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Differential effects of D600, nifedipine and dantrolene sodium on excitation-secretion coupling and presynaptic β-adrenoceptor responses in rat atria

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1 The stimulation-evoked release of tritium was measured from rat atria labelled with [^{3}H]-noradrenaline. The calcium dependence of evoked release and the facilitation of this release via activation of presynaptic β-adrenoceptors were examined using D600 (methoxyverapamil), nifedipine and dantrolene sodium.

2 Both D600 and nifedipine at dose levels of 20 and 100 μM inhibited evoked release. Dantrolene (20, 100 μM) reduced release by 25%, the effect being maximal at 20 μM.

3 In the presence of 20 nM isoprenaline, a facilitation of evoked release occurred, which was blocked by 0.1 μM (−)-propranolol.

4 The facilitatory action of isoprenaline was abolished by omission of calcium from the buffer, or by D600 or nifedipine, (100 μM). In contrast, the response to isoprenaline was not modified by dantrolene (20, 100 μM).

5 It is concluded that (a) the evoked release of noradrenaline (NA) utilizes Ca from both intra- and extracellular sources and that (b) isoprenaline increases NA secretion by promoting the depolarization-induced influx of Ca.

Introduction

The depolarization-induced secretion of noradrenaline (NA) from noradrenergic neurones and from the adrenal medulla occurs by exocytosis, a process which requires extracellular Ca (Trifaro, 1977). For activation of the secretory process the external Ca must first enter the cell (Miledi, 1973) through voltage-sensitive Ca channels in the nerve terminal membrane (Baker, 1974). It has been shown that drugs which elevate intracellular cyclic AMP levels induce the exocytotic release of catecholamines from the adrenal medulla (Boonyaviroj & Gutman, 1977) and since external Ca is not required for the action of these drugs it has been suggested that cyclic AMP interferes with the storage in, or promotes the release of calcium from subcellular organelles, thereby increasing intracellular levels of ionized Ca (Cohen & Gutman, 1979). It is, therefore, possible that the exocytotic release of NA from sympathetic nerves could involve a contribution from the release of intracellular Ca.

The quantity of NA released during depolarization of the sympathetic nerves supplying certain tissues can be enhanced by activation of β-adrenoceptors located presynaptically on the nerve terminal (Adler-Graschinsky & Langer, 1975; Stjärne & Brundin, 1975; Majewski *et al.*, 1980). It has been proposed that the presynaptic β-receptor response is mediated by cyclic AMP (Adler-Graschinsky & Langer, 1975) but it is not clear whether Ca is involved.

In the present study the site(s) in sympathetic nerves where Ca is made available for excitation-secretion coupling were investigated using the slow Ca channel blocking agents D600 (methoxyverapamil) and nifedipine (Fleckenstein, 1977) and dantrolene which interferes with the release of intracellular bound Ca (Ellis & Carpenter, 1972). In addition, the role of Ca in the presynaptic β-adrenoceptor-mediated facilitation of NA release was examined.

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Methods

Wistar rats of either sex (200–400 g) were stunned by a blow to the head and their necks broken. Hearts were removed and the atria carefully dissected free from surrounding tissue. Atria were preincubated for 10 min at 37°C in gassed Locke solution of the following composition (mM): NaCl 154.0, KCl 5.6, $CaCl_2$ 2.2, $NaHCO_3$ 6.0, glucose 11.1, ascorbic acid 0.11 and EDTA 0.04. Each pair of atria was then transferred to 1 ml Locke solution and incubated for a further 30 min with 7 μCi 1-[7,8-^{3}H] noradrenaline (0.5–0.9 μM), specific activity 8–15 Ci mmol^{-1} (Amersham). Following the incubation period each tissue was washed rapidly in a 10 ml organ bath. Superficially bound radioactivity was washed off by replacing the fluid in the bath with fresh solution at 5 min intervals for 65 min. After the washing period the fluid in the bath was further replaced at 5 min intervals throughout the experiment. Each sample was analysed for tritium in an Intertechnique SL 20 liquid scintillation counter, using a scintillation mixture of the following composition (per litre): toluene 666 ml, Triton X-100 333 ml, PPO (2,5-diphenyloxazole) 5.5 g and POPOP (1,4-bis- 2-(5-phenyloxazolyl) -benzene) 0.1 g. Field stimulation was applied using stainless steel wire electrodes placed above and below the atria.

Three stimulation periods were applied during each experiment at t = 15 min (S_1), t = 40 min (S_2) and t = 65 min (S_3). Each stimulation period consisted of 300 shocks delivered at a frequency of 1 Hz, 0.2 ms pulse width and output voltage 50V. At the end of each experiment tissues were solubilized for determination of tissue radioactivity. Antagonist drugs were administered 15 min before S_2 and were present for the remainder of the experiment. Isoprenaline was administered 15 min before S_3.

Calculations and statistics

Results are expressed as means ± s.e.mean for the fractional release (△t) of tritium per shock for S_2 or S_3 relative to that for S_1 (Langer, 1977). Significant differences between group means were assessed using the analysis of variance model (one way). In one comparison (100 μM nifedipine vs 100 μM nifedipine plus 20 nM isoprenaline) the Fisher-Behrens test was used because data failed to satisfy the condition of homogeneity of variance (Campbell, 1974).

Drugs

The following drugs were used: (−)-isoprenaline(+)-bitartrate (Sigma), (−)-propranolol (I.C.I.), D600 HCl (Knoll), nifedipine (Bayer), dantrolene-sodium (Eaton Laboratories), tetrodotoxin (Sigma). All drugs were made up in distilled water immediately before use, except for nifedipine and dantrolene which were dissolved in propylene glycol: water, 9:1. Concentrations of propylene glycol up to 1% (v/v) did not interfere with transmitter release; the highest concentration of propylene glycol used in the present experiments was 0.9%. All experiments with nifedipine were carried out in a darkened room with a sodium light source.

Results

Evoked release: effects of isoprenaline

Electrical stimulation of rat atria preloaded with [^{3}H]-NA evoked the secretion of transmitter as judged by the increase over background of tritium overflowing into the bathing solution. Addition of 2 μM tetrodotoxin to the bath abolished the response to nerve stimulation, indicating that the tritium was of neuronal origin. In a series of control experiments the evoked release of tritium did not decline significantly over three successive stimulation periods. Administration of isoprenaline (20 nM) resulted in a significant increase in the amount of [^{3}H]-NA released (control $S_3/S_1 = 0.93 \pm 0.04$, $n = 9$; isoprenaline $S_3/S_1 = 1.24 \pm 0.09$, $n = 5$. $P < 0.001$, see Figure 1). The release-enhancing response to isoprenaline was abolished by prior administration of 0.1 μM (−)-propranolol.

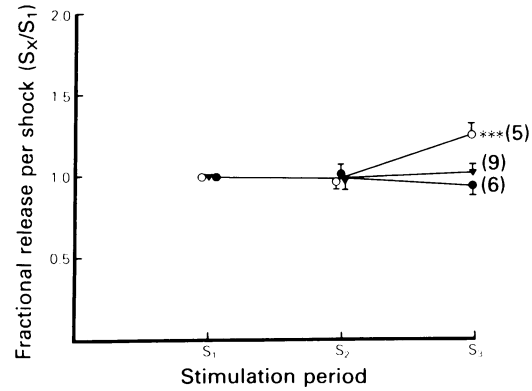

Figure 1 The enhancement of stimulation-evoked release of [^{3}H]-noradrenaline from rat atria by 20 nM isoprenaline and its antagonism by 0.1 μM (−)-propranolol. S_1–S_3 indicate stimulation periods. Results are expressed as the ratios of fractional release per shock between each period of stimulation (S_x) and the first (S_1). (▼) Control; (○) isoprenaline; (●) isoprenaline plus (−)-propranolol. Vertical bars show the s.e.mean and parentheses indicate the number of experiments. ***$P < 0.001$ compared with control.

Table 1 Effects of Mn and EGTA on the fractional release of ^{3}H-transmitter ($\times 10^{-5}$) from rat atria exposed to Ca-free Locke solution.

Solution	S_1 (Δt/shock)	S_2 (Δt/shock)	S_3 (Δt/shock)
Ca-free	3.08±0.30	0.11±0.04	0.72±0.15**
Ca-free + Mn	1.91±0.52	0.05±0.05	0.11±0.08
Ca-free + EGTA	2.40±0.51	0.20±0.12	0.27±0.18

The data represent the fraction of total tissue radioactivity released during each stimulation period when Ca-free buffer was substituted for normal Locke solution 15 min before S_2 and was present for the remainder of the experiment. The concentrations of Mn and EGTA used were 2.2 and 0.1 mM respectively. Values given are the means±s.e.mean of 4 experiments. $^{**}P<0.01$ when compared with S_2 (Student's t test).

Effects of Ca depletion

When tissues were bathed in Ca-free Locke solution, the fraction of total tissue [^{3}H]-NA released per shock during nerve stimulation ($\times 10^{-5}$) fell from 3.08±0.3, $n=4$ for S_1 to 0.11±0.04 for S_2. However, 25 min later the value for S_3 had recovered somewhat to 0.72±0.15 although it was still significantly depressed ($P<0.01$). Inclusion of 2.2 mM $MnCl_2$ or 0.1 mM EGTA in the Ca-free Locke solution prevented the partial recovery of the response to nerve stimulation (Table 1). When Ca was omitted from the bathing solution, isoprenaline did not facilitate transmitter release (see Figure 2).

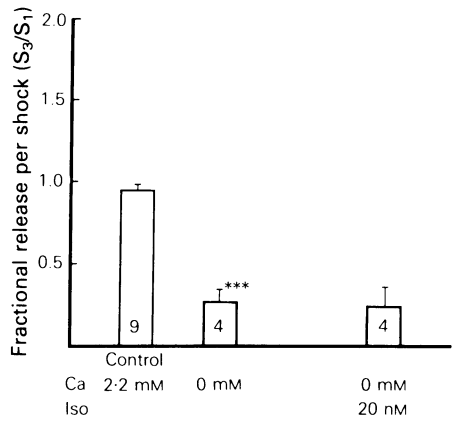

Figure 2 Effects of Ca depletion on the stimulation-evoked overflow of [^{3}H]-noradrenaline and on the response to isoprenaline (Iso). Columns represent mean ratios of fractional release per shock between the third and first stimulation periods (S_3/S_1) under the different treatments. Vertical bars indicate the s.e.mean. The number of experiments in each group is indicated in the column. $^{***}P<0.001$ when compared with control group.

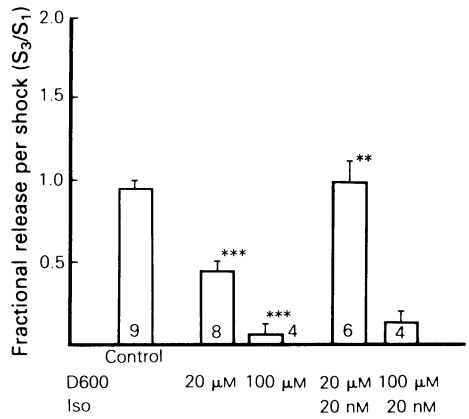

Figure 3 Effects of D600 on the stimulation-evoked overflow of [^{3}H]-noradrenaline and on the response to isoprenaline (Iso). Columns represent the mean ratio of fractional release per shock between the third and first stimulation periods (S_3/S_1) for the different treatments. Vertical bars indicate the s.e.mean. The number of experiments in each group is indicated in the column. $^{***}P<0.001$ when compared with control group. $^{**}P<0.01$ when compared with antagonist alone.

Effects of the Ca channel inhibitors D600 and nifedipine

D600 (20, 100 μM) dose-dependently reduced the stimulation-evoked overflow of ^{3}H-transmitter by 47% and 96%, respectively (see Figure 3). In the presence of the lower dose, isoprenaline significantly increased transmitter overflow ($P<0.01$). However the response to isoprenaline was abolished by pretreatment with 100 μM D600 (Figure 3).

A significant reduction in the amount of [^{3}H]-NA released by electrical stimulation was obtained with 20 and 100 μM nifedipine (Figure 4). The response to stimulation compared to that obtained in a control period was reduced to 0.77±0.04 ($n=5$) and 0.67±0.06 ($n=6$) for the low and high doses respectively. When isoprenaline was administered with the low dose of nifedipine, a small but statistically significant ($P<0.05$) increase in the fractional release of [^{3}H]-NA occurred. In the presence of 100 μM nifedipine, isoprenaline had no effect (Figure 4).

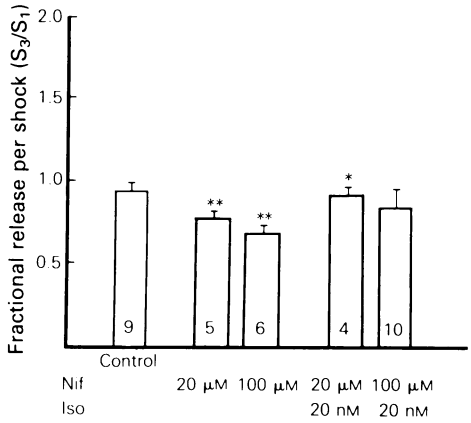

Figure 4 Effects of nifedipine (Nif) on the stimulation-evoked overflow of [^{3}H]-noradrenaline and on the response to isoprenaline (Iso). Columns represent mean ratios of fractional release per shock between the third and first stimulation periods (S_3/S_1). Vertical bars indicate the s.e.mean. The number of experiments in each group is indicated in the column. $^{**}P<0.01$ when compared with control group, $^{*}P<0.05$ when compared to antagonist alone.

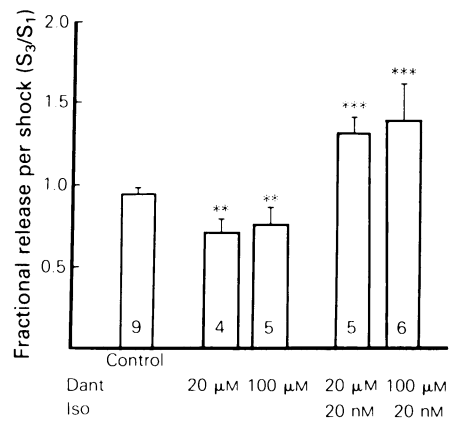

Figure 5 Effects of dantrolene sodium (Dant) on the stimulation-evoked overflow of [^{3}H]-noradrenaline and on the response to isoprenaline (Iso). Columns represent the mean ratios of fractional release per shock between the third and first stimulation periods (S_3/S_1) for the different treatments. Vertical bars indicate the s.e.mean. The number of experiments in each group is indicated in the column. $^{**}P<0.01$ when compared with control group. $^{***}P<0.001$ when compared with antagonist alone.

Effects of dantrolene

The two concentrations of dantrolene used (20, 100 μM) inhibited the stimulation-evoked overflow of [^{3}H]-NA to the same extent (S_3/S_1 values were 0.70 ± 0.09, $n=4$ and 0.75 ± 0.10, $n=5$ respectively; see Figure 5). The isoprenaline-induced increase in evoked release was not affected by dantrolene (Figure 5).

Discussion

Noradrenaline secretion

It is well known that the presence of Ca in the external medium is essential for the release of transmitter NA by a depolarizing stimulus (Hukovic & Muscholl, 1962; Boullin, 1967). Thus the finding that removal of Ca from the bathing Locke solution inhibited evoked [^{3}H]-NA release from rat atria was expected. However, the effect of Ca depletion on the stimulation-induced overflow of NA was time-dependent; evoked release was abolished 15 min after removal of Ca but had recovered to 22% of the control value after a further 25 min. A possible explanation for the partial recovery of the release response to nerve stimulation is that mere omission of Ca from the external medium is not sufficient to deplete completely extracellular sources of Ca. This explanation is supported by the findings that inclusion of Mn or EGTA in the Ca-free Locke solution prevented the partial recovery of the response to nerve stimulation (see Table 1).

In the present experiments it was decided to investigate whether the Ca used in the exocytotic release of NA was derived solely from extracellular sources or whether intracellular Ca was also involved. To clarify this question, two drug types were used: (i) D600 and nifedipine which are reported to inhibit the transmembrane influx of Ca through voltage-dependent 'slow' Ca channels (Fleckenstein, 1971; Kohlhardt *et al.*, 1972) and (ii) dantrolene-sodium which has been reported to inhibit the depolarization-induced release of Ca from intracellular storage sites (Ellis & Carpenter, 1972; Desmedt & Hainaut, 1979).

The Ca channel blockers D600 and nifedipine both inhibited the release of [^{3}H]-NA evoked by nerve stimulation (Figures 3 and 4), the effect being most clearly dose-dependent for D600. Previous reports have indicated that the concentration of D600 required to inhibit NA secretion (Göthert *et al.*, 1979) or neuronal Ca fluxes (Baker *et al.*, 1973), is of the order of 10–100 μM. In the present study the concentration of D600 required to produce half-maximal inhibition of [^{3}H]-NA release (IC_{50}) was estimated to be 30 μM, as judged from the data presented in Figure 3. As with D600, high concentrations of nifedipine (20–100 μM) were needed to in-

hibit evoked release. In a study by Högestätt *et al.* (1982) nifedipine failed to alter K-evoked [^{3}H]-NA efflux from arteriolar preparations. However, the highest concentration of nifedipine used in the latter experiments was 3 μM, and in view of the present results a higher concentration might have been effective. Ca currents in smooth and cardiac muscle are generally blocked by 0.01–0.1 μM D600 and nifedipine (Triggle & Swamy, 1980). By comparison, neuronal Ca currents are far less sensitive to slow channel inhibitors. At the higher concentrations required to affect neuronal Ca fluxes, some loss of specificity occurs. Thus concentrations of D600 above 10 μM have pronounced membrane stabilizing effects relating to the inhibition of fast Na channels (Baker *et al.*, 1973; Galper & Catterall, 1979). The method used in the present experiments to evoke the secretion of NA was electrical stimulation. It is a condition of impulse propagation that Na channels must be functional. Therefore, the local anaesthetic property of D600 is likely to contribute to the observed inhibition of NA release, whereas the inhibition of NA release by nifedipine was probably solely caused by decreased inward Ca movement during depolarization.

The evoked release of [^{3}H]-NA was partially inhibited by dantrolene, the effect being maximal at the lower concentration used (20 μM) (see Figure 5). According to Desmedt & Hainaut (1979), 35 μM dantrolene does not alter Ca fluxes at the plasma membrane of barnacle muscle cells. If this is also true of neuronal membranes, then the inhibition of NA release obtained is consistent with a dantrolene-induced reduction of Ca release from intracellular Ca-storing organelles. A similar mechanism has been proposed to explain the inhibition by dantrolene of spontaneous acetylcholine release from amphibian nerve terminals (Statham & Duncan, 1976). Since the exocytotic release of NA from sympathetic nerves is entirely dependent on the presence of extracellular Ca (Boullin, 1967), it can be postulated that the release of Ca from intracellular sites is secondary to depolarization-induced Ca entry i.e. it is not caused by depolarization *per se*.

The results obtained with D600, nifedipine and dantrolene indicate that the Ca utilized during the exocytotic release of NA from sympathetic nerves comes from both extra- and intra-cellular sources. Ca entry into the cell seems to be a prerequisite for the release of further Ca from storage sites. Both pools must be accessible for maximal secretion to occur.

Presynaptic β-adrenoceptors

Facilitatory presynaptic β-adrenoceptors have been found in many sympathetically innervated tissues (Majewski, 1983). The criteria adopted for the identification of these receptors include a β-agonist-induced facilitation of NA released on low frequency nerve stimulation, and blockade of this effect by β-adrenoceptor blocking agents. When rat isolated atria prelabelled with [^{3}H]-NA were exposed to 20 nM isoprenaline, the amount of ^{3}H-transmitter released by nerve stimulation increased by 25% (Figure 1). Furthermore, the facilitatory response to isoprenaline was abolished by 0.1 μM (−)-propranolol. It can therefore be concluded that presynaptic β-adrenoceptors are present in this preparation.

The facilitatory action of isoprenaline on NA release differs from that of indirectly acting sympathomimetics in that isoprenaline only facilitates release evoked by depolarizing stimuli such as electrical stimulation (Adler-Graschinsky & Langer, 1975; Majewski *et al.*, 1980; 1981; Dahlöf *et al.*, 1980) or high K^+ (Weinstock *et al.*, 1978) and does not affect background efflux. Furthermore nerve terminal depolarization is not a sufficient condition for isoprenaline to promote NA secretion because when Ca is omitted from the external medium isoprenaline no longer enhances release (Figure 2). This suggests that the response to isoprenaline is Ca-dependent.

In order to see whether the release-enhancing response to isoprenaline required intra- or extracellular Ca, the response to isoprenaline was tested in the presence of the Ca channel blockers D600 and nifedipine and in the presence of dantrolene. When 20 μM D600 was present in the bathing fluid, isoprenaline caused a significant enhancement of evoked [^{3}H]-NA release. In the presence of 100 μM D600, isoprenaline had no effect (Figure 3). The results obtained with nifedipine were qualitatively similar: in the presence of 20 μM nifedipine isoprenaline produced a small but significant increase in the evoked release of [^{3}H]-NA whereas 100 μM nifedipine completely blocked the isoprenaline response (Figure 4). It is unlikely that the effect of D600 is due to β-adrenoceptor blockade since D600 has been found not to compete with [^{3}H]-dihydroalprenolol for β-adrenoceptor binding sites in rat ventricular membranes (Karliner *et al.*, 1982). The present results suggest that isoprenaline may enhance the stimulation-evoked release of NA by facilitating the opening of Ca channels in the nerve terminal membrane in response to a depolarizing stimulus.

The contribution of Ca from intracellular sites to the release-enhancing effect of isoprenaline was assessed using dantrolene. In concentrations up to 100 μM dantrolene had no effect on the response to isoprenaline (Figure 5), suggesting that the latter does not mobilize the release of Ca from subcellular storage organelles.

Central to the interpretation of the foregoing re-

sults is the assumption that the antagonists used influence Ca movements in accordance with their reported mechanisms of action. In support of this assumption, Cohen & Gutman (1979) demonstrated that raised extracellular K levels and acetylcholine stimulated catecholamine release from the adrenal medulla by a verapamil-sensitive mechanism. They further showed that theophylline induced the release of catecholamines by a dantrolene-sensitive mechanism which was unaffected by verapamil. Stjärne & co-workers (1978) have proposed that NA secretion can be regulated in two ways, (1) by controlling the invasion of the terminal varicosities of each neurone by impulses (recruitment) or (2) by adjusting the local secretory efficiency of each nerve terminal to depolarizing stimuli. The results presented here provide evidence that isoprenaline increases NA secretion using the latter mechanism, by promoting the depolarization-induced influx of Ca.

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