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THE DIFFERENT EFFECTS OF D-600 (METHOXYVERAPAMIL) ON THE RELEASE OF ADRENAL CATECHOLAMINES INDUCED BY ACETYLCHOLINE, HIGH POTASSIUM OR SODIUM DEPRIVATION

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1 Bovine adrenal glands were perfused with Locke solution and catecholamine release was induced by acetylcholine, by a depolarizing concentration of potassium, or by omission of sodium from the perfusion fluid.

2 D-600 (methoxyverapamil) at the concentration of 30 μM produced a 23% inhibition of catecholamine release evoked by acetylcholine (0.1 mM) in the presence of physostigmine (10 μM).

3 A concentration of D-600 of 0.3 mM produced 86% and 85% inhibition in the output of catecholamines in response to acetylcholine and high potassium respectively.

4 D-600 (0.3 mM) failed to block the release of catecholamines evoked by sodium deprivation.

5 The results suggest the involvement of intracellular calcium in the exocytotic release of catecholamines induced by sodium omission.

Introduction

When the adrenal medulla is stimulated by either acetylcholine or depolarizing concentrations of K^+, catecholamines and other soluble components of the chromaffin granules are released to the cell exterior by a process of exocytosis (Smith & Winkler, 1972). The release of catecholamines induced by the above stimuli requires the presence of extracellular Ca^{2+} (Douglas, 1968). In contrast to the above observations, we have recently shown that the omission of Na^+ from the extracellular environment evokes release of catecholamines by exocytosis in the absence of extracellular Ca^{2+} (Lastowecka & Trifaró, 1974).

Methoxyverapamil (D-600) is a drug that blocks both calcium influx in excitable tissues (Fleckenstein, 1971; Fleckenstein, Grun, Tritthart & Byon, 1971; Mayer, van Breemen & Casteels, 1972; Baker, Meves & Ridgway, 1973) and hormone release from the neurohypophysis (Dreifuss, Grau & Nordmann, 1973; Russell & Thorn, 1974). Therefore, the present study was undertaken to examine the effect of D-600 on the release of catecholamines evoked by all of the above stimuli.

Methods

Bovine adrenal glands obtained from a local slaughterhouse were perfused and stimulated *in vitro* as described by Trifaró, Poisner & Douglas (1967).

Perfusion fluids

(a) The main perfusion fluid was phosphate-buffered-Locke solution of the following composition (mM): NaCl 154, $CaCl_2$ 2.2, KCl 2.6, K_2HPO_4 2.15, KH_2PO_4 0.85 and dextrose 10, Na^+-free Locke solution was of the same composition as the main perfusion fluid except that NaCl was replaced by an osmotically equivalent concentration of sucrose; (c) high potassium Locke solution contained 56 mM K^+ of which 53 mM was as KCl and 3 mM as K_2HOP_4 and KH_2PO_4. In this solution NaCl was reduced by an equivalent amount (50.4 mM). All the above solutions contained 0.06% ethanol. This was the final concentration of ethanol required in the experiments with D-600. This concentration of ethanol did not affect either the spontaneous or the evoked release of catecholamines. All solutions were equilibrated with 5% CO_2 in O_2, and the final pH of the solutions was 7.2. The glands were perfused at room temperature (25°C) by means of a multichannel peristaltic pump (Büchler) at a constant rate of flow of 10 ml/minute.

Samples of the perfusate were collected at 1 min intervals in ice-chilled tubes containing 10 μl of 1.9 N

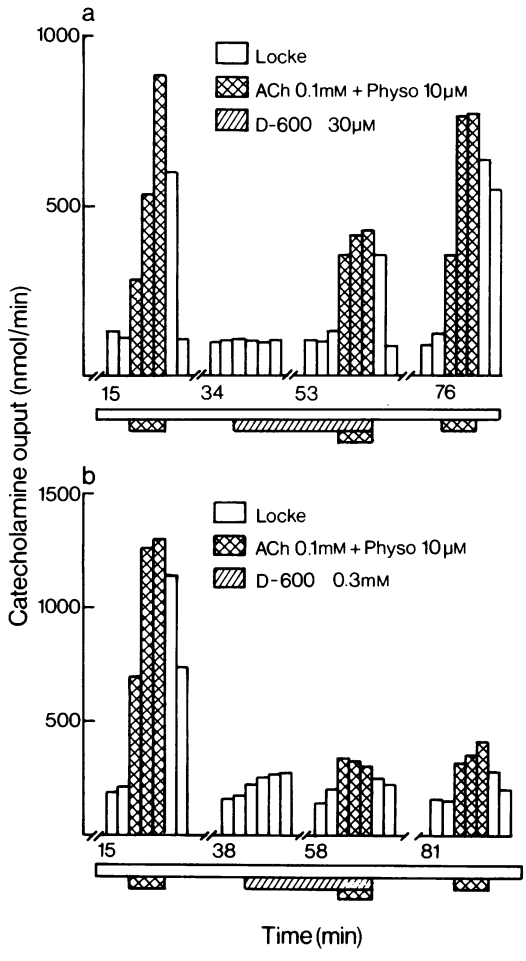

Figure 1 Effect of D-600 on acetylcholine-evoked release of catecholamines. In this, and in all subsequent figures, the graphs show the rate of catecholamine output (nmol/min) from perfused bovine adrenal glands. (a) and (b) show the stimulant effect of acetylcholine (0.1 mM) in the presence of physostigmine (Physo, 10 μM) (cross-hatched areas), during perfusion with Locke solution (open areas) or Locke solution containing D-600 at the concentration of 30 μM (diagonally hatched areas) in (a) and 0.3 mM (diagonally hatched areas) in (b). Glands were perfused at room temperature (25°C) with a flow rate of 10 ml/minute. The perfusing solutions were gassed with a mixture of 5% CO_2 in O_2. Samples were collected from the perfusates at 1 min intervals; these were assayed for catecholamine content as indicated in the methods section.

HCl per ml perfusate. The catecholamine content of the perfusates was determined by the trihydroxyindole fluorometric method (Anton & Sayre, 1962).

Chemicals

The chemicals were obtained from the following sources: physostigmine (eserine) sulphate, Sigma Chemical Company; acetylcholine chloride, Welcker Laboratories; and sucrose (density gradient grade), Schwarz-Mawn. Methoxyverapamil (D-600) was a generous gift from Drs Oberdorf and Sharma, Knoll A.C., Ludwigshafen.

Results

Seven adrenal glands were perfused for three successive periods of 3 min each with Locke solution containing acetylcholine (0.1 mM) and physostigmine (10 μM). Each of these 3 min stimulation periods was separated from the next by 20–25 min perfusion with normal Locke solution. When compared to the first acetylcholine stimulation, catecholamine outputs during the second and third stimulation periods decreased by 10–20 and 20–30% respectively. This decrease in the adrenal medullary responses to successive exposures to secretagogues has been reported previously (Douglas & Rubin, 1961; Lastowecka & Trifaró, 1974). Seven other adrenal glands were also perfused and stimulated as indicated above, but during the second acetylcholine stimulation, D-600 (0.3 mM) was present in the perfusion fluid. D-600 was introduced into the medium 20 min before the second acetylcholine stimulation and it was withdrawn from the perfusion fluid together with the acetylcholine (Figure 1). Perfusion of the adrenals with normal Locke solution containing 0.3 mM D-600 did not produce any significant change in the spontaneous release of catecholamines. Figure 1b shows that the response to acetylcholine was almost completely blocked. Furthermore, it should also be noticed that 20 min after the removal of D-600 from the perfusion medium, the catecholamine output in response to acetylcholine stimulation did not reach the expected values (Figure 1b). Table 1 shows outputs of catecholamines in response to acetylcholine stimulations performed before and during the exposure to D-600. Under these conditions, that is, in the presence of D-600, the catecholamine outputs in response to acetylcholine stimulation were 0.9–17.2% of that obtained during the preceding stimulations in the absence of D-600. This inhibition of the acetylcholine-evoked release of catecholamines was highly significant, as indicated in Table 2. When the concentration of D-600 was one order of magnitude smaller (30 μM) the inhibition of the catecholamine release induced by acetylcholine was 23% ($n=3$) (Figure 1a). Moreover, in contrast to the results obtained in the presence of 0.3 mM D-600 (Figure 1b), the response to the subsequent acetylcholine stimulation completely recovered (Figure 1a).

As in the case of acetylcholine stimulation, the adrenal medullary responses to depolarizing concentrations of K^+ (56 mM) were blocked by D-600. Figure 2 shows that the release of catecholamines in response to 56 mM K^+ was inhibited by D-600 (0.3 mM), and as during acetylcholine stimulation, the responses to high K^+ did not recover until 60 min after the removal of D-600 from the perfusion medium. The degree of inhibition produced by D-600 during stimulation either by acetylcholine or by high K^+ was similar (Table 2).

In contrast to the above observations, D-600 failed to inhibit the release of catecholamines produced by the omission of Na^+ from the extra-cellular medium (Figure 3). The values of the output of catecholamines obtained during Na^+-deprivation, either in the presence or in the absence of D-600, are shown in Table 1. As Table 2 also indicates, D-600 produced no

Table 1 Effect of D-600 on the output of catecholamines (CA) evoked either by acetylcholine (ACh 0.1 mM) or by Na^+ omission. Adrenal glands were perfused during the second stimulation period with Locke solution containing D-600 (0.3 mM)

		Increase in catecholamine release (nmol/min)		
Expt. No.	*Condition*	*1st Stimulation*	*2nd Stimulation + D-600*	*CA released during 2nd stimn. as % of release during 1st stimn. (=100%)*
1	ACh Locke	+ 305	+ 43	14.1
2	ACh Locke	+ 408	+ 70	17.2
3	ACh Locke	+ 511	+ 71	13.9
4	ACh Locke	+ 565	+ 5	0.9
5	ACh Locke	+ 682	+ 30	4.4
6	ACh Locke	+ 767	+ 75	9.8
7	ACh Locke	+ 1079	+ 157	14.6
8	Na^+-free Locke	+ 842	+ 792	94.1
9	Na^+-free Locke	+ 876	+ 933	106.5
10	Na^+-free Locke	+ 939	+ 848	90.3
11	Na^+-free Locke	+ 1428	+ 780	68.6

Table 2 Effect of D-600 on the release of catecholamines (CA) from the adrenal medulla. Adrenal glands were perfused with Locke solution and stimulated in the presence or in the absence of D-600 (0.3 mM) by acetylcholine (0.1 mM) in the presence of physostigmine (10 μM), by 56 mM KCl and by Na^+ deprivation

Condition		*CA released during 2nd stimn. as % of release during 1st stimn. (=100%)*
1st Stimulation	*2nd Stimulation*	
ACh	ACh	84.5 ± 1.2* (n=7)
ACh	ACh + D-600	10.7 ± 2.3† (n=7)
K^+	K^+	91.9 ± 2.2 (n=4)
K^+	K^+ + D-600	13.6 ± 4.5† (n=3)
Na^+-free	Na^+-free	91.1 ± 11.1 (n=13)
Na^+-free	Na^+-free + D-600	89.8 ± 7.9 (n=4)

* Mean ± s.e. mean; n = number of tests.
† $P < 0.001$

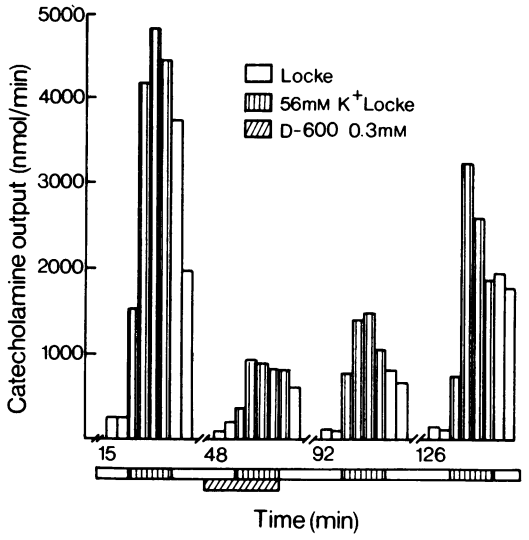

Figure 2 Effect of D-600 on the output of catecholamines induced by a depolarizing concentration of KCl. A bovine adrenal gland was perfused for 17 min with Locke solution (open areas) and was subjected to four periods of stimulation by 56 mM K^+ Locke solution (vertically hatched areas). Twenty min before the second stimulation, D-600 (0.3 mM) was added to the perfusion fluid (diagonal hatching). D-600 was removed from the medium at the end of the second stimulation period. Similar results were obtained with two other glands. Other conditions were as described in Figure 1.

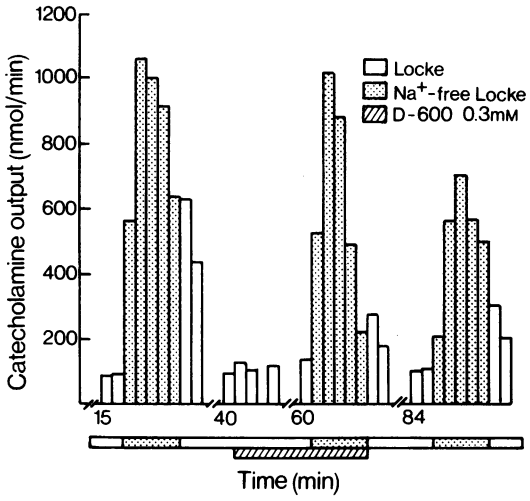

Figure 3 The lack of effect of D-600 on the release of catecholamines produced by the omission of Na^+ from the perfusion fluid. A bovine adrenal gland was perfused alternately with Locke solution (open areas) and Na^+-free Locke solution (stippled areas). During the second perfusion period with Na^+-free Locke solution, 0.3 mM D-600 (diagonally hatched areas) was present in the fluid. Similar results were obtained in three other experiments. Other conditions were as described in Figure 1.

significant changes in the catecholamine output induced by Na^+ omission.

In order to appreciate better the contrasting effects of D-600 on the release of catecholamines induced by either acetylcholine or by Na^+-deprivation, a series of combined experiments were also carried out. One of these experiments is depicted in Figure 4, which clearly shows that D-600 (0.3 mM) blocked acetylcholine-evoked amine release without affecting the release induced by Na^+ omission. Furthermore, the catecholamine output in response to the acetylcholine stimulation which followed the stimulation by Na^+-deprivation, was still depressed and only reached normal values of output during the subsequent acetylcholine stimulation. This stimulation was applied approximately 60 min after the removal of D-600 from the perfusion medium (Figure 4).

Discussion

The requirement of extracellular Ca^{2+} for the release of catecholamines from the adrenal medulla induced by acetylcholine or by depolarizing concentrations of K^+ is well established (Douglas, 1968; Smith & Winkler, 1972). Furthermore, the blocking effect of Mg^{2+} in the release process of the adrenal medulla is also well documented (Douglas, 1968; Smith & Winkler, 1972). Similar results on the need of extracellular Ca^{2+} and on the blocking effect of Mg^{2+} have been obtained for other secretory tissues (Rubin, 1974). Although it is still not clear where calcium ions are required in the secretory process, there is a suggestion that under physiological conditions, Ca^{2+} entry might be the first step in stimulus-secretion coupling. However, under certain experimental conditions, as for example during Na^+-deprivation, it is possible to induce release of catecholamines from the adrenal medulla during perfusion with solutions devoid of Ca^{2+} (Lastowecka & Trifaró, 1974). Since Na^+-deprivation, like acetylcholine, induces release by exocytosis, calcium ions might be involved in this process (Lastowecka & Trifaró, 1974). These calcium ions could originate from either intracellular, or perhaps even extracellular sources, since it is difficult to produce a state in which the tissue is free of extracellular Ca^{2+} because some Ca^{2+} may leak from adjacent tissue structures (Thorn, 1974). This holds for perfusion experiments with calcium-free medium, even in the presence of chelating agents

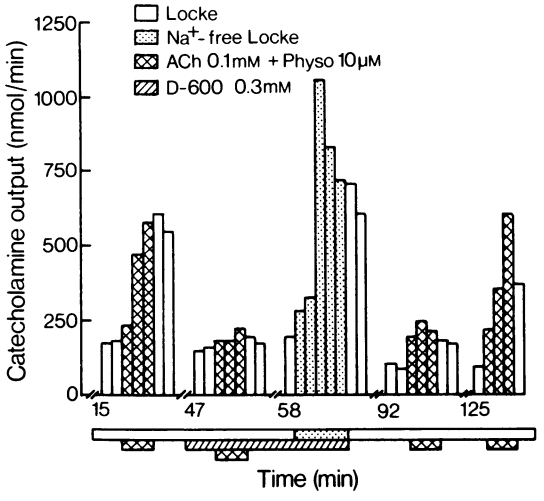

Figure 4 Contrasting effects of D-600 on the release of catecholamines induced by acetylcholine and by Na^+-deprivation. A bovine adrenal gland was perfused with Locke solution (open areas) and was subjected to four periods of stimulation by acetylcholine (cross hatched areas). Twenty min before the second acetylcholine stimulation 0.3 mM D-600 (diagonally hatched areas) was added to the perfusion fluid. During the last 5 min of perfusion with Locke solution containing D-600, the NaCl of the medium was replaced by an osmotic equivalent amount of sucrose (stippled areas). Similar results were obtained in three other experiments. Other conditions were as described in Figure 1.

(EDTA or EGTA). Although the amount of Ca^{2+} may be small, it could be large enough to enter the cell and trigger catecholamine release, especially in a condition such as Na^+-deprivation. Experiments carried out in other excitable tissues, as for example the squid axon and the heart muscle, have shown that, during Na^+ omission from the extracellular environment, there is an increased Ca^{2+} entry (Baker & Reuter, 1975). Because of all of the above reasons, it was thought that methoxyverapamil (D-600) might be a useful tool in order to obtain more information on the role of calcium ions in the release of catecholamines evoked by different agents and conditions. D-600 has been shown to inhibit both the release of vasopressin and oxytocin from the neurohypophysis (Dreifuss *et al.*, 1973; Russell & Thorn, 1974; Thorn, 1974). In addition, D-600 blocks the ^{45}Ca uptake by the neurohypophysis (Dreifuss *et al.*, 1973), the transmembrane slow calcium current in cardiac fibres (Kohlhardt, Baüer, Krause & Fleckenstein, 1972) and the influx of calcium through the 'late calcium channel' in the squid axon (Baker *et al.*, 1973; Baker & Reuter, 1975).

Our results provide new evidence on the blocking effect of D-600 on another endocrine tissue. Perfusion of adrenal glands with D-600 at a concentration of 0.3 mM produced a 85–86% inhibition of the responses to both acetylcholine and high K^+ stimulation. The concentration of D-600 required to produce a blockage in our experiments was somewhat greater than that necessary to block the responses in other tissues: 0.19 mM D-600 blocked calcium entry in the squid axon (Baker *et al.*, 1973); and D-600 concentrations of 20 μM and 50 μM inhibited the release of hormones from the neurohypophysis in response to depolarizing concentrations of potassium and to electrical stimulation respectively (Dreifuss *et al.*, 1972; Russell & Thorn, 1974). The lower sensitivity of the adrenal medullary tissue to D-600 may be due either to a low affinity of D-600 for the chromaffin cell or to a high density of calcium channels in this tissue. If this latter possibility were the case, it would provide an explanation for the discrepancy between the findings in the neurohypophysis and in the adrenal medulla. A very small amount of calcium seems to be required for the release of hormone in the neurohypophysis (Russell & Thorn, 1974; Thorn, 1974), whereas release of catecholamines from the adrenal medulla is accompanied by a large uptake of calcium (Douglas & Poisner, 1962).

In spite of the differences between the optimal blocking concentrations of D-600 in the neurohypophysis (Russell & Thorn, 1974; Thorn, 1974) and in the adrenal medulla, the slow reversibility (about 60 min) of the blocking effect of the drug was similar in both tissues.

Since Ca^{2+} entry through the late calcium channel of the squid axon is blocked by the same agents (i.e., D-600, Mg^{2+}, Mn^{2+}, etc.) which block secretion, Baker *et al.* (1973) have suggested that Ca^{2+} may enter the secretory cells through a channel of properties similar to those of the late calcium channel of the squid axon. The present results, showing that D-600 blocks both acetylcholine and high K^+-induced release of catecholamines, are in agreement with Baker's suggestion. The failure of D-600 to block the release of catecholamines in response to Na^+-deprivation would suggest that a mechanism other than Ca^{2+} entry might be involved in this process. Therefore, if we assume that, in response to different types of stimulation, release by exocytosis involves similar cellular and molecular mechanisms, the possibility of an increase in intracellular Ca^{2+} during Na^+-omission must be considered. Recently published results which show that iontophoretic administration of Ca^{2+} into nerve terminals (Miledi, 1973) and into mast cells (Kanno, Cochrane & Douglas, 1973) induces acetylcholine and histamine release respectively, seem to indicate that an increase in the intracellular levels of Ca^{2+} is a necessary event in

stimulus-secretion coupling. Therefore, if the omission of Na^+ from the extracellular environment induces exocytotic release of catecholamines by increasing intracellular Ca^{2+}, the lack of a blocking effect of D-600 is to be expected.

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