 to 60 min showed some accumulation of catecholamine in the medium. There was no activation of the cyclic AMP generating system. Histamine, serotonin and angiotensin II continued to stimulate catecholamine-release up to the 60 min-incubation and changes in cyclic AMP levels were not observed (Table 2).

Incubation with unusually large amounts of epinephrine for 10 to 60 min had no effect on the cyclic AMP accumulation (Table 2).

DISCUSSION

Acetylcholine and other secretogogues such as nicotine, histamine, serotonin and angiotensin II inducing the catecholamine-release from the adrenal medulla slices had no stimulatory effect on the cyclic AMP concentration (Figs. 1 to 3), suggesting that the cyclic AMP system(s) is not involved in the process of catecholamine-secretion, or only a part of cyclic AMP fractions is coupled to the medullary secretion. The concept of 'active' compartment of cyclic AMP pool(s) responsive to biological stimulants cannot however, be ruled out (11-13). On the other hand, activation of the adenylylate cyclase by carbachol was observed in plasma membrane from the bovine adrenal medulla (14). These workers reported that the enzyme increased by only 50 to 60%, which was less than that observed in other stimulated biological systems. The present experiments on the adenylylate cyclase activity in cell-free preparations of 1,100 x g and 43,500 x g particles, presumably rich in the storage granules, showed a lack of response to various secretogogues including acetylcholine (Table 1), thus supporting the findings of Hurko et al (7) and Serck-Hanssen et al (6). Of interest is the data that accumulation of cyclic AMP occurred some time after an initial increase of the catecholamine-release evoked by acetylcholine (Fig. 4). This delayed increase in levels of cyclic AMP is unlikely to be involved in 'stimulus-secretion coupling process' (15) in the adrenal medulla. Table 2 shows that there was no increase in cyclic AMP levels so long as stimulation of the medullary secretion was continued by the long-acting secretogogues, such as histamine, serotonin and angiotensin II. These findings favor the assumption that the stimulated release of catecholamine/or the replenishment of catecholamine storage is related to activation of the medullary adenylylate cyclase linking to synthesis (16) or restoration of the amines (17), with an increase of tyrosine hydroxylase activity (5).

Cyclic AMP system(s) in the stimulated sympathetic nervous system was reportedly involved in hyperpolarization on the postsynaptic membrane, coupled to a slow inhibitory postsynaptic potential after depolarization (18-20). These changes in cyclic nucleotides system(s) related to the rapid membranous alteration do not appear to be applicable to chromaffin cells of the medulla. There are many reports (21-25) that various biogenic amines and chemical agents act directly to increase cyclic AMP levels in the brain tissue. In the case of chromaffin cells, there is apparently no evidence that agents stimulating the
catecholamine-secretion act directly on the adenylate cyclase system. The present data suggest that the rise in cyclic AMP could be caused by/or after the release of intracellular catecholamine. Ferrendelli et al (26) observed an increase in both cyclic AMP and GMP levels after the release of intracellular substances in mouse cerebellum slices stimulated by depolarizing agents.

In the perfused cat adrenal gland, cyclic AMP, its dibutyrate and theophylline stimulated the catecholamine-release by translocating intracellular bound calcium or by a mechanism independent of calcium (27). Exogenous cyclic AMP and GMP were found to be potent releasers of catecholamine from medulla slices and the stimulatory effect of cyclic nucleotides was observed even in a calcium free medium (28). Poisner (29) stressed that stimulation by aminophylline of the catecholamine-release from the perfused bovine adrenal involved two different mechanisms, one for depolarization of the chromaffin cell with increased influx of calcium triggering secretion, and one for release of intracellular calcium from the membranes stores. In the present experiment, addition of theophylline for 10 min incubation failed to increase either in catecholamine-release or the endogenous cyclic AMP levels (Fig. 1). This discrepancy could be attributed to the experimental conditions we employed. The experiment using adrenal medulla slices is not an ideal system, and the kinetics of cyclic AMP production are largely dependent on the diffusion of the stimulatory agents into and out of the slices (30). Other factors such as accumulation of 'leaked out' cyclic AMP, the catecholamine released, or reabsorption of the amines during incubation may also be involved.

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