Stimulation-Evoked Cyclic Nucleotides Efflux from Isolated Perfused Dog Adrenals and Possible Involvement of Calcium

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Abstract—Nicotine and muscarine caused the transient increase in cAMP and cGMP efflux from dog adrenal glands followed by a small but lasting increase in the nucleotide efflux. The initial increase preceded catecholamine (CA) release and the latter slowly developed. Nicotine and muscarine caused the maximal increase of cAMP and cGMP levels in adrenal medulla 15 sec after the treatment, and these effects were antagonized by hexamethonium and atropine, respectively. Hexamethonium in combination with atropine, verapamil and an omission of Ca\(^{2+}\) in the medium prevented ACh from producing the increase in cyclic nucleotide efflux and CA release. Reintroduction of Ca\(^{2+}\) (1.3 mM) in fluid after perfusion with Ca\(^{2+}\) and Mg\(^{2+}\)-free fluid caused the transient increase in cyclic nucleotide efflux and CA release. Adenylate cyclase activity in adrenal medulla was activated by Ca\(^{2+}\). These results may suggest that cAMP formation was increased by activation of adenylate cyclase as a result of increased influx of Ca\(^{2+}\) when adrenal medulla were stimulated.

Materials and Methods

Adrenal perfusion: Dogs of both sexes, weighing 14–25 kg, were anesthetized with sodium pentobarbital (30 mg/kg, i.v.), and the bilateral adrenals were removed. Retrograde perfusion of the isolated adrenal glands was made according to the method of Robinson (5). The details have been described previously (4). Briefly, the isolated adrenal glands were perfused with aerated modified Krebs-Ringer phosphate solution (pH 7.3) of the following composition in mM: NaCl, 137; KCl, 5.5; CaCl\(_2\), 1.3; MgSO\(_4\), 1.38; KH\(_2\)PO\(_4\), 1.38; Na\(_2\)HPO\(_4\), 10.6 and glucose, 8.5. When excess K\(^+\) was used, the equivalent amount of Na\(^+\) was replaced. Five drops (0.3 ml) to 16 drops (0.96 ml) of perfusate were collected sequentially every 15 sec in glass tubes containing ice cold trichloroacetic acid (TCA, with a final concentration of approximately 6%). The aliquot was used for CA and cyclic nucleotide assay.

Catecholamine and cyclic nucleotides
determination: The total CA was measured fluorometrically by the methods of Von Euler and Lishajko (6) with adrenaline as the standard.

Cyclic nucleotides were determined as described previously (4). Samples were centrifuged at 3500 rpm for 15 min. 1 N HCl was added to the supernatant, which was shaken with water saturated ethyl ether to remove TCA. The water layer was separated from the ether layer, heated in a water bath at 60°C for 20 min and then neutralized. Cyclic AMP and guanosine cyclic 3',5'-monophosphate (cGMP) were measured by radioimmunoassay using a cAMP RIA kit and a cGMP RIA kit, respectively.

For determination of tissue levels of cyclic nucleotides, the adrenal glands were plunged into liquid nitrogen after the perfusion. The medulla separated from the glands were homogenized with 5 volumes of 6% TCA solution in a Potter-Elvehjem's teflon-glass homogenizer. Samples were treated as described above.

**Assay of adenylate cyclase:** Adrenal medulla were separated from cortex and homogenized in nine volumes of cold 0.25 M sucrose/10 mM Tris-HCl buffer, pH 7.4. For determination of adenylate cyclase activity, reaction mixtures containing the enzyme preparation, 50 mM Tris-HCl buffer (pH 7.4), 10 mM theophylline, 4 mM MgCl2 and 1 mM ATP in a final volume of 100 μl were incubated for 10 min at 37°C, and the reaction was terminated by the addition of 1 ml of 50 mM sodium acetate buffer (pH 4) and heated at 90°C for 3 min. cAMP formed was determined with the radioimmunoassay as described above. The reagents used were obtained commercially as described previously (4).

**Results**

**Effects of nicotine, muscarine and excess K+ on cyclic nucleotide efflux and CA release:** Nicotine increased output of cyclic nucleotides and CA from isolated perfused dog adrenal glands. A typical pattern of the time course is shown in Fig. 1. Increased levels of cyclic nucleotides in the perfusate were observed within 10 sec and reached a maximum within 30 sec, and these were followed by a sharp falling phase. Subsequently, efflux of cyclic nucleotides gradually increased during exposure to the drug, while CA release increased 15 sec after the start of perfusion with nicotine and reached a peak within 2 min and then attenuated during perfusion with the drug. The changes in cyclic nucleotide levels in adrenals exposed to nicotine is shown in Fig. 2. cAMP levels in adrenal medulla, but not in cortex, significantly increased with a peak attained in 15 sec, and the time courses were similar to those of cAMP efflux, but no increase was detected after 30 sec. Muscarine also caused a large and transient increase in cAMP and cGMP efflux followed by a small but lasting increase in the nucleotides (Fig. 1). Amount of cAMP efflux in response to muscarine was similar with that to nicotine, but the amount of cGMP efflux was much greater. The peak response of the initial cyclic nucleotide efflux induced by nicotine and muscarine preceded the maximum increase in CA release caused by these treatments. Similar results were obtained by perfusing adrenal glands with 65...
mM K⁺ (data was not shown).

Effects of various treatments on the cyclic nucleotide levels and CA release: Changes in tissue levels of cyclic nucleotides during stimulation in the presence or absence of various drugs were determined (Table 1). Both cAMP and cGMP levels in adrenal medulla were significantly elevated by ACh, nicotine and muscarine treatment after 15 sec. Hexamethonium and atropine antagonized the effect of nicotine and muscarine, respectively. In the presence of hexamethonium plus atropine, verapamil or absence of Ca²⁺ in the perfusion medium, ACh failed to cause the increase in cyclic nucleotide efflux and CA release (Fig. 3).

Effects of Ca²⁺-reintroduction after the perfusion with Ca²⁺-free medium were examined (Fig. 4). During perfusion of glands with Ca²⁺ and Mg²⁺-free solution, the basal level of cAMP was 1.82±0.06 pmoles/ml in perfusate which was higher than the level of 0.91±0.02 pmoles/ml in perfusate from glands perfused with normal fluid. Reintroduction of Ca²⁺ (1.3 mM) into the perfusion medium produced increases in cAMP and cGMP with a sharp peak at 15 sec by more than 100% and 230% over the basal level, respectively, but the second phase in increase was not manifest (Fig. 4). The patterns of the time course of the initial cyclic nucleotide efflux and CA release induced by Ca²⁺ reintroduction were very similar to those induced by a nicotinic cholinergic agonist.

Effect of Ca²⁺ on adenylate cyclase activity in adrenal medulla: Adenylate cyclase activity from adrenal medulla was reduced to 60% of control by the addition of EGTA (Table 2). The addition of 50 and 100 μM of Ca²⁺ stimulated the activity by 65 and 87%, respectively. The addition of ACh, nicotine and bethanechol in the concentration range

![Fig. 2. Changes in cAMP concentrations in perfusate (A), adrenal medulla (B) and cortex (C) induced by nicotine: □ control, ● nicotine. Perfusion with solution containing nicotine (5×10⁻⁵ M) was started at the arrow. *P<0.05, **P<0.01: Significantly different from spontaneous cAMP efflux (n=5). †P<0.05, ††P<0.001: Significantly different from the corresponding control. Numbers in parentheses are numbers of experiments.](image)

**Table 1. Effects of acetylcholine, nicotine and muscarine on cyclic nucleotide levels in adrenal medulla**

| Drugs               | Conc. M | cAMP pmol/mg protein | cGMP pmol/mg protein |
|---------------------|---------|----------------------|----------------------|
| None                |         | 34.1±0.8             | 4.2±0.1              |
| Acetylcholine       | 10⁻⁴    | 58.5±5.0*            | 8.8±0.5*             |
| Nicotine            | 5×10⁻⁵  | 56.3±3.7*            | 8.5±0.3*             |
| Hexamethonium       | 5×10⁻⁵  | 34.1±2.9             | 4.8±0.6              |
| Hexamethonium+Nicotine | 6×10⁻⁵ | 29.7±1.6             | 4.6±0.6              |
| Muscarine           | 5×10⁻⁴  | 72.4±5.5*            | 11.1±0.3*            |
| Atropine            | 2×10⁻⁵  | 33.2±3.3             | 4.2±0.3*             |
| Atropine+Muscarine  |         | 37.6±2.8             | 4.0±0.4              |

Adrenal glands were perfused with secretagogues for 15 sec. Perfusion with hexamethonium or atropine was started 5 min before stimulants. Values represent the mean±S.E.M. of 4 to 10 experiments. *P<0.001: significant difference from the control.
of 5 to 500 μM had no effect on the activity (data was not shown).

Discussion

Davoren and Sutherland demonstrated extrusion of cAMP from pigeon erythrocytes following stimulation of adenylate cyclase with epinephrine (7). Subsequently, the presence of a cAMP transport system was confirmed in a variety of cells and organs including isolated superior cervical ganglion of rats (8–12). From these studies, the significance of cAMP efflux to the regulation of cellular cAMP levels became evident. Wiemer et al. (13) drew the conclusion from a kinetic study of extrusion of cAMP and changes in cellular cAMP concentration in red blood cells that extrusion of cAMP is a saturable and energy-dependent process which regulates the intracellular cAMP concentration. It has been suggested that cAMP efflux is coupled to cAMP synthesis or proportional to intracellular cAMP levels in adrenal medulla (4) and some other tissues (7, 9, 13), while efflux of cAMP has been also demonstrated in response to low concentration of hormones in perfused liver without a detectable elevation in cellular cAMP concentrations and was well correlated with glucose mobilization (11). It is reported that a close correlation existed between augmentation of the mechanical performance of the heart in response to norepinephrine and the amount of released cAMP, but not the tissue cAMP level (12). These evidences suggest that cAMP efflux...
provides a reliable measure in relation to the corresponding metabolic or pharmacologic effects.

The present results showed that the concentration of cAMP in adrenal medulla rapidly increased after the exposure to nicotine, and the time courses of the changes in tissue cyclic nucleotide level were quite similar to those of efflux of the nucleotide. The changes of cAMP concentration in the perfusate were more remarkable than those in tissues. Thus, the measurement of cyclic nucleotides in the perfusate may provide a sensitive indicator of the intracellular cyclic nucleotide response to stimulants in adrenals as suggested previously (4).

The consecutive analysis of changes in cAMP and cGMP in the perfusate during exposure to nicotine and muscarine revealed that time course of changes in cyclic nucleotide efflux was composed of two phases. The initial large increase, which appeared within 10 sec and was transient, preceded to the onset of an increase in CA release. The second phase in increase slowly developed, attained a plateau at about 1.5 min and lasted during perfusion with stimulants. Jannus and Rubin (3) described the discrepancy between the time course of an increase in CA release and that of cAMP efflux. Namely, during exposure to secretagogues, a peak CA release occurred earlier than maximal increase in cAMP level. These changes in cAMP may correspond to the second phase observed in the present study.

It has been recently reported that secretagogues-evoked CA release from dog adrenals and PC12 cells was enhanced by adenylate cyclase activator- and phosphodiesterase inhibitor-induced elevation of cAMP (14, 15). The direct addition of cAMP also can potentiate the evoked CA release in perfused dog adrenals (16) and isolated bovine adrenal medullary cells (17). These evidences suggest the possible role of cAMP as a modulator of CA release in adrenals. It is well accepted that CA release from chromaffin cells in adrenal medulla is associated with an increase in intracellular Ca$^{2+}$ concentration, which is known to result from increased Ca$^{2+}$ influx through voltage-dependent or ACh-receptor linked Ca$^{2+}$ channels (18–20). Another pathway for a rise in intracellular Ca$^{2+}$ concentration is Ca$^{2+}$ influx in exchange for internal Na$^{+}$ (21–23). The reintroduction of Ca$^{2+}$ to the medium after the various treatments which cause an increase in intracellular Na$^{+}$ concentration results in an increased intracellular Ca$^{2+}$ concentration via the Na$^{+}$-Ca$^{2+}$ exchange mechanism (22, 24). The present results show that Ca$^{2+}$ reintroduction after perfusion of adrenals with Ca$^{2+}$- and Mg$^{2+}$-free fluid caused an increase in cAMP and cGMP efflux followed with an increase in CA release. Patterns of the time courses of cyclic nucleotides efflux and CA release were very similar with those produced by nicotine except in the following points: During perfusion of adrenals with Ca$^{2+}$-free fluid, the basal cyclic nucleotides levels in perfusate were higher than the levels obtained in Ca$^{2+}$-containing medium, and the second phase of increase in cAMP efflux was not manifest. These may be explained as follows: phosphodiesterase activity may be reduced by an

### Table 2. Effect of Ca$^{2+}$ on adenylate cyclase activity from adrenal medulla

| Conc. $\mu$M | Cyclic AMP formed (pmol/mg protein per min) |
|--------------|-------------------------------------------|
| None         | 18.5±0.8                                  |
| EGTA 100     | 11.2±0.4                                  |
| EGTA 100     | 18.5±0.6                                  |
| $+$CaCl$_2$ 50 | 21.0±1.1                                  |
| $+$CaCl$_2$ 100 | 21.0±1.1                                  |

Cyclic AMP formation was determined as described in Materials and Methods. Reactions were started by adding ATP to the incubation mixture with or without Ca$^{2+}$ or glycoetheriamine-tetra acetic acid (EGTA). Values are means±S.E.M. of triplicate incubations.
omission of Ca\(^{2+}\), resulting in the higher basal levels of cyclic nucleotides and reactivation of the enzyme activity by Ca\(^{2+}\) introduction may mask the second phase of changes. The adenylate cyclase activity from bovine adrenal medulla is stimulated by calmodulin in the presence of calcium (25). The present results showed that a Ca\(^{2+}\) antagonist and the omission of Ca\(^{2+}\) in the perfusion medium prevented the increase in cyclic nucleotides efflux and that adenylate cyclase activity in a crude preparation of adrenal medulla was reduced by EGTA and activated by Ca\(^{2+}\). From these evidences, it is suggested that an increase in intracellular Ca\(^{2+}\) concentration induced by secretagogues or Ca\(^{2+}\) reintroduction results in an increase in cAMP formation by an activation of adenylate cyclase via interaction with calmodulin. Thus it may be possible that physiologically increased cAMP level preceding to CA release in adrenal chromaffin cells may modulate CA release. For the role of cAMP which increased after the peak CA release, it may be possible as discussed by Gutman and Boonyaviroj (2) that adrenal medullary adenylate cyclase is activated by released CA and the resulting increase of cAMP would serve as a positive feedback loop for CA release.

The present results suggest that an increased Ca\(^{2+}\) concentration induced by secretagogues result in an increase of cAMP formation and support the hypothesis that cAMP may play a modulating role in CA release from adrenals.

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