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THE INVOLVEMENT OF Ca^{2+} AND Mg^{2+} IN THE SPONTANEOUS AND DRUG INDUCED RELEASE OF [^{3}H]NORADRENALINE FROM MESENTERIC ARTERIES

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Abstract— The efflux of ^{3}HNA from mesenteric tissue was studied using the perfused rat mesenteric artery preparation preloaded with ^{3}HNA. In the absence of Mg^{2+} there was no increase in ^{3}HNA efflux, but during Ca^{2+}-free perfusion the efflux was increased and during Ca^{2+}- and Mg^{2+}-free perfusion there was an even greater increase over control values. During normal Krebs perfusion, injection of each of the amines noradrenaline 200 ng, octopamine 50 μg, metaraminol 20 μg, and tyramine 100 μg produced transient increases in efflux above the rate obtained in the absence of the drug. The potency of the drugs in this respect was metaraminol > octopamine > tyramine > noradrenaline. The absence of Mg^{2+} had no effect on the ^{3}HNA releasing potency of any of the drugs. During Ca^{2+}-free perfusion each drug released more ^{3}HNA but there was a differential increase in potency such that the ratio became tyramine > metaraminol > octopamine > noradrenaline. A further increase in potency occurred during Ca^{2+}- and Mg^{2+}-free perfusion and the ratio became tyramine > octopamine > metaraminol > noradrenaline. It is concluded that Ca^{2+} has a controlling influence on the release of ^{3}HNA by sympathomimetic amines.

It has now been established that extracellular Ca^{2+} is essential for the release of adrenaline from the adrenal medulla by nerve stimulation [1] and for sympathetic neurotransmission [2, 3]. However, little attention has been paid to the effect of ions on the release of neurotransmitter by sympathomimetic amines though it has been shown that Ca^{2+} ions are necessary for tyramine evoked noradrenaline release from the adrenal medulla [4].

It has been shown that responses to sympathomimetic amines in the rat mesentery are differentially potentiated when the Ca^{2+} or Mg^{2+} concentration of the perfusion fluid is reduced [5, 6]. It was suggested that this phenomenon might involve changes in the mechanism by which noradrenaline (NA) is released from the sympathetic neurone since a preliminary study demonstrated that tyramine released more [^{3}H]noradrenaline from rat mesenteric arteries during Ca^{2+}-free perfusions than during perfusion with normal Krebs solutions [7]. An attempt has therefore been made to study the [^{3}H]noradrenaline release pattern in response to administration of four sympathomimetic amines and the effects of alteration of Ca^{2+} and Mg^{2+} concentrations on these patterns.

METHODS

Extracellular space. The extracellular space and extracellular washout time was initially determined using the [^{14}C]sorbitol method [8, 9]. The rat mesentery was prepared for perfusion as previously described [5]. [^{14}C]D-sorbitol (50 mCi/m-mole), obtained from the Radiochemical Centre, Amersham, was assessed for purity using paper chromatography [10]. Mesentery preparations were perfused as described [5] for periods of 2, 5, 10, 15 and 20 min respectively with normal Krebs solution containing [^{14}C]D-sorbitol (0·05 Ci/ml) and D-sorbitol (B.D.H.) to give a final sorbitol concentration of 250 mg/ml. At the end of the perfusion period, the [^{14}C]sorbitol tissue content was estimated by extracting [^{14}C]sorbitol from the tissue [8] and assaying a 0·75-ml aliquot sample of each extract by scintillation counting using 10 ml Bray's scintillant [11] for each sample. The determinations were repeated using perfusion solutions containing [^{14}C]sorbitol and D-sorbitol dissolved in Ca^{2+}- and Mg^{2+}-free Krebs solution.

The [^{14}C]sorbitol extracellular washout time from the mesentery was determined by prior loading of the tissue with [^{14}C]sorbitol for 20 min as described above. Washout was commenced with [^{14}C]sorbitol and carrier sorbitol-free Krebs solution, and the effluent perfusate was collected at 1-min intervals for up to 20 min using a fraction collector. A 0·75-ml aliquot from each sample collected at 1-min intervals was assayed for [^{14}C]sorbitol as described above.

Release of noradrenaline. Release of [^{3}H]noradrenaline was determined by first loading the tissue with [^{3}H]noradrenaline (sp. act. 5125 mCi/m-mole), obtained from Radiochemical Centre, Amersham, using the method and precautions described [7]. The mesentery was prepared as previously described [5, 7], and perfused for 60 min with [^{3}H]noradrenaline (^{3}HNA) diluted with normal Krebs solution containing L-NA to give a final concentration of 4·2 μCi/ml of ^{3}HNA and 200 ng/ml L-NA respectively plus ascorbic acid 20 mg/l. and EDTA 10 mg/l. Perfusion with this solution was continued for 60 min and the effect of perfusion with normal and modified Krebs on the spontaneous and drug induced release of ^{3}HNA was measured over a further 60-min period.

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Spontaneous release of ^{3}HNA after initial loading was estimated by the collection of perfusate samples at 1-min intervals after transfer to perfusion with non-radioactive and carrier NA-free Krebs solution. The samples were cooled to 4° until subsequent assay. For assay purposes, the ^{3}HNA in each 1-min sample was separated from its metabolites by column chromatography as described [12], but using Dowex 50 × 840 resin. The eluate containing the ^{3}HNA was neutralised to pH 6·5 and assayed by liquid scintillation counting using a Triton X100/toluene scintillant containing Triton X100 33% (v/v).

In addition, the effects of changes in ionic composition of the perfusing solution on the release of ^{3}HNA were investigated.

The release of ^{3}HNA caused by injection of 200 ng NA, 20 μg metaraminol, 50 μg octopamine, and 100 μg tyramine, and the effect of changes in ionic composition of the perfusing solution were also studied. In this series of experiments the first administration of a given amine was made 20 min after transfer to isotope and carrier NA-free perfusion; this period being chosen so as to ensure a steady state of spontaneous ^{3}HNA efflux. Repeated injection of the same amine was then made at 15-min intervals for up to three administrations. Sympathomimetics were injected in doses corresponding to their previously determined 50 per cent maximal pressor response dose for the rat mesentery preparations [5, 7]. The responses to each amine were examined on separate mesentery preparations.

RESULTS

The effect of changes in Mg^{2+} *and* Ca^{2+} *on the extracellular space*

From the results obtained with [^{14}C]sorbitol, the extracellular space was calculated to be 420 μl. An analysis of the time of course for the incorporation of [^{14}C]sorbitol into mesenteric tissue (Fig. 1) showed that the extracellular space was saturated with [^{14}C]sorbitol after 12 min perfusion using normal Krebs solution delivered at a rate of 4 ml/min. Furthermore, the saturation time and saturation kinetics were unaffected by alteration of the Ca^{2+} and Mg^{2+} concentrations of the perfusion solution. Figure 1 also shows the pattern of [^{14}C]sorbitol washout determined over a 20-min period during normal Krebs perfusion using the method previously described [9]. The results are expressed as the concentration of [^{14}C]sorbitol present in the perfusate effluent as dis/min per ml. After only 5 min perfusion with normal Krebs, 95 per cent of the [^{14}C]sorbitol had been washed out from the tissue, indicating that 95 per cent of the extracellular volume was exchanged with sorbitol free perfusate after only a 5-min perfusion with normal Krebs. It is also apparent (from Fig. 1) that the [^{14}C]sorbitol washout time was unaltered by perfusion with Ca^{2+}- and Mg^{2+}-free Krebs.

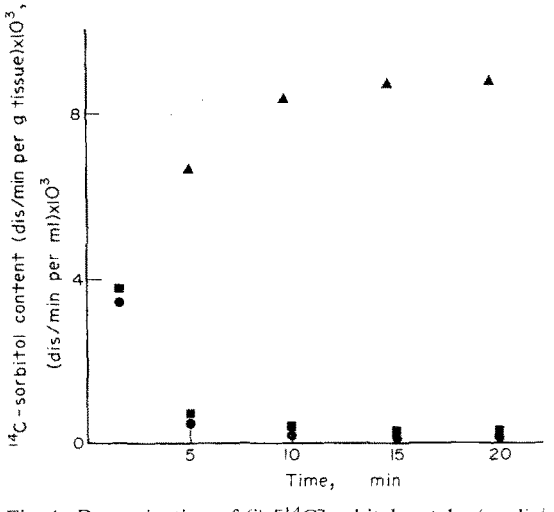

Fig. 1. Determination of (i) [^{14}C]sorbitol uptake (as dis/min per g tissue) × 10^3 in mesenteric arteries following perfusion with 0·05 μCi/ml [^{14}C]sorbitol in: ▲ normal Krebs; (ii) [^{14}C]sorbitol washout (as dis/min per ml) × 10^3 from mesenteric arteries (following [^{14}C]sorbitol uptake) during: ■ normal Krebs, ● Ca^{2+}- and Mg^{2+}-free Krebs.

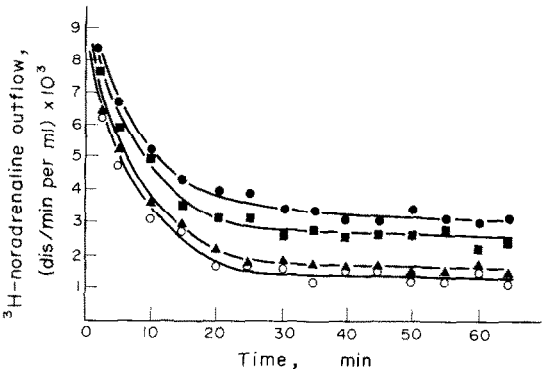

Fig. 2. The spontaneous efflux of ^{3}HNA from rat mesenteric arteries (following pre-loading with normal Krebs containing 0·42 μCi/ml ^{3}HNA) measured during perfusion with: ○ normal Krebs, ▲ Mg^{2+}-free Krebs, ■ Ca^{2+}-free Krebs, ● Ca^{2+}- and Mg^{2+}-free Krebs.

Spontaneous [^{3}H]*noradrenaline release.* The graph (Fig. 2) shows the [^{3}HNA] efflux per min from zero perfusion time when commencement of radioactive and carrier-free noradrenaline perfusion was first substituted (see Methods section) up to 70 min after commencement. Figure 2 shows that the rate of ^{3}HNA loss followed a two-stage pattern. After an initial rapid rate of loss of ^{3}HNA, the ^{3}HNA outflow declined to an approximately steady rate of 1849 (dis/min) within 20 min after commencement of noradrenaline-free perfusion. During Mg^{2+}-free Krebs perfusion (Fig. 2), no significant changes occurred in the rate of ^{3}HNA outflow from the tissue compared with normal Krebs perfusion. However, during Ca^{2+}-free perfusion, there was a considerable increase in the rate of ^{3}HNA efflux (Fig. 2, Table 1) compared to normal Krebs perfusion, whilst the use of Ca^{2+}- and Mg^{2+}-free Krebs solution resulted in an even greater increase (55 per cent) in the rate of ^{3}HNA efflux compared to that occurring during normal Krebs perfusion, (Fig. 2, Table 1).

Effect of sympathomimetic administration of ^{3}HNA *release.* Administration of sympathomimetic amines increased the rate of ^{3}HNA efflux when administered to ^{3}HNA-loaded preparations 20 min after changing to NA-free perfusion solution; these effects were repeatable at subsequent 15-min intervals. A quantitative expression of this increase in ^{3}HNA concentration present in 1-min collection samples was obtained by comparing the total increase in amounts of ^{3}HNA in

Table 1. The mean increase in [^{3}H]noradrenaline outflow ± S.E. following injection of each of the amines during perfusion with normal and modified Krebs solution

Perfusate composition	Mean spontaneous release (dis/min per ml)	Total mean increase in [^{3}H]noradrenaline outflow (dis/min per ml) ± S.E. following each injection of Noradrenaline 200 ng	Octopamine 50 μg	Metaraminol 20 μg	Tyramine 100 μg
Normal	1849	950 ± 135	2127 ± 202	3807 ± 320	2516 ± 231
Mg^{2+}-free	1976	969 ± 129	2793 ± 256	3961 ± 301	2807 ± 276
Ca^{2+}-free	2618	1009 ± 145	6271 ± 481	4921 ± 384	10,031 ± 641
Ca^{2+}- and Mg^{2+}-free	3549	1117 ± 172	10,098 ± 836	9551 ± 522	16,728 ± 1062

Each result is the mean of six experiments.

dis/min min^{-1} released by the amine with that occurring in control preparations in which the spontaneous efflux was relatively constant for the corresponding period. The injection of tyramine (100 μg) at 15-min intervals caused a significant and consistent increase in ^{3}HNA output above the background rate of spontaneous release (Fig. 3).

Injection of 50 μg octopamine, or 20 μg metaraminol, caused a transient but significant rise in ^{3}HNA efflux and although injection of 100 ng noradrenaline caused a transient increase, its magnitude was seen to be much less than that resulting from injection of any of the other amines (Fig. 4, Table 1). It can be seen from Table 1 that during normal Krebs perfusion, metaraminol releases more ^{3}HNA (3,500 dis/min) than any of the other amines, while injection of L-noradrenaline brings about a release equivalent to only 950 dis/min.

Effect of changes of ionic composition on ^{3}HNA *release.* The effect of changes of ionic composition of the perfusion fluid on the release of ^{3}HNA by each of the sympathomimetic amines was then investigated and the results are also shown in Table 1. During Mg^{2+}-free perfusion, individual injections of 100 μg tyramine, 50 μg octopamine, 20 μg metaraminol, or 200 ng noradrenaline, were still able to release ^{3}HNA from the mesentery preparation.

Injections of 100 μg tyramine, and 50 μg octopamine, each produced approximately the same increase in ^{3}HNA efflux as each did during normal Krebs perfusion (Table 1). The noradrenaline releasing potency of metaraminol was unchanged during Mg^{2+}-free perfusion and under these conditions noradrenaline still possessed the weakest ^{3}HNA releasing potency.

During Ca^{2+}-free perfusion, however, each of the three injections of metaraminol (20 μg) released 32 per cent more ^{3}HNA than the corresponding amounts released during normal Krebs perfusion. For octopamine, the release was increased by 147 per cent and on injection of tyramine the release was 295 per cent greater than that obtained during normal Krebs perfusion (Fig. 3, Table 1). However, there was no significant difference between the amounts of ^{3}HNA released by L-noradrenaline in Ca^{2+}-free solution compared with its effect during normal Krebs perfusion (Fig. 4, Table 1). Thus, tyramine was seen to be the most

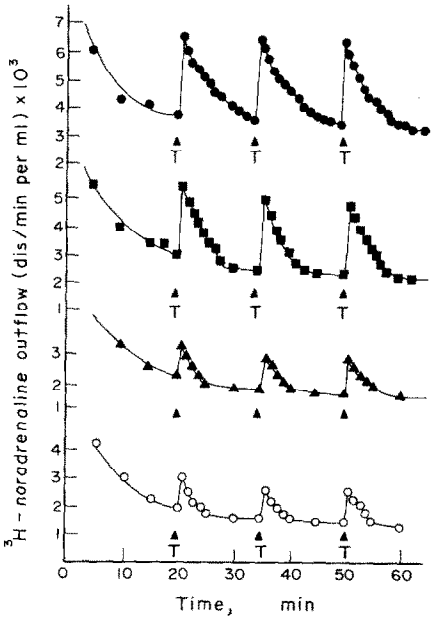

Fig. 3. The effect of injection of tyramine (T) 100 μg at 15-min intervals on the spontaneous efflux of ^{3}HNA from mesenteric arteries during perfusion with: ○ normal Krebs, ▲ Mg^{2+}-free Krebs, ■ Ca^{2+}-free Krebs, ● Ca^{2+}- and Mg^{2+}-free Krebs.

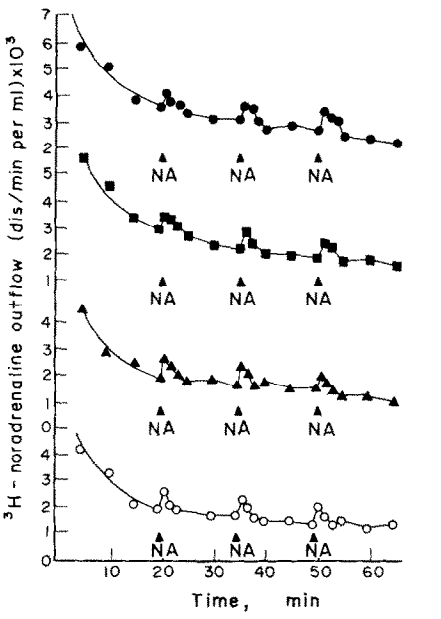

Fig. 4. The effect of noradrenaline injection (NA) 200 ng at 15-min intervals on the spontaneous efflux of ^{3}HNA from rat mesenteric arteries during perfusion with: ○ normal Krebs, ▲ Mg^{2+}-free Krebs, ■ Ca^{2+}-free Krebs, ● Ca^{2+}- and Mg^{2+}-free Krebs.

potent of the four amines, in terms of ^{3}HNA released per injection, during Ca^{2+}-free perfusion.

Under Ca^{2+}- and Mg^{2+}-free perfusion conditions, no difference could be seen between the amount of ^{3}HNA released upon injection of noradrenaline compared with the amount released during normal Krebs perfusion. Injection of octopamine during Ca^{2+}- and Mg^{2+}-free perfusion caused a 362 per cent greater release of ^{3}HNA than during normal Krebs perfusion. Similarly, injection of metaraminol under these conditions caused a release of 170 per cent more ^{3}HNA than during normal Krebs perfusion. Tyramine, (100 μg), however, caused a 565 per cent greater release of ^{3}HNA during Ca^{2+}- and Mg^{2+}-free perfusion than during normal Krebs perfusion (Fig. 3, Table 1).

DISCUSSION

The results show that the spontaneous release of ^{3}HNA from rat mesenteric arterial vessels after preloading the tissue with ^{3}HNA follows a two-stage kinetic pattern upon perfusion with NA-free normal Krebs. As the time course of the initial rapid phase of NA efflux corresponded to the washout time of [^{14}C]sorbitol from the extracellular space, it is concluded that the rapid phase consisted of ^{3}HNA washed out of the extracellular space plus residual ^{3}HNA contained in the lumen of the mesenteric vessels. The exponential decay stage of the rate of ^{3}HNA efflux occurred after the washing out of the extracellular space; this effect is likely to be attributable to release of ^{3}HNA from neuronal storage sites and not to the washing action of the saline, since a similar ^{3}HNA release pattern has been shown to occur in the cat iris preparation *incubated* in normal Krebs solution [13]. It is concluded from the results that a reduction in the Ca^{2+} concentration of the perfusion solution produces an increase in the spontaneous release of the ^{3}HNA from the mesentery while reduction in the Mg^{2+} concentration has no significant effect. Nevertheless, Mg^{2+} appears to be capable of interacting with Ca^{2+} since the absence of both Ca^{2+} and Mg^{2+} causes a greater increase in the amount of ^{3}HNA release than that occurring in the absence of Ca^{2+} alone. Similar results to these have been obtained using cat irides [13], rat brain synaptosomes [14], and rat heart [15, 16]. Calcium ions must be considered to exert an important role in the neuronal storage process for noradrenaline entering the sympathetic neurones of the mesentery via the uptake process, since it has also been demonstrated that Ca^{2+} is as important as Na and K in the storage of noradrenaline in the rat atria [17] and ventricle [15, 18]. Since the uptake of noradrenaline into the sympathetic neuronal vesicle operates via a Ca^{2+}-dependent flux [19], it is possible that absence of Ca^{2+} in the extracellular fluid causes an inhibition of noradrenaline uptake into the vesicle and results in an increased efflux from the vesicle and eventually through the neuronal membrane.

It is evident from the results that tyramine, octopamine, metaraminol and even noradrenaline, are all capable of releasing ^{3}HNA from its store within the rat mesenteric arteries. During normal Krebs perfusion, the order of potency of the four amines in terms of ^{3}HNA releasing ability is metaraminol > tyramine and octopamine > noradrenaline.

Perfusion with Mg^{2+}-free solution caused no significant alteration in either relative potency ratios or the amounts of ^{3}HNA which each amine released. However, when the Ca^{2+} perfusion concentration was reduced there was a differential increase in ^{3}HNA releasing potency for the four amines; the greatest increase occurring for tyramine and the least for noradrenaline, such that the relative potency ratio under these perfusion conditions became tyramine > octopamine > metaraminol > noradrenaline.

During Ca^{2+}- and Mg^{2+}-free perfusion, the potency ratio was found to be tyramine > octopamine > metaraminol > noradrenaline.

It is evident that some change in the mechanism by which sympathomimetic amines release noradrenaline from the sympathetic neurone must have occurred during Ca^{2+}-free perfusion, since noradrenaline remained a very weak releaser of ^{3}HNA, as previously demonstrated [20] while the tyramine potency increased. Most knowledge concerning the release of noradrenaline from sympathetic nerve endings is confined to release of neurotransmitter by nerve stimulation, a process which has been shown to be Ca^{2+}-dependent [2, 3] but it is not known at exactly what neurophysiological stage the Ca^{2+} participates [21]. In addition, Ca^{2+} has been shown to be essential for both adrenaline release from the adrenal medulla by nerve stimulation [1] and by the action of sympathomimetic amines [4]. Thus it appears that there is an important difference between the ionic requirements for sympathomimetic release of noradrenaline from the sympathetic neurone, and for adrenaline from the adrenal medulla. It would also seem that the mechanisms by which noradrenaline is released from sympathetic nerve endings by either nerve stimulation or sympathomimetic amines are different. The essential difference between the two release systems may be that release of sympathetic transmitter by nerve stimulation from sympathetic neurones may be a quantal Ca^{2+}-dependent process [23, 23], while release by sympathomimetic amines may be a non-quantal process independent of extracellular Ca^{2+} concentration. Recent evidence has suggested that another important difference between the two release systems may be the presence of an active efflux transport system pump responsible for the spontaneous release of noradrenaline from the neurone [18]. This flux has been demonstrated in sympathetic neurones [18, 24–26] and has been shown to be Na^+- and K^+-sensitive [18, 24]. A theoretical model of the noradrenaline flux, involving K^+ and Ca^{2+} has also been evaluated [17, 25]. If the noradrenaline efflux is Ca^{2+}-sensitive, then the mechanism of increased ^{3}HNA efflux from mesenteric arteries during Ca^{2+}-free perfusion could be readily accounted for. In addition, if sympathomimetic amines stimulated the outward flux of noradrenaline, it is possible that the differential increase in noradrenaline releasing potency that occurred during Ca^{2+}-free perfusion may be the result of changes in the configuration of a binding site for sympathomimetic amines. The configuration of this site could well be controlled allosterically by changes in Ca^{2+} concentration.

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