ACTIVATION OF PREJUNCTIONAL β-ADRENOCEPTORS IN RAT ATRIA BY ADRENALINE APPLIED EXOGENOUSLY OR RELEASED AS A CO-TRANSMITTER

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1 Adrenaline (10 nM) significantly enhanced the stimulation-induced efflux of radioactivity from rat atria previously incubated with [^{3}H]-noradrenaline ([^{3}H]-NA). This effect was abolished by metoprolol (0.1 μM).

2 Adrenaline in a higher concentration (1 μM) and NA (1 μM) significantly reduced the stimulation-induced efflux of radioactivity. However, in the presence of phenoxybenzamine (10 μM), adrenaline (1 μM) enhanced the efflux, whereas NA (1 μM) had no effect.

3 In rat isolated atria pre-incubated with adrenaline and then incubated with NA, both catecholamines were taken up and were released by field stimulation. When pre-incubation was with adrenaline and incubation was with [^{3}H]-NA, metoprolol decreased the stimulation-induced efflux of radioactivity. This effect did not occur if the atria were pre-incubated with NA instead of adrenaline, suggesting that neuronally released adrenaline activates prejunctional β-adrenoceptors.

4 In conscious rats, intravenously administered adrenaline (6.0 and 0.6 nmol/kg) was taken up and retained in the atria and could be released by field stimulation. The release was calcium-dependent from these rats up to 24 h after administration.

Introduction

There is now a substantial body of evidence supporting the existence at sympathetic nerve terminals of catecholamine receptor-mediated mechanisms which modulate the amount of transmitter noradrenaline (NA) released by each nerve impulse (see review by Rand, McCulloch & Story, 1980). One of these mechanisms is a facilitatory system mediated through prejunctional β-adrenoceptors. These prejunctional β-adrenoceptors resemble the β$_2$-subtype (Stjärne & Brundin, 1976; Dahlöf, Ljung & Åblad, 1978). NA is a much weaker β$_2$-adrenoceptor agonist than adrenaline (Lands, Arnold, McAuliff, Luduena & Brown, 1967), and the latter would be expected to be more potent on prejunctional β-adrenoceptors than NA. Indeed, adrenaline but not NA facilitated the release of transmitter NA from rabbit carotid sinus (Pham, In Sokan, Pham & Basile, 1978) and from rat portal vein in the presence of phenoxybenzamine (Westfall, Peach & Tittermary, 1979). However, in the phenoxybenzamine-treated rat portal vein, Dahlöf et al. (1978) were able to demonstrate a facilitation of transmitter NA release by NA itself but it was about 100 times less potent than adrenaline. Low concentrations (0.5–10 nM) of adrenaline have been shown to activate prejunctional β-adrenoceptors in human omental blood vessels (St-

järne & Brundin, 1975), rat portal vein (Westfall et al., 1979), rabbit carotid sinus (Pham et al., 1978) and guinea-pig atria (Majewski, McCulloch, Rand & Story, 1980a). The aim of the first part of this study was to determine whether adrenaline or NA could activate prejunctional β-adrenoceptors in rat isolated atria incubated with [^{3}H]-NA.

Adrenaline can be taken up into sympathetic nerves (Iversen & Whitby, 1962) and once incorporated into the nerve terminals, may be released as a co-transmitter with NA. When adrenaline is released along with NA, the feedback modulation of transmission may differ from that observed when only NA is released, since adrenaline has a different spectrum of pharmacological activity, being a more potent β$_2$-adrenoceptor agonist than NA (Lands et al., 1967). This possibility has been investigated in vitro in guinea-pig atria preincubated with adrenaline and then incubated with [^{3}H]-NA: in this preparation, several β-adrenoceptor blocking drugs reduced the stimulation-induced efflux of radioactivity, but had no effect when the pre-incubation was with NA (Rand, Majewski, McCulloch & Story, 1978; Majewski et al., 1980a). This result suggests that neuronally-released adrenaline can activate prejunctional β-adrenoceptors, thus completing a positive

feedback system which facilitates transmitter release. The second part of the present study is concerned with the uptake and release of adrenaline in rat atria *in vitro* and with the operation of a β-adrenoceptor-coupled facilitatory feedback loop in transmitter release in rat atria.

Adrenaline released as a co-transmitter may contribute to the facilitatory effect of circulating adrenaline on sympathetic transmitter release since it is possible that circulating adrenaline may be taken up and retained in sympathetic nerve endings and then be released in sufficient quantities to activate prejunctional β-adrenoceptors. It is possible that during brief periods of increased secretion of adrenaline from the adrenal medullae there would be increased adrenaline uptake by sympathetic nerves and its subsequent release as a co-transmitter could activate prejunctional β-adrenoceptors well after the circulating adrenaline had returned to basal levels. This hypothesis was investigated in conscious rats which were given intravenous injections of [³H]-adrenaline. The time courses of the disappearance of the [³H]-adrenaline from the circulation and its retention in the atria were determined and the subsequent release of the [³H]-adrenaline from the atria was also studied.

Methods

Rat atria incubated *in vitro with adrenaline and noradrenaline*

In these experiments atria from untreated rats were dissected free and placed in an organ bath containing Krebs-Henseleit solution. The solutions in the organ bath and in the reservoir supplying the organ bath were aerated with a mixture of 5% CO_2 in O_2 and maintained at 37°C. The atria were then incubated with either [³H]-NA, [³H]-adrenaline or a combination of either [³H]-adrenaline and NA or adrenaline and [³H]-NA.

[³H]-noradrenaline and [³H]-adrenaline labelling

After 15 min of equilibration the atria were incubated with either [³H]-(−)-NA (2 μCi/ml, 0.1 μM) for 20 min or (−)-adrenaline-[N-methyl-³H] (4.6 μCi/ml, 0.1 μM) for 10 min and then washed repeatedly for 60 min with catecholamine-free Krebs-Henseleit solution to remove loosely bound tritiated compounds. In some experiments, cocaine (100 μM) and in some cases phenoxybenzamine (10 μM) was added 15 min before the end of the washing procedure and then remained present for the duration of the experiment. The cocaine was used to prevent displacement of tritiated catecholamines

from the transmitter storage vesicles by the unlabelled catecholamine which was used subsequently.

Combined incubation with adrenaline and noradrenaline

These experiments were designed to incorporate both adrenaline and NA into the transmitter stores of the sympathetic nerves of rat atria and then to investigate the effects of drugs on [³H]-NA release.

[³H]-adrenaline labelling Rat atria were dissected and equilibrated as described above. The atria were then pre-incubated with [³H]-(−)-adrenaline (0.46 μCi/ml, 2 or 10 nmol/l) for 30 min and washed with drug-free solution for 30 min to remove loosely bound adrenaline. Then the atria were incubated with unlabelled NA (0.3 μM) for 10 min and washed in drug-free solution for 60 min to remove loosely bound catecholamines.

[³H]-noradrenaline labelling Rat atria were dissected and equilibrated as described above, pre-incubated in either unlabelled adrenaline (2 or 10 nM) or NA (10 nM), after which they were washed for 30 min, and incubated in [³H]-(−)-NA (4 μCi/ml, 0.3 μM) for 10 min, then washed in drug-free Krebs-Henseleit solution for 60 min.

Stimulation procedure and measurement of radioactive efflux

The stimulation-induced (S-I) efflux of radioactivity from tissues labelled with [³H]-NA represents the sum of [³H]-NA efflux and the efflux of ³H-metabolites which are formed subsequent to its release; this provides a better index of actual transmitter release than measurements of [³H]-NA (Langer, 1970). In all experiments in which the S-I efflux was measured, the intramural nerves of the atria were stimulated through a pair of parallel platinum electrodes (situated on either side of the atria) at a frequency of 2 Hz for 30 s with 1 ms square wave pulses at supramaximal voltage for stimulating the nerves. The first period of stimulation was given 12 min after the 60 min period of washing and a second period of stimulation was given 30 min after the first. The effect of drugs on the S-I efflux of radioactivity was determined by adding them to the Krebs-Henseleit solution bathing the atria 24 min before the second period of stimulation.

The Krebs-Henseleit solution bathing the atria was collected after 3 min periods of contact with the atria for the determination of the efflux of radioactivity from the tissue, five consecutive collections being made, starting 9 min before each stimulation was applied. Radioactivity was measured by liquid scintil-

lation counting. The resting radioactive efflux was taken as the mean radioactive content of the sample of the bathing solution collected during the three 3 min periods immediately preceding the period of stimulation. The stimulation-induced component of the radioactive efflux was calculated by subtracting the resting efflux from the radioactive content of the samples collected in the 3 min period in which stimulation was applied. In each experiment the S-I efflux for the second period of stimulation was calculated as a percentage of the corresponding efflux for the first period ($\%S_2/S_1$). In some experiments the S-I efflux was expressed as a percentage of the total radioactivity present in the tissue at the start of the stimulation procedure. The radioactivity present in the atria was determined by adding the atria to a vial containing 1 ml of Soluene 350 (Packard Instruments) and when solubilized, 10 ml of liquid scintillation fluid was added and the radioactivity estimated by liquid scintillation counting. The results are expressed as radioactivity (d/min) per atria or, where specifically stated, per g of tissue (wet wt.).

There were minor procedural differences in experiments with atria which were incubated with [³H]-NA alone. The first period of stimulation was given 15 min after the 60 min period of washing and the second stimulation was 35 min after the first. Drugs were added 28 min before the second period of stimulation. Further, only three consecutive samples of the bathing solution were made, starting 6 min before each stimulation, and the resting efflux was defined as the mean of the radioactive content of the two samples of bathing solution collected before stimulation.

Estimation of radioactivity in collections of tissue bathing solution

The amount of radioactivity present in the samples of the Krebs-Henseleit solution bathing the atria was estimated by placing 1 ml aliquots in vials containing 0.1 ml HCl (6 M) and 10 ml of scintillation solution. The radioactivity (counts/min) was measured in a Packard Tricarb 3380 scintillation counter and expressed as disintegrations per min (d/min) after correction for counting efficiency. Counting efficiency as determined by automatic external standardization (AES) ranged from 20 to 25%.

Adrenaline administration and blood sampling in conscious rats

Female hooded rats (180 to 220 g) were anaesthetized with an intraperitoneal injection of a mixture of sodium amylobarbitone (62.4 mg/kg) and sodium methohexitone (24.9 mg/kg). Polyethylene cannulae were inserted into the left common carotid artery and

the right external jugular vein and were exteriorized at the back of the neck. The rats recovered from the anaesthesia within 4 h; 24 h later the rats were given 0.2 ml of ($-$)-adrenaline-[N-methyl-³H] (0.6 or 6.0 nmol/kg; 2.5 µCi/ml, i.v.). At various times after administration of [³H]-adrenaline, 0.2 ml of blood was taken from the carotid artery and added to a centrifuge tube containing 1 ml of ice cold saline (with 250 i.u. of heparin and 0.1 mg of ascorbic acid). The plasma was separated by centrifugation at 1300 g for 10 min at 4°C, and the [³H]-adrenaline was extracted by an alumina adsorption technique.

Estimation of adrenaline accumulation and stimulation-induced efflux in atria

Rats were anaesthetized with ether at various times after [³H]-adrenaline administration, exsanguinated, and the heart was swiftly removed. The atria were dissected free and set up in an organ bath containing 2.5 ml of Krebs-Henseleit solution. The solutions in the organ bath and in the reservoir supplying the organ bath were aerated with a mixture of 5% CO_2 in O_2 and maintained at 37°C. The atria were held in place by means of two hook-shaped platinum electrodes, the anode (uppermost) piercing the apex of the right atrium and the cathode piercing the apex of the left atrium. The isolated atria were stimulated with monophasic square wave pulses of 1 ms duration at a frequency of 2 or 5 Hz for 30 s. The voltage was supramaximal for stimulating the intramural nerves (12 V/cm). During stimulation, the level of the solution in the organ bath was lowered to prevent the anode making direct contact with the bathing solution, and raised immediately after stimulation: this was to minimize the oxidation of tritiated adrenaline (Langer, 1970). Samples of the bathing solution were taken at 3 min contact periods, two before stimulation and one 3 min after the start of stimulation. The S-I component of the [³H]-adrenaline efflux was calculated by subtracting the mean content of [³H]-adrenaline in the two samples in the bathing solution collected just before the start of stimulation from the amount of [³H]-adrenaline in the sample of the bathing solution collected 3 min after the start of stimulation. Immediately after collecting the stimulation sample, the atria were homogenized in 2 ml of 0.4 M perchloric acid containing disodium edetate (2.7 mM). The homogenate was centrifuged at 1300 g for 10 min and the supernatant (100 µl) was analysed by paper chromatography for [³H]-adrenaline according to the method of Majewski & Story (1977).

Some experiments were designed to test whether [³H]-adrenaline efflux from the atria was calcium-dependent. In these experiments rats were killed 1 h after [³H]-adrenaline administration (2.5 µCi/ml, 6 nmol/kg, i.v.). The atria were placed in an organ bath

containing either Krebs-Henseleit solution or calcium-free Krebs-Henseleit solution containing ethylene-glycol-bis-(β-amino-ethyl ether) N,N'-tetra-acetic acid (EGTA, 3 mM), a calcium chelating agent, and the S-I efflux of [³H]-adrenaline was measured as described above. The S-I efflux of [³H]-adrenaline in these experiments was expressed as a percentage of the tissue content of [³H]-adrenaline at the time of stimulation.

Analysis of [³H]-adrenaline

For estimation of [³H]-adrenaline, samples of tissue bathing fluid, tissue homogenate supernatants, or plasma were added to tubes containing 4 ml of 0.5 M Tris buffer pH 8.6 containing 2% (w/v) disodium edetate and 200 mg of alumina (BDH) which was prepared as described by Anton & Sayre (1962). The tubes were shaken vigorously for 5 min, then the alumina was allowed to settle. The supernatant was decanted and the alumina was washed three times with 7 ml of distilled water. [³H]-adrenaline was eluted from the alumina by shaking with 2 ml of 0.1 M perchloric acid for 15 min. The eluate was placed in a plastic vial containing 10 ml of scintillation fluid. The radioactivity was measured in a Packard Tricarb 3380 scintillation counter. The overall efficiency of alumina extraction was about 60%; corrections were made for efficiency of extraction. Possible contaminants in the estimation of [³H]-adrenaline using the alumina adsorption technique are deaminated catechol metabolites; however, these are not radioactive in the present series of experiments because the [³H]-adrenaline was radiolabelled at the N-methyl position. In some experiments, the final acid eluate from the alumina was further analysed by paper chromatography by the method of Majewski & Story (1977). This procedure revealed that the only radioactivity present was as [³H]-adrenaline.

Statistical analysis of results

The data were analysed by the unpaired 2-tailed Student's *t* test. Probability levels of less than 0.05 were taken to indicate statistical significance.

Materials

The Krebs-Henseleit solution had the following composition (mM): NaCl 118, KCl 4.7, CaCl$_2$ 2.5, MgSO$_4$ 0.45, NaHCO$_3$ 25.0, KH$_2$PO$_4$ 1.03, D-(+)-glucose 11.1, disodium edetate 0.067 and ascorbic acid 0.07. The scintillation solution had the following composition: 5.5 g of 2,5-diphenyloxazole, 0.1 g of 1-3-bis-2-(5-phenyloxazolyl) benzene, and 333 ml of Triton X-100 made up to 1 l in toluene.

The following drugs were used: (−)-adrenaline bitartrate (Calbiochem); metoprolol tartrate (Ciba); (−)-noradrenaline hydrochloride (Sigma); ethylene-glycol-*bis*-(β-amino ethyl ether)-N,N'-tetra-acetic acid (Sigma); cocaine hydrochloride (Sigma); phenoxybenzamine hydrochloride (Smith, Kline & French); (−)-7,8-[³H]-noradrenaline (13 or 15 Ci/mmol) (Radiochemical Centre, Amersham); (−)-adrenaline-[N-methyl-³H] (40.8 Ci/mmol or 45.9 Ci/mmol) (New England Nuclear Corp, Boston).

Results

Effect of adrenaline on S-I efflux from rat atria incubated with [³H]-noradrenaline

These experiments were conducted in the presence of cocaine (100 μM). After the atria were incubated with [³H]-NA and washed for 60 min, the mean amount of radioactivity retained was 2.2×10^6 d/min per atrial preparation (s.e.mean 0.4×10^6, $n = 6$). The atria were subjected to two periods of field stimulation (2 Hz for 30 s), 35 min apart. The mean amount of radioactivity released by the first period of stimulation was 22,382 d/min (s.e.mean = 2244, $n = 6$). The S-I efflux expressed as a percentage of the total radioactivity in the tissue was 1.26% (s.e.mean = 0.29, $n = 6$). The S-I efflux of radioactivity for the second period of stimulation was 55.5% (s.e.mean = 2.6, $n = 6$) of that for the first. The effects of adrenaline on the S-I efflux of radioactivity were assessed by adding it to the atrial bathing solution after the first stimulation period. In a concentration of 1.0 nM it was without effect, 10 nM significantly enhanced the S-I efflux of radioactivity, whereas 1.0 μM markedly reduced the efflux. The enhancement in S-I efflux produced by 10 nM adrenaline was prevented by metoprolol (0.1 μM); thus the enhancement may be due to activation of prejunctional β-adrenoceptors. These results are shown in Figure 1.

A second series of experiments was conducted in the presence of cocaine (100 μM) and also phenoxybenzamine (10 μM) in order to block prejunctional α-adrenoceptors. The atria were subjected to two periods of stimulation (2 Hz for 30 s), 35 min apart. The S-I efflux of radioactivity expressed as a percentage of the total radioactivity in the tissue during the first period of stimulation was 2.62% (s.e.mean = 0.5, $n = 7$). This was significantly ($P < 0.05$) greater than the corresponding figure obtained in the previous experiment in the absence of phenoxybenzamine and indicates that there is blockade of transmitter NA activation of inhibitory prejunctional α-adrenoceptors by phenoxybenzamine. The S-I efflux of radioactivity for the second period of stimulation was 52.5% (s.e.mean = 6.9, $n = 7$) of that of the first. In a concentration of 1.0 μM, adrenaline now signific-
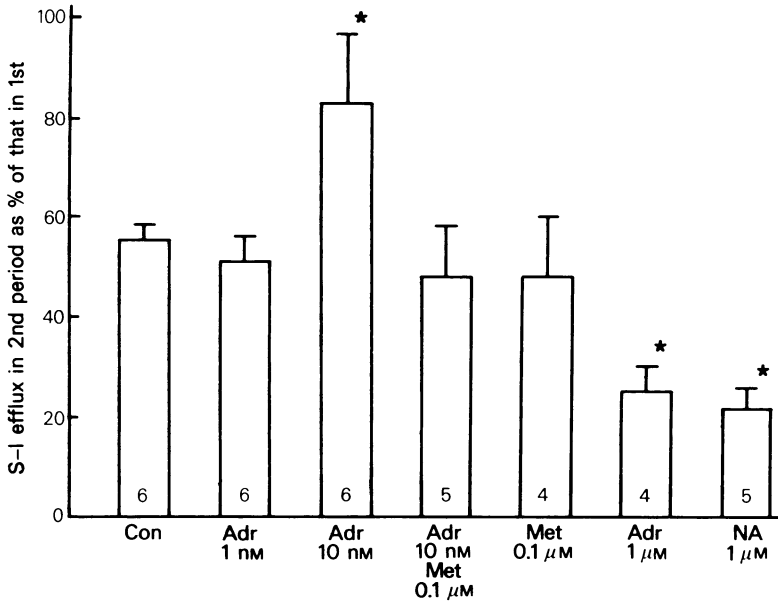
Figure 1   The effect of drugs on the S-I efflux of radioactivity from rat atria incubated with [³H]-noradrenaline. There were two periods of stimulation (2 Hz for 30 s). Cocaine (100 μM) was present throughout both periods of stimulation. Other drugs (Adr = adrenaline, Met = metoprolol and NA = noradrenaline) were present only during the second period of stimulation. The S-I efflux in the second period of stimulation is expressed as a percentage of that in the first. The vertical bars represent the standard errors of means. The number at the base of each histogram refers to the number of experiments performed. Statistically significant difference from control (Con) and from the effect of adrenaline in the presence of metoprolol: $*P < 0.05$.

antly enhanced the S-I efflux of radioactivity. The enhancement was prevented by metoprolol (0.1 μM) and therefore may be due to activation of prejunctional β-adrenoceptors. These results are shown in Figure 2. The facilitatory effect of adrenaline (1.0 μM) in the presence of phenoxybenzamine (10 μM) contrasts with its inhibitory effect in the absence of phenoxybenzamine.

NA (1 and 10 μM) neither alone nor in combination (10 μM) with metoprolol (0.1 μM) had any effect on the S-I efflux of radioactivity in the presence of phenoxybenzamine (Figure 2); this indicates that NA does not activate prejunctional β-adrenoceptors. However, NA (1 μM) inhibited the S-I efflux in the absence of phenoxybenzamine, probably by activation of inhibitory prejunctional α-adrenoceptors (Figure 1).

[³H]-adrenaline content of rat isolated atria

In guinea-pig isolated atria, after incubation with a ³H-catecholamine and subsequent to a period of washing in drug-free Krebs-Henseleit solution, almost all of the radioactivity in the tissue is present as the unmetabolized ³H-catecholamine (Majewski *et al.*, 1980a). In the present study similar results were

obtained in rat atria incubated with [³H]-adrenaline (2 μCi/ml, 0.1 μM) for 20 min and then washed repeatedly with drug-free Krebs-Henseleit solution for 60 min. After washing, radioactivity in the atria was measured as before by the method of Majewski & Story (1977). There was only one peak of radioactivity on the developed chromatogram representing [³H]-adrenaline which comprised 98.7% (s.e.mean = 0.2, $n = 6$) of the total radioactivity on the chromatogram. Therefore, after washing, the radioactivity in the rat atria can be attributed almost solely to unchanged adrenaline.

Effect of metoprolol on the S-I efflux of radioactivity from rat atria incubated with [³H]-adrenaline

Rat atria were isolated and then incubated with [³H]-adrenaline. The atria were washed by repeated changes of fresh radioactivity-free Krebs-Henseleit solution for 60 min. The mean total atrial content of radioactivity was 1,035,554 d/min (s.e.mean = 117,254, $n = 4$). There were two periods of stimulation, each at a frequency of 2 Hz for 30 s, 30 min apart. The S-I efflux of radioactivity during the second period of stimulation was 77.1% (s.e.mean = 8.4, $n = 15$) of the first; that is, the mean S_2/S_1 was
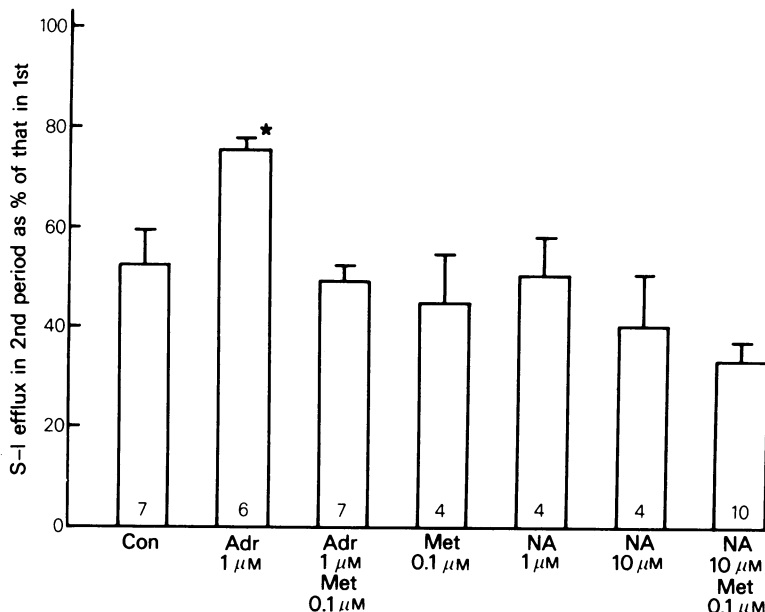
Figure 2   The effect of drugs on the S-I efflux of radioactivity from rat atria incubated with [³H]-noradrenaline. There were two periods of stimulation (2 Hz for 30 s). Cocaine (100 μM) and phenoxybenzamine (10 μM) were present throughout both periods of stimulation. Other drugs (Adr = adrenaline, Met = metoprolol and NA = noradrenaline) were present only during the second period of stimulation. The S-I efflux in the second period of stimulation is expressed as a percentage of that in the first. The vertical bars represent the standard errors of means. The number at the base of each histogram refers to the number of experiments performed. Statistically significant difference from control (Con) and from the effect of adrenaline in the presence of metoprolol: $*P < 0.05$.

77.1%. Metoprolol (0.1 μM), present during the second period of stimulation, significantly reduced the S-I efflux of radioactivity, the mean of S_2/S_1 being reduced to 50.3% (s.e.mean 8.5, $n = 14$) ($P < 0.05$). These results indicate that neuronally released adrenaline activates prejunctional β-adrenoceptors to facilitate its own release.

Incubation of atria with both adrenaline and noradrenaline in vitro

In the first set of experiments, atria were pre-incubated with [³H]-adrenaline (10 nM or 2 nM) and then incubated with unlabelled NA (0.3 μM). The concentration of [³H]-adrenaline in the atria following the adrenaline (10 nM) incubation and after the final 60 min period of washing was 4.2×10^6 d/min per g of tissue (s.e.mean = 0.4×10^6, $n = 4$), which represents 45.9 pmol/g of tissue (s.e.mean = 4.6, $n = 4$) of adrenaline. The S-I efflux as a percentage of total tissue radioactivity during the first period of stimulation (at 2 Hz for 30 s) was 0.64% (s.e.mean = 0.08, $n = 4$). Following the adrenaline (2 nM) incubation, the concentration of [³H]-adrenaline in the atria was 1.4×10^6 d/min per g of tissue (s.e.mean = 0.1×10^6, $n = 3$) which represents 13.9 pmol/g

(s.e.mean = 1.1, $n = 3$) of adrenaline.

In the second set of experiments, atria were incubated with adrenaline (10 nM) or NA (10 nM) followed by [³H]-NA. After pre-incubation with adrenaline (10 nM) the mean atrial content of radioactivity after the final 60 min period of washing was 2.2×10^6 d/min (s.e.mean = 0.2×10^6, $n = 5$). The S-I efflux of radioactivity during the first period of stimulation was 6346 d/min (s.e.mean = 1008, $n = 5$). The S-I efflux during the second period of stimulation was 105.7% (s.e.mean = 7.7, $n = 5$) of the first. Metoprolol (0.1 μM), present during the second period of stimulation, significantly reduced the S-I efflux of radioactivity ($P < 0.05$) (See Figure 3). After pre-incubation with NA (10 nM), the mean atrial content of radioactivity after the 60 min period of washing was 1.7×10^6 d/min (s.e.mean = 0.3×10^6, $n = 4$). The S-I efflux of radioactivity during the first period of stimulation was 6070 d/min (s.e.mean = 2857, $n = 4$). The S-I efflux during the second period of stimulation was 94.3% (s.e.mean = 13.6, $n = 4$) of that of the first. Metoprolol (0.1 μM) present during the second period of stimulation, had no effect on the S-I efflux of radioactivity ($P > 0.05$). These results are shown in Figure 3.
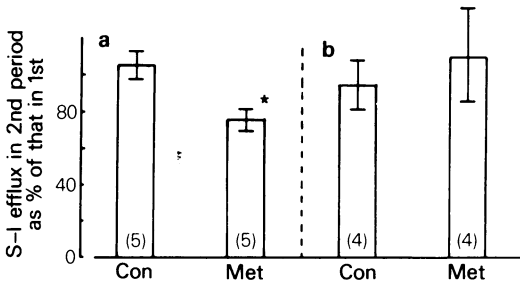
In the third set of experiments, atria were pre-

Figure 3 Effect of metoprolol (0.1 μM; Met) on the stimulation-induced (S-I) efflux of radioactivity from rat atria preincubated with either adrenaline (Adr 10 nM) (a) or noradrenaline (NA 10 nM) (b) and then [³H]-noradrenaline (0.3 μM) (a and b). There were two periods of stimulation each at a frequency of 2 Hz for 30 s. The stimulation-induced efflux of radioactivity in the second period of stimulation is expressed as a percentage of that in the first and is represented by the vertical axis. Metoprolol was present during the second period of stimulation. The vertical bars represent the standard errors of means and the number in parentheses at the base of each histogram refers to the number of experiments performed. Statistically significant difference from control: *P < 0.05.

incubated with a lower concentration of adrenaline (2 nM) and then incubated with [³H]-NA. The mean atrial content of radioactivity after the 60 min period of washing was 2.7×10^6 d/min (s.e.mean = 0.3×10^6, n = 4). The S-I efflux of radioactivity during the first period of stimulation was 10,072 d/min (s.e.mean = 815, n = 4). The S-I efflux during the second period of stimulation was 81.4% (s.e.mean = 4.8, n = 4) of that of the first. In this case, metoprolol (0.1 μM) present during the second period of stimulation had no effect on S-I efflux, the mean % S_2/S_1 being 107.2% (s.e.mean = 6.6, n = 4) (P > 0.05).

These experiments confirm that adrenaline is taken up and released as a co-transmitter. The findings with metoprolol suggest that when there is sufficient adrenaline released, that is after pre-incubation with 10 nM adrenaline, but not 2 nM adrenaline or 10 nM NA, this adrenaline activates the β-adrenoceptor-coupled facilitatory feedback loop.

Retention of adrenaline by atria after administration in vivo

Conscious rats with chronically implanted jugular vein and carotid artery cannulae were given intravenous injections of [³H]-adrenaline (0.6 or 6.0 nmol/kg). Samples of arterial blood were taken at various times for measurement of plasma [³H]-adrenaline concentration. As can be seen from Figure 4, the plasma level of [³H]-adrenaline fell rapidly. The pattern of decline was similar for both doses,

with an initial rapid decline lasting 10 min, followed by a prolonged steady state level.

With the 0.6 nmol/kg dose, there was no change in blood pressure, whereas with the 6.0 nmol/kg dose the mean blood pressure was increased transiently by a mean peak response of 52 mmHg (s.e.mean = 8, n = 3).

The [³H]-adrenaline concentrations in the atria were measured at various times after administration. The concentration was highest shortly after injection and declined slowly thereafter; however, [³H]-adrenaline could be detected 24 h after administration for both doses. These results are shown in Figure 5.

The release of [³H]-adrenaline from atria after uptake in vivo

In some experiments the atria of rats previously given [³H]-adrenaline (0.6 or 6.0 nmol/kg, i.v.) were placed in an organ bath and subjected to one period of field stimulation (5 Hz for 30 s). The S-I efflux of [³H]-adrenaline was measured and, as can be seen from Figure 5, declined with time; however, even 24 h after administration [³H]-adrenaline release from the atria could be demonstrated. The S-I efflux as a percentage of total tissue radioactivity was about 2% with either dose at all periods after administration of [³H]-adrenaline.

Some experiments were designed to see if [³H]-adrenaline release from the atria was calcium-dependent. In these experiments rats were given
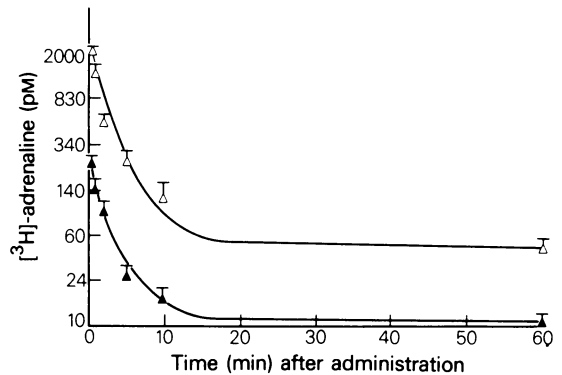

Figure 4 Time course of the disappearance of [³H]-adrenaline in plasma after its intravenous administration in conscious rats. The open symbols represent plasma levels in rats given [³H]-adrenaline (6.0 nmol/kg). The closed symbols represent plasma levels in rats given [³H]-adrenaline (6.0 nmol/kg). Each point represents the mean of 3 to 7 observations. The vertical bars indicate the standard errors of means. Horizontal axis: Time after [³H]-adrenaline administration (min). Vertical axis: Plasma concentration of [³H]-adrenaline (pM).
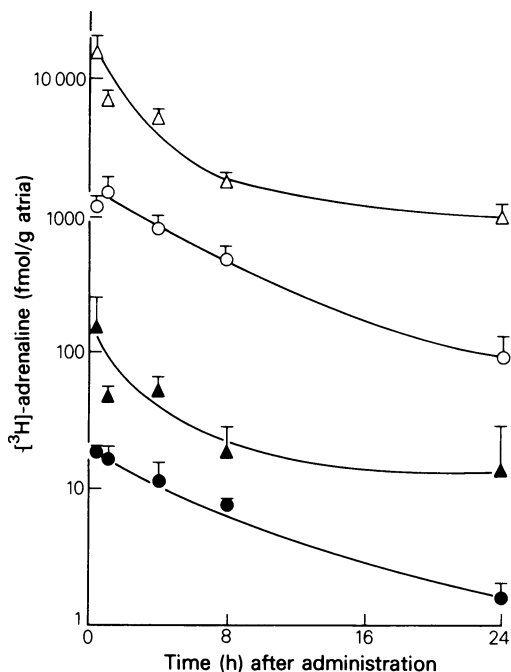
Figure 5 The time course of the accumulation and release of [^{3}H]-adrenaline in atria following its intravenous administration in conscious rats. The open symbols refer to the accumulation of [^{3}H]-adrenaline in the atria. The closed symbols refer to the release of [^{3}H]-adrenaline by field stimulation. Atria were stimulated once at a frequency of 5 Hz for 30 s with 1 ms square waves of supramaximal voltage for stimulating the intramural nerves. The circles represent results from rats treated with [^{3}H]-adrenaline (6 nmol/kg) and the triangles represent results from rats treated with '[^{3}H]-adrenaline (0.6 nmol/kg). Each symbol represents the mean of 3 to 4 experiments. The vertical bars represent the standard errors of means. The vertical axis represents the amount of [^{3}H]-adrenaline (fmol per g of atria) and the horizontal axis represents the time after adrenaline administration (h).

[^{3}H]-adrenaline (6.0 nmol/kg, i.v.), and 1 h later the atria were placed in an organ bath containing either Krebs-Henseleit solution or calcium-free Krebs-Henseleit solution. The tissue content of [^{3}H]-adrenaline in the normal atria was 25,455 d/min (s.e.mean = 2126, $n = 10$) which was not significantly different from atria in the calcium-free medium; the mean in this case was 29,094 d/min (s.e.mean = 4930, $n = 5$) ($P > 0.05$). The S-I efflux of [^{3}H]-adrenaline as a percentage of total tissue radioactivity in the normal bathing solution was 2.5% (s.e.mean = 0.4%, $n = 10$), which was significantly greater than that of the calcium-free group, the mean being 0.74% (s.e.mean = 0.03%, $n = 5$) ($P < 0.05$).

Thus the release of [^{3}H]-adrenaline appears to be to a large extent calcium-dependent.

Discussion

The effect of adrenaline on the S-I efflux of radioactivity from rat atria which were incubated with [^{3}H]-NA depended upon its concentration. A low concentration (1.0 nM) of adrenaline had no effect, whereas adrenaline in a concentration of 10 nM significantly enhanced the S-I efflux. This enhancement was abolished by metoprolol (0.1 μM), indicating the involvement of β-adrenoceptors. Such a facilitatory effect of adrenaline on noradrenergic transmission has also been demonstrated in human omental blood vessels (Stjärne & Brundin, 1975), rabbit carotid sinus (Pham *et al.*, 1978), rat portal vein (Westfall *et al.*, 1979) and guinea-pig atria (Majewski *et al.*, 1980a). The concentrations of adrenaline which exert this effect (0.5 nM to 10 nM) are within the range found in human plasma during periods of enhanced adrenomedullary secretion (Vendsalu, 1960). Therefore, adrenaline activation of facilitatory prejunctional β-adrenoceptors constitutes a possible hormonal mechanism for the modulation of noradrenergic transmission.

The effects of NA on prejunctional β-adrenoceptors are less clear. Reports (Adler-Graschinsky & Langer, 1975; Yamaguchi, De Champlain & Nadeau, 1977) that β-adrenoceptor blocking drugs reduce transmitter release, have been interpreted as evidence that neuronally released NA activates prejunctional β-adrenoceptors. However, studies in human omental blood vessels (Stjärne & Brundin, 1975), human oviduct (Hedqvist & Moawad, 1975) and guinea-pig atria (Rand, Law, Story & McCulloch, 1976) revealed no such effect. In the present study, in rat isolated atria, transmitter NA did not appear to activate prejunctional β-adrenoceptors to facilitate its own release, since metoprolol (0.1 μM) by itself did not reduce the S-I efflux of radioactivity. This may be due to transmitter NA activation of inhibitory prejunctional α-adrenoceptors obscuring its effects on prejunctional β-adrenoceptors. There is evidence that the facilitatory effect of β-adrenoceptor activation is inversely proportional to the degree of activation of prejunctional α-adrenoceptors (Majewski & Rand, 1980a). However, when prejunctional α-adrenoceptors were blocked with phenoxybenzamine (10 μM), metoprolol still did not reduce the S-I efflux.

This problem was also investigated by determining the effects of exogenous NA on the S-I efflux. NA in a concentration of 1.0 μM inhibited the S-I efflux, as did the same concentration of adrenaline (1.0 μM). These effects are due to activation of prejunctional x-adrenoceptors since in the presence of phenoxy-

benzamine (10 μM), though NA (1.0 μM) was ineffective, adrenaline (1.0 μM) significantly enhanced the S-I efflux. This enhancement was abolished by metoprolol (0.1 μM) and therefore appears to be due to activation of prejunctional β-adrenoceptors. A higher concentration (10 μM) of NA was tried to see if in the presence of phenoxybenzamine, it could activate prejunctional β-adrenoceptors to enhance the S-I efflux; however, no effect was observed. In this case it may be that the high concentration of NA broke through the prejunctional α-adrenoceptor blockade produced by phenoxybenzamine which may mask its effect on prejunctional β-adrenoceptors. However, this is unlikely since metoprolol (0.1 μM) had no effect on the S-I efflux in the presence of both NA (10 μM) and phenoxybenzamine (10 μM), indicating that prejunctional β-adrenoceptors were not activated.

Adrenaline but not noradrenaline has also been shown to activate prejunctional β-adrenoceptors in other tissues. In the rabbit carotid sinus, NA (1 nM to 1 μM) was ineffective but adrenaline in concentrations of 5 nM and 10 nM significantly enhanced noradrenaline release (Pham et al., 1980). In the rat portal vein, adrenaline (10 nM) in the presence of phenoxybenzamine significantly enhanced transmitter NA release by activation of prejunctional β-adrenoceptors whereas under the same conditions NA (10 nM and 100 nM) did not (Westfall et al., 1979). In contrast, in the phenoxybenzamine-treated rat portal vein, NA (10 μM) increased transmitter NA release; however, adrenaline in a concentration of only 0.05 μM produced the same degree of enhancement (Dahlöf et al., 1978).

Taken together, these findings indicate that facilitatory prejunctional β-adrenoceptors are much more sensitive to adrenaline than to NA. This would be expected if the prejunctional β-adrenoceptors are of the β_2-subtype, since NA is a much weaker β_2-adrenoceptor agonist than adrenaline (Lands et al., 1967). On the basis of the action of relatively selective β_1- and β_2-adrenoceptor agonists and antagonists, Stjärne & Brundin (1975) and Westfall et al. (1979) have suggested that the prejunctional β-adrenoceptors are of the β_2-subtype. However, the relatively selective β_1-adrenoceptor blocking drug metoprolol blocked the facilitatory effect of adrenaline in guinea-pig atria (Majewski et al., 1980a), and the β-adrenoceptor-coupled facilitation of transmitter NA release in the blood vessels of the cat hind limb (Dahlöf, Åblad, Borg, Ek & Waldeck, 1975). In the present study, in rat atria, metoprolol blocked the facilitatory effects of adrenaline on transmitter release; therefore some caution may be necessary in the classification of the prejunctional β-adrenoceptor sub-type.

Adrenaline rather than NA may be important in

the activation of prejunctional β-adrenoceptors; the significance of the facilitation of sympathetic transmitter release by adrenaline depends upon the time and extent by which circulating adrenaline levels exceed the threshold concentration necessary for the activation of prejunctional β-adrenoceptors. Adrenaline could also activate prejunctional β-adrenoceptors after its uptake and release by sympathetic nerve endings. Circulating adrenaline can be accumulated by sympathetic nerve endings and can be released by nerve stimulation (Iversen & Whitby, 1962; Andén, 1964). Increases in the adrenaline content of peripheral sympathetically innervated tissues occur when large amounts of adrenaline are released from the adrenal medulla by splanchnic nerve stimulation (Raab & Gigee, 1953). Therefore after brief periods of increased adrenaline release from the adrenal medullae there could be increased adrenaline accumulation and release from sympathetic nerve endings. This could lead to activation of prejunctional β-adrenoceptors well after the circulating adrenaline level falls to the basal value and thus neuronally released adrenaline could contribute significantly to the modulation of sympathetic transmitter release.

This hypothesis was investigated in conscious rats. The half-life of [^{3}H]-adrenaline injected intravenously was measured in both plasma and atria. The doses of adrenaline used (0.6 nmol/kg and 6.0 nmol/kg) were smaller than the dose used (16.4 nmol/kg) by Iversen & Whitby (1962). Further, the lower dose (0.6 nmol/kg) had no effect on blood pressure and thus can be described as a tracer amount. The disappearance of [^{3}H]-adrenaline was biphasic with an initial rapid decline lasting about 6 min followed by a much slower fall. Ferreira & Vane (1967) estimated the plasma half-life of adrenaline in cats as less than 30 s. The sites of loss of adrenaline from the plasma include uptake into noradrenergic nerve terminals. Once taken up into sympathetically innervated tissues, the decrease in content of [^{3}H]-adrenaline is far less rapid than that in plasma; in this case, the half-time for loss from atria is about 4 h. Further, the [^{3}H]-adrenaline retained could be released from atria by field stimulation up to 24 h after administration. The release of [^{3}H]-adrenaline from the atria by field stimulation was reduced in the absence of calcium to 29% of control values, suggesting that it is to a large extent calcium-dependent. Thus adrenaline can be released from sympathetically innervated tissues in a calcium-dependent manner after its uptake from the circulation and this release persists for a much longer period than its circulatory life span.

To investigate the hypothesis that adrenaline once incorporated into sympathetic nerves can be released by nerve stimulation and activate prejunctional β-

adrenoceptors, experiments were designed to incorporate both adrenaline and NA into the sympathetic transmitter stores of rat atria by pre-incubation with adrenaline followed by incubation with NA. In preparations pre-incubated with [³H]-adrenaline, there was a release of radioactivity in response to field stimulation, that is, adrenaline was released together with NA. In preparations pre-incubated with adrenaline (10 nM) followed by [³H]-NA the β-adrenoceptor blocking drug metoprolol (0.1 μM) significantly reduced the S-I efflux. If the atria were pre-incubated with lower concentrations of adrenaline (2 nM instead of 10 nM), metoprolol (0.1 μM) had no significant effect. In atria in which the adrenaline was replaced with NA (10 nM) in the pre-incubation period, metoprolol (0.1 μM) did not alter the S-I efflux of radioactivity. These results suggest that in rat atria the facilitatory mechanism mediated by prejunctional β-adrenoceptors is not activated by transmitter NA but when the transmitter stores contain adrenaline in a sufficient amount, the facilitatory mechanism is activated by the adrenaline released as a co-transmitter. Similar results were obtained in guinea-pig atria by Rand et al. (1979) and Majewski et al. (1980a) and in dog saphenous vein strips by Guimarães, Brandão & Paiva (1978). However, in the present experiments, the rat atria were incubated in a much lower concentration of adrenaline (10 nM) compared to that (3 μM) in the guinea-pig atria used by Majewski et al. (1980a). There is a critical level of adrenaline necessary to show this effect. In the present study, in atria pre-incubated with adrenaline (10 nM) and then incubated with [³H]-NA, the adrenaline level in the tissue due to this incubation was 45 pmol/g and neuronally released adrenaline activated prejunctional β-adrenoceptors. However, if the atria were pre-incubated with adrenaline (2 nM), the adrenaline level in the tissue due to the incubation was only 14 pmol/g and no facilitatory effect was observed. The endogenous adrenaline concentration of normal rat atria is about 109 pmol/g (Head & Berkowitz, 1979). In the present experiments the amount of endogenous adrenaline in the atria was not measured therefore it is difficult to assess the total tissue concentration of adrenaline necessary before prejunctional β-adrenoceptors are activated since during the in vitro incubation with adrenaline and noradrenaline, endogenous adrenaline may be displaced from the tissue. Nevertheless, it does seem possible that adrenaline could be taken up from the circulation in sufficient amounts such that when released by sympathetic nerve stimulation it could activate facilitatory prejunctional β-adrenoceptors. Further, to achieve the tissue level of adrenaline necessary to activate prejunctional β-adrenoceptors, the atria were incubated with adrenaline in a concentration of 10 nM for 10 min. This concentration of adrenaline is within the range found in plasma during enhanced adrenomedullary excretion in man (Vendsalu, 1960).

The facilitatory effect produced by adrenaline does not apply to noradrenaline release alone. In isolated atria incubated with [³H]-adrenaline, metoprolol reduced the S-I efflux of radioactivity, suggesting that neuronally released adrenaline is also subject to feedback modulation through prejunctional β-adrenoceptors.

It has been well documented that stress is a component of the aetiology of human essential hypertension. During periods of stress, secretion of adrenaline from the adrenal medullae is increased and it is possible that this adrenaline is implicated in the development of hypertension. Although the increased adrenaline secretion during stressful situations is an acute response, because adrenaline can be accumulated in sympathetic nerves and released by nerve stimulation well after its initial entry into the circulation, it may exert a prolonged facilitatory effect on sympathetic transmitter release leading to sustained increases in blood pressure. Indeed increased adrenaline release has been demonstrated in some patients with essential hypertension (De Champlain, Farley, Cousineau & van Ameringen, 1974; Franco-Morselli, Elghozi, Joly, di Giulio & Meyer, 1977). Further, chronic administration of adrenaline to rats results in a sustained increase in blood pressure even after the adrenaline administration ceases (Majewski, Tung & Rand, 1980b). This increase in blood pressure is prevented by metoprolol and therefore appears to be due to activation of β-adrenoceptors. Majewski & Rand (1980b) have suggested that β-adrenoceptor blocking drugs may owe part of their antihypertensive action to blockade of adrenaline activation of prejunctional β-adrenoceptors.

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