DIFFERENTIAL EFFECTS OF D 600 ON RELEASE OF CATECHOLAMINES BY ACETYLCHOLINE, HISTAMINE, TYRAMINE AND BY CYCLIC AMP FROM CANINE ADRENAL MEDULLA

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Abstract- The isolated canine adrenal glands were perfused retrogradely with Locke's solution, and the catecholamine contents of the effluents were measured by the trihydroxyindole-fluorimetric method. Stimulation of the glands by acetylcholine, histamine, tyramine and cyclic AMP caused an increase in release of catecholamines from the glands. Introduction of D 600 to the perfusion medium reduced release of catecholamines in response to acetylcholine, and this reduction was overcome by raising calcium ion concentrations of the perfusion medium. Similarly, D 600 reduced release of catecholamines in response to histamine. The release of catecholamines evoked by tyramine was also inhibited by D 600, although to a lesser degree than the release by acetylcholine. In contrast, D 600 was entirely ineffective on the catecholamine release in response to cyclic AMP. D 600 had no effect on the spontaneous catecholamine output. From these results it was concluded that release of catecholamines from adrenal chromaffin cells by acetylcholine and histamine, and by tyramine in part requires the entry of calcium ions across the cell membrane, whereas that by cyclic AMP does not.

Many investigations carried out so far concerning the release mechanism of catecholamines (CA) from the adrenal medulla have made it clear that extracellular calcium ions (Ca\(^{2+}\)) play an important role in stimulus-secretion coupling there. Acetylcholine (ACh), the physiological secretagogue of the adrenal medulla (1, 2), and histamine, serotonin, angiotensin and bradykinin (3) require the presence of extracellular Ca\(^{2+}\) to release CA from there. Sympathomimetic amines such as tyramine have also been reported to require extracellular Ca\(^{2+}\) for release of CA from the perfused adrenal medulla (4), although these amines are capable of releasing CA from isolated medullary granules in the absence of environmental Ca\(^{2+}\) (5, 6). Adenosine 3',5'-cyclic monophosphate (cyclic AMP), the implication of which is suggested in several secretory processes, has been shown to release CA from the perfused adrenal medulla in the absence of extracellular Ca\(^{2+}\) (7), whereas Jaanus and Rubin (8) failed to demonstrate the release of CA from there by cyclic AMP. Thus, the requirement of extracellular Ca\(^{2+}\) for release of CA from the adrenal medulla appears to depend upon experimental conditions and secretagogues. Recently, D 600 (methoxyverapamil) which blocks an inward current carried by Ca\(^{2+}\) in heart muscle (9) and other excitable tissues (10–12) has also been demonstrated to reduce secretory responses of the neurohypophysis (13, 14), and of the adrenal medulla to ACh (15).
We designed the present experiments in an attempt to obtain further insight into the possible role of Ca\(^{2+}\) in the secretory process of CA in adrenal chromaffin cells. In the present experiments, we examined the effects of D 600 on the release of CA evoked by ACh, histamine, tyramine and cyclic AMP, since the effects of D 600 on the release of CA evoked by the latter three substances have not been investigated in the perfused adrenal medulla as yet.

MATERIALS AND METHODS

Mongrel dogs of either sex, weighing 6-16 kg, were anesthetized with sodium pentobarbital (30 mg/kg, i.v.), given sodium heparin (500 units/kg, i.v.) and exsanguinated. One or both adrenal glands were isolated together with the adrenolumbar vein. In phosphate-buffered Locke's solution the adrenolumbar vein was cannulated, and all detectable side branches entering it were tied. The isolated glands were placed in a funnel-shaped water jacket warmed at 37°C, and perfused retrogradely at a constant flow rate with phosphate-buffered Locke's solution by virtue of a peristaltic pump (Harvard Apparatus, 1210). The composition (mM) of the solution was as follows: 154 NaCl; 5.6 KCl; 2.2 CaCl\(_2\); 2.15 Na\(_2\)HPO\(_4\); 0.85 NaH\(_2\)PO\(_4\); 10 dextrose. In the case of high Ca\(^{2+}\), bicarbonate-buffered Locke's solution of the following composition (mM) was used: 130 NaCl; 5.6 KCl; 14 CaCl\(_2\); 6 NaHCO\(_3\); 10 dextrose. These solutions were warmed at 37°C and equilibrated with 95% O\(_2\)-5% CO\(_2\) (pH was about 7.0). The flow rate was adjusted to be 1.3-2.6 ml/min depending upon the wet weight of the gland, and the gland was perfused for more than 60 min for equilibration before start of the experiment. The effluents from the gland were collected in glass tubes as samples. Before each stimulation one or two samples were collected and these served as an estimate of the spontaneous CA release. Then, a stimulant solution was injected into the perfusion circuit close to the gland, and the effluents from the gland were collected 10 sec after the injection at one min intervals. The CA contents of the effluents were measured by the trihydroxyindole-fluorimetric method after separation by use of the phosphorylated-cellulose column separation technique (16). The differential estimation of adrenaline and noradrenaline was carried out by use of the different pH technique (17), and the sum of them represented the total CA (simply called CA below) which were expressed in \(\mu\)g/min. The fluorescence was measured at excitation-emission wavelengths of 420-520 nm by a spectrofluorometer (Hitachi, MPF-4).

In each experiment, only one kind of stimulant was given three times at 60 min intervals. D 600 was added to the perfusion medium at a concentration of 1 \(\mu\)g/ml or 3 \(\mu\)g/ml from 20-30 min before and to 5 min after the second stimulation. Prior to these experiments, control experiments were carried out without D 600. In the present paper, a peak increase in CA output produced by a given stimulant, that is, the peak CA release minus the spontaneous CA release in the preceding sampling period, was defined as the CA release response, or simply response.

The drugs used were acetylcholine chloride (Daiichi Seiyaku Co. Ltd.), histamine dihydrochloride (Wako Pure Chemical Industries Ltd.), tyramine hydrochloride (Wako...
Pure Chemical Industries Ltd.), sodium salt of adenosine 3',5'-cyclic monophosphate (Seishin Seiyaku Co. Ltd.), methoxyverapamil hydrochloride (D 600, Knoll AG). These drugs except cyclic AMP were dissolved in Locke’s solution, and cyclic AMP was dissolved in 0.9% saline. Drug solutions of ACh, histamine and tyramine were injected into the perfusion circuit in a volume of 10 μl in 6–8 sec with an individual microsyringe, and cyclic AMP was given in a volume of 0.2 ml in 6–8 sec with a 0.5-ml syringe. Doses of all drugs were expressed in terms of their bases.

Values of CA release were expressed in terms of mean±S.E. Significance in difference between mean values was analysed by Student’s t-test and expressed by p values. The difference was regarded to be significant at p<0.05.

RESULTS

In the present experiments, the isolated gland was perfused for 3 hr or more, during which the spontaneous (Fig. 1A) and the evoked CA release decreased gradually (Fig. 1A and Table 1). When the gland was stimulated by ACh, a peak increase in CA release was obtained at the first minute, and then the CA release declined rapidly to return to the spontaneous level within 3–4 min (Fig. 1A). Essentially the same time course of changes in CA

![Fig. 1. Changes in catecholamine (CA) output from canine adrenal glands evoked by acetylcholine (ACh) in the absence (A) and in the presence (B) of D 600. The glands were perfused with Locke's solution. Numerals below vertical columns indicate time which elapsed from start of perfusion in minutes. ACh (30 μg) was injected into the perfusion circuit close to the gland at time indicated by arrows. A period in which D 600 was present in the perfusion medium was indicated by a solid horizontal bar. A peak increase in CA output (dotted columns) over the spontaneous CA output in the preceding sampling period is defined as the CA release response (see Method).](image-url)
release was observed with histamine and tyramine. On the other hand, the CA release evoked by cyclic AMP developed slowly, reached a peak at the second minute and declined gradually; the CA release at the first and the third minute being close to that at the second minute. A single injection of 10 μl of Locke’s solution or 0.2 ml of 0.9% saline had virtually no effect on the spontaneous CA release.

### TABLE 1. Spontaneous and evoked CA release from canine adrenal glands perfused with Locke’s solution

| Stimulant     | Dose (μg) | Number of preparations | Spontaneous release (ng/min) | 1st stimulation Peak release (ng/min) | 2nd stimulation Response* in % of 1st response | 3rd stimulation Response in % of 1st response |
|---------------|-----------|------------------------|------------------------------|--------------------------------------|---------------------------------------------|---------------------------------------------|
| Acetylcholine | 30        | 5                      | 0.30±0.03                    | 2.48±0.40                            | 74.4±0.7                                    | 61.5±1.8                                    |
|               | 100       | 6                      | 0.34±0.08                    | 2.54±0.55                            | 80.4±6.8                                    | 71.1±9.0                                    |
| Histamine     | 300       | 5                      | 0.29±0.07                    | 0.97±0.12                            | 70.3±2.8                                    | 55.6±2.8                                    |
| Tyramine      | 500       | 5                      | 0.32±0.06                    | 1.17±0.22                            | 73.1±2.9                                    | 60.7±4.3                                    |
| Cyclic AMP    | 10**      | 5                      | 0.26±0.09                    | 0.53±0.15                            | 71.1±4.5                                    | 62.1±6.8                                    |

*Response—peak release evoked by stimulation — spontaneous release. **μg. Values are mean ± S.E.

Effects of D 600 on catecholamine output evoked by acetylcholine and histamine

Control experiments with ACh were carried out without addition of D 600 in 11 adrenal glands. Values of the spontaneous CA release and the peak CA release evoked by the first stimulation by 30 μg and 100 μg of ACh, and the peak increases in CA output (CA release responses) caused by the second and the third stimulation in the control experiments are presented in Table 1. As can be seen in this table and Fig. 1A, the CA release in response to ACh was decreased by repeated injections of ACh. The results are consistent with those reported by Douglas and Rubin (1). The effect of D 600 was examined in other adrenal glands. D 600 was added to the perfusion medium 20–30 min before the second ACh stimulation and was present until 5 min after the stimulation. D 600, 1 μg/ml failed to reduce the CA release responses to the second stimulation by 30 μg and 100 μg of ACh (Fig. 2). However, when the concentration of D 600 was increased to 3 μg/ml, a significant reduction of the CA release response to the second stimulation occurred with 30 μg of ACh (Figs. 1B and 2A). A similar reduction was observed in the CA release response to the second stimulation by 100 μg of ACh (Fig. 2B). This reduction of the CA release response to the second ACh stimulation lasted for about 20 min at the shortest and for about 60 min at the longest after withdrawal of D 600 from the perfusion medium (data not shown). The responsiveness of the adrenal glands recovered after withdrawal of D 600, as the CA release response to the third stimulation shows (Figs. 1B and 2).

Essentially similar results were obtained with 300 μg of histamine and 3 μg/ml of D 600. The values of the spontaneous CA release and a peak CA release in response to histamine obtained in control experiments are tabulated in Table 1, and the results obtained with D 600 are summarized in Fig. 3.
**FIG. 2.** Effect of D 600 on the catecholamine (CA) release responses of canine adrenal glands to acetylcholine (ACh). The CA release responses to the second and the third stimulation by ACh are expressed as a percent of the first CA release response. The glands were perfused with Locke's solution, and were stimulated three times at 60 min intervals by 30 μg (A) and 100 μg (B) of ACh in the presence (2nd stimulation) and after withdrawal (3rd stimulation) of D 600 (1 μg/ml and 3 μg/ml). Control experiments were carried out without introduction of D 600, as shown in Fig. 1A. In other experiments, D 600 was introduced to Locke's solution from 20–30 min before and to 5 min after the second stimulation by ACh. Values are expressed in terms of mean ± S.E. The number of experiments is shown in parentheses. *: p<0.001 against control.

**Fig. 3.** Effect of D 600 on the catecholamine (CA) release response of canine adrenal glands to histamine. The second and the third CA release responses are expressed as a percent of the first CA release response. Values are expressed in terms of mean ± S.E. The number of experiments is shown in parentheses. *: p<0.02 against control. Otherwise, the same as in Fig. 2.

**Fig. 4.** Effect of D 600 on catecholamine (CA) release response of canine adrenal glands to tyramine. The second and the third CA release responses are expressed as a percent of the first CA release response. *: p<0.03 against control. Otherwise, the same as in Fig. 2.

**Effect of high Ca\(^{2+}\) concentration on inhibitory action of D 600**

The effect of an increase in concentration of Ca\(^{2+}\) in the perfusion medium from 2.2 mM to 14 mM was examined in 10 adrenal glands. With perfusion of the glands with Locke's solution containing 14 mM Ca\(^{2+}\), the spontaneous and the evoked CA release by ACh was
increased in comparison with those obtained with the normal solution. In 5 control experiments, values of the spontaneous and the peak CA release evoked by the first stimulation by 30 μg of ACh were 0.50 ±0.12 μg/min and 4.89 ±1.04 μg/min, and the CA release response to the second ACh stimulation was 78.3±1.6% of the first response. The reduction caused by 3 μg/ml of D 600 of the CA release response to the second ACh stimulation as observed under the condition of normal Ca2+ concentration disappeared under the condition of high Ca2+ concentration; the CA release response to the second ACh stimulation was 78.3±3.3% (n=5) (p>0.9 against the second CA release response in control experiments).

**Effect of D 600 on catecholamine output evoked by tyramine**

The spontaneous and the peak CA release from 5 adrenal glands in response to three successive stimulations by 500 μg of tyramine is shown in Table 1. The CA release response to the second tyramine stimulation was not affected by the lower concentration of D 600 (1 μg/ml) (75.4±2.8%, n=5), but was significantly reduced by the higher concentration of D 600 (3 μg/ml) (55.1±4.7%, n=5, p<0.03 against control) (Fig. 4). The reduction of the tyramine-evoked CA release disappeared when D 600 was withdrawn (Fig. 4).

**Effect of D 600 on catecholamine output evoked by cyclic AMP**

The spontaneous CA output and the peak CA release in response to 10 mg of cyclic AMP are shown in Table 1. The higher concentration of D 600 (3 μg/ml), which was effective in reducing the ACh-, histamine- and tyramine-evoked CA release, failed to affect the CA output evoked by 10 mg of cyclic AMP; the CA release response to the second stimulation was 71.6±4.4% (n=5) (p>0.9 against control).

**Effects of D 600 on the spontaneous catecholamine output and on the ratio of adrenaline to total catecholamines**

Introduction of D 600 to the perfusion medium in final concentrations of 1 and 3 μg/ml caused no significant change in the spontaneous CA release (p>0.4, n=42). The ratios of adrenaline (A) to total catecholamines (CA) of the spontaneous and the evoked CA

| Table 2. Adrenaline/total CA ratio |
|-----------------------------------|
|                                  |
| **n** | **spontaneous** | **evoked** |
|-----------------------------------|
| Acetylcholine 30 μg               |
| †D 600 1 μg/ml                   |
| †D 600 3 μg/ml                   |
| Acetylcholine 30 μg (14 mM Ca2+)  |
| †D 600 3 μg/ml                   |
| Histamine 300 μg                 |
| †D 600 3 μg/ml                   |
| Tyramine 500 μg                  |
| †D 600 3 μg/ml                   |
| Cyclic AMP 10 mg                 |
| †D 600 3 μg/ml                   |

**n**: Number of preparations. *Otherwise 2.2 mM Ca2+. Values are mean±S.E.
release are presented in Table 2. Only with stimulation by ACh, was the A/CA ratio of
the evoked CA release lower than that of the spontaneous CA release (p<0.02, n=32; results with ACh 30 μg and 100 μg). With the other stimulants examined, the A/CA ratios
were not different between the spontaneous and the evoked CA output. D 600 did not
affect the A/CA ratios.

DISCUSSION

The fact that extracellular Ca²⁺ is essential for release of CA by ACh from the adrenal
medulla has been well established (1, 18, 19). In this case the entry of Ca²⁺ across the
membrane would be the first step of a chain of events leading to release of CA. Indeed,
Ca²⁺ uptake into chromaffin cells is distinctly increased during stimulation by ACh (18),
and this increase is thought to be due to changes in permeability of the cell membrane to
common species of ions of chromaffin cells accompanied by depolarization. Deprivation
of Ca²⁺ from the environmental medium reduces the increase in Ca²⁺ uptake resulting in
reduction of CA release from the adrenal medulla (1). The release of CA by ACh from the
adrenal medulla is also inhibited by an organic substance, D 600 (15), which blocks the slow
inward current carried by Ca²⁺ in heart muscle (9) and in squid axons (12). D 600 also
blocks ⁴⁵Ca uptake in the neurohypophysis (14), and reduces the secretion of vasopressin
and oxytocin (13, 14).

In agreement with the results obtained by Pinto and Trifaró (15) from the bovine
adrenal gland, D 600 distinctly reduced the release of CA by ACh without affecting the
spontaneous CA release from the canine adrenal gland, although the effective concentration
of D 600 in their experiments (0.3 mM = 145 μg/ml) was higher than ours (3 μg/ml). This
discrepancy may to some extent be due to the difference in species and experimental con-
ditions. The effective concentration of D 600 in the present experiments is close to those
which reduced the release of hormones from the neurohypophysis induced by high K⁺ and
electrical stimulation, respectively (13, 14). The reduction of CA release by D 600 was
overcome by an increase in extracellular Ca²⁺ concentration to 14 mM (normal concen-
tration was 2.2 mM). In the present experiments, D 600 also reduced CA release induced
by histamine. Such was expected in view of the facts that histamine, like ACh belongs to
a group of substances which cause depolarization of chromaffin cells by changing perme-
ability of the cell membrane (20), and that the histamine-induced release of CA from the
adrenal medulla requires extracellular Ca²⁺ (3).

The release of CA by tyramine from the adrenal medulla was also inhibited by D 600
although inhibition was weaker with tyramine than with ACh. This can be taken to indicate
that the entry of Ca²⁺ into chromaffin cells is necessary for the release of CA by tyramine.
Thus, the present results are in accord with those obtained by Rubin and Jaanus (4) that
the presence of Ca²⁺ in perfusion medium was required for the release of CA from the feline
adrenal gland. Indirectly acting sympathomimetic amines including tyramine are capable
of releasing CA from bovine adrenal medullary granules in the absence of extracellular
Ca²⁺ (5, 6), and the displacement of CA by these amines from the storage site in the granules
has been suggested as a release mechanism (6, 21). Thus, the apparent requirement of
the entry of Ca\textsuperscript{2+} for release of CA from the intact adrenal medulla by tyramine should be
explained. Ca\textsuperscript{2+} may play some role in transport of tyramine from the membrane of chro-
maffin cells to that of medullary granules, or in transport of released CA from medullary
granules to outside of chromaffin cells, although in adrenergic nerve terminals tyramine-
induced release of CA has been reported to be independent of extracellular Ca\textsuperscript{2+} (22, 23).
In the splenic nerve, the release process of CA by tyramine does not involve exocytosis (24).
However, data are available indicative of the Ca\textsuperscript{2+}-dependent exocytosis involved in the CA
release by amphetamine from the bovine adrenal medulla (25). Therefore, a Ca\textsuperscript{2+}-dependent
part of the CA release by tyramine as revealed in the present experiments may reflect involve-
ment of exocytosis.

Cyclic AMP caused CA release from the adrenal medulla. However, the release was
slow in onset and long-lasting in duration, which contrasted with the release by ACh, histi-
amine and tyramine. The slow release of CA by cyclic AMP can be attributed to its poor
access to the intracellular site of action. As to the role of cyclic AMP in the secretion of
CA from adrenal chromaffin cells, Berridge (26) has mentioned the following: (1) An increase
in intracellular concentration of cyclic AMP would release Ca\textsuperscript{2+} from intracellular calcium
reservoirs, e.g., mitochondria, and released Ca\textsuperscript{2+} would trigger the release of CA. (2) The
increase in intracellular concentration of cyclic AMP would also stimulate the biosynthesis
of CA. Accepting this hypothesis, the entry of Ca\textsuperscript{2+} across the cell membrane of adrenal
chromaffin cells would not be necessary for CA release by cyclic AMP even if Ca\textsuperscript{2+} is involved
in the secretory process of CA. The ineffectiveness of D 600 in reducing the CA release
by cyclic AMP as demonstrated in the present experiments can be interpreted in a similar
way. The results that even when the gland was perfused with Ca\textsuperscript{2+}-free medium, cyclic
AMP still had the ability to release CA (7) are in good accordance with ours. As clearly
demonstrated by Miledi (27) regarding the release of transmitter from the presynaptic
terminal in the giant synapse of the squid, an increase in intracellular concentration of Ca\textsuperscript{2+}
in adrenal chromaffin cells would be only a prerequisite for release of CA whatever Ca\textsuperscript{2+}
may enter from the extracellular medium or may be released from intracellular reservoirs.
Jaanus and Rubin (8) has reported that cyclic AMP was unable to release CA from the
perfused adrenal medulla, and that an increase in cyclic AMP level followed an increase
in CA release when the medulla was stimulated by ACh. These authors, therefore, concluded
that cyclic AMP would not be directly involved in the medullary secretion in comparison
with the cortical secretion evoked by ACTH in which an increase in cyclic AMP level pre-
ceded an increase in hormone secretion (28). The difference in results concerning the
secretory activity of cyclic AMP between their experiments and ours may be attributed to
difference in methods and experimental animals used.

The ratio of A/CA decreased only when the gland was stimulated by ACh, but not by
histamine, tyramine and cyclic AMP and this decrease in A/CA ratio is in accordance with
the result of Tsujimoto and Nishikawa (29). D 600 did not affect the A/CA ratio of the
spontaneous or evoked CA release; the reduced A/CA ratio of CA evoked by ACh remained
unchanged by D 600.

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