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RELEASE OF NORADRENALINE FROM THE CAT SPLEEN BY NERVE STIMULATION AND POTASSIUM

BY A. G. GARCIA, S. M. KIRPEKAR AND P. SANCHEZ-GARCIA

From the Department of Pharmacology, State University of New York, Downstate Medical Center, Brooklyn, New York 11203, U.S.A. and Facultad de Medicina, Departmento de Farmakologia y Terapeutica, Valladolid, Spain

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SUMMARY

1. Release of noradrenaline from the perfused cat spleen, or from isolated spleen slices, in response to prolonged nerve stimulation or maintained depolarization by potassium was measured.

2. Prolonged stimulation of the splenic nerves at 2, 10 and 30 Hz for 10 min evoked release, which was maximum during the first 2 min, and then declined during the remaining period of stimulation. When noradrenaline release was induced by high potassium from the perfused spleen or from isolated slices, it followed a similar time course to nerve stimulation. Similar results were obtained from phenoxybenzamine-treated spleens, using both modes of stimulation.

3. Stimulation of the splenic nerves in calcium-free Krebs solution did not release noradrenaline. If calcium was introduced at a later stage during stimulation, the release was markedly diminished. In phenoxybenzamine- or phentolamine-treated spleens, stimulation of the nerves in the presence of calcium evoked a secretory response which was comparable to the one produced by introduction of calcium after a few minutes of nerve stimulation.

4. Simultaneous application of calcium plus high potassium always produced a much greater secretion of noradrenaline than application of calcium after a few minutes of potassium depolarization. Release of noradrenaline by potassium from phenoxybenzamine-treated spleens was also much greater if calcium and potassium were added simultaneously than addition of calcium after a few minutes of potassium depolarization.

5. In the presence of maintained depolarization by potassium, tyramine was effective in causing release of noradrenaline.

6. The relationship between noradrenaline release by repetitive nerve stimulation or by maintained depolarization with high potassium to influx of calcium ions has been discussed.

INTRODUCTION

During prolonged high-frequency (10–30 Hz) stimulation of the sympathetic nerves to the cat spleen, the amount of noradrenaline overflowing into the venous effluent declines progressively after the first few minutes, and is then maintained at a low constant level over long periods (Davies, 1963; Dearnaley & Geffen, 1966; Kopin, Breese, Krauss & Weise, 1968; Hedqvist & Stjärne, 1969; Kirpekar & Yamamoto, 1971). The cause for this marked reduction in noradrenaline release has not been well understood. Dearnaley & Geffen (1966) attributed it to depletion of a readily releasable store of noradrenaline and neither synthesis nor enzymatic degradation of transmitter was quantitatively important in regulating release. Kopin *et al.* (1968) concluded, on the basis of their studies on the release of noradrenaline and [^{14}C] noradrenaline synthesized from [^{14}C] tyrosine, that during the early phase of stimulation, mobilization of transmitter contributed mostly to release while, at a later stage, over two thirds of noradrenaline released was newly synthesized. According to these authors, the steady-state release was therefore largely dependent on the availability of newly synthesized noradrenaline. Kirpekar & Yamamoto (1971) suggested that during nerve stimulation, whether short or prolonged, mobilization of stored noradrenaline plays a much more important role in maintaining release than synthesis.

Kirpekar, Prat, Puig & Wakade (1972) showed that acetylcholine, protoveratrine and potassium ions cause inhibition of the evoked release of noradrenaline from the perfused spleen of the cat. One of the suggestions offered to explain the inhibition of release by these agents and potassium was that these treatments, by depolarizing the nerve endings, partially inactivated the potential-dependent calcium entry into the axon terminal and thus inhibited evoked release. Lorenz & Vanhoutte (1975) also showed inhibition of adrenergic transmission in isolated dog veins by potassium. Baker, Meves & Ridgeway (1973*a*, *b*) have shown in giant squid axons that sustained depolarization of the axon has a phasic effect on the tetrodotoxin-resistant calcium entry; that is an initial rapid activation of calcium channels followed by their inactivation. If depolarization of sympathetic nerve endings affects the calcium entry in a similar manner, then it appears that the main reason for the reduction in transmitter output during prolonged depolarization or during repetitive nerve stimulation may be related to the calcium inactivation process. The present investiga-

tion was undertaken to test this possibility and, additionally, to determine the relative importance of both transmitter mobilization and *de novo* synthesis in regulating release during prolonged sympathetic nerve stimulation. In order to study the effect of intermittent as against maintained depolarization on refractoriness of release, noradrenaline release was evoked by repetitive elctrical stimulation of sympathetic nerves or by infusion of potassium into the perfused spleen of the cat. A preliminary report on some of the present findings has been published (Kirpekar, Garcia & Sanchez-Garcia, 1976).

METHODS

Experiments were carried out on the *in situ* perfused cat spleen or on splenic slices.

Perfused spleen. Cats weighing about 2–3 kg were anaesthetized with chloralose (60 mg/kg, I.V.) after induction with ether. The spleen was isolated and perfused *in situ* with Krebs-bicarbonate solution according to a procedure previously described (Kirpekar & Misu, 1967). The perfusion rate was about 5 ml. per minute and temperature of the effluent about 33° C. Splenic nerves were repetitively stimulated at 2, 10 and 30 Hz, respectively, with supramaximal monophasic rectangular pulses of 1-msec duration for a total of 10 min. In some experiments noradrenaline release was evoked by perfusing the spleen with isotonic potassium (140 mM) Krebs solution for 10 min. Venous samples were collected for 2 min and control samples without nerve stimulation were taken 2 min before stimulation. At the end of the stimulation period, a 2-min sample was also collected. In some experiments, spleens were initially perfused with Krebs solution containing phenoxybenzamine (10 μg/ml.) for half an hour and then with normal Krebs solution for an additional half-hour. In others, spleens were perfused with Krebs solution containing phentolamine (3 μg/ml.) during the entire experiment.

In general, the spleen was initially perfused with normal Krebs solution for about 90 min, and nerves were stimulated once at 30 Hz for 5 sec in order to expel any trapped blood in the venous sinuses. After about 30 min the nerves were repetitively stimulated at different frequencies or a high-potassium solution was infused. In some experiments, after the initial equilibration procedure perfusion was begun with Krebs solution containing either 0·1 mM or no calcium for half an hour. Nerve stimulation or high-potassium perfusion was started in low-calcium medium, and at different times normal calcium (2·5 mM) was introduced. After the initial period of nerve stimulation or perfusion with high potassium, the spleen was perfused with normal Krebs solution for an additional 60-min period, and a second stimulation or high-potassium perfusion of 10-min duration was carried out in the presence of calcium ions.

Spleen slices. Cats were anaesthetized with ether; spleens were quickly removed and cut into transverse sections of about 0·5 mm thickness with a McIlwain slicer. Slices were washed twice and stored in cold Krebs-bicarbonate solution for further use. The slices were then divided into four groups in beakers containing 8 ml. different bathing solutions and incubated at 37° C in a water bath with continuous shaking under an atmosphere of 95% O_2–5% CO_2. Each beaker contained about 1 g slices.

Slices were subjected to the following pre-treatments: (*a*) one group of slices was initially incubated in normal Krebs-bicarbonate solution for 30 min, during which

three washes were given; (*b*) the second group was incubated in calcium-free Krebs solution during 30 min with three washes; (*c*) the third group was incubated for 30 min in the presence of phenoxybenzamine 10 μg/ml., and then thoroughly washed three times with fresh Krebs solution over a period of 30 min; and (*d*) the last group was treated with phenoxybenzamine as in Group (*c*) but the slices were washed with calcium-free solution for 30 min as in Group (*b*).

After these treatments, a 2-min control sample of bathing medium (normal Krebs in the case of Groups (*a*) and (*c*), calcium-free Krebs in the case of Groups (*b*) and (*d*)) was collected, and secretion of noradrenaline was evoked by adding, every 2 min, 8 ml. fresh high-potassium (35–140 mM) Krebs solution either in the presence (Groups (*a*) and (*c*)) or in the absence (Groups (*b*) and (*d*)) of calcium ions. After 16-min stimulation with high-potassium solution (Groups (*a*) and (*c*)), two additional 2-min control samples were collected in normal Krebs. In Groups (*b*) and (*d*) stimulation with high potassium was carried out for a 12-min period, then calcium was introduced and slices were further stimulated with high potassium for an additional 4-min period; finally, two control samples were collected in normal Krebs. In other experiments, calcium was introduced at the 2nd, 4th or 6th min of incubation in high-potassium solution.

Perfusion or incubation solutions. The composition of the normal Krebs-bicarbonate solution was as follows (mM concentrations); NaCl, 119; KCl, 4·7; $CaCl_2$, 2·5; $MgSO_4.7H_2O$, 1·2; KH_2PO_4, 1·2; $NaHCO_3$, 25; and glucose, 11. This solution was equilibrated with 95% O_2–5% CO_2, and the final pH was 7·4–7·5. High-potassium solutions were prepared by adding 35, 70 or 140 mM potassium (as K_2SO_4) and proportionately reducing NaCl to maintain isotonicity. Low calcium (0·1 mM) or calcium-free solutions were prepared by removing $CaCl_2$.

Assay of noradrenaline. Aliquots of perfusates and the incubation media were cooled and immediately acidified with concentrated perchloric acid to a final acid concentration of 0·4 N. Sodium meta-bisulphate and the disodium salt of ethylenediaminetetra-acetic acid (EDTA) were added to a final concentration of 0·1% and 0·05%, respectively. Samples were then centrifuged, and the noradrenaline present in the supernatant was determined after purification on alumina according to Shellenberger & Gordon (1971). Mean recovery of standard amounts of noradrenaline in concomitant assays was 80% and all values were corrected for recovery. The amount of noradrenaline released is expressed as ng/2-min sample or ng/g wet weight per 2-minute sample for perfusates and incubation media, respectively.

RESULTS

The release of noradrenaline during prolonged nerve stimulation

Fig. 1 shows that stimulation of the splenic nerves at 2, 10, or 30 Hz for 10 min resulted in the release of noradrenaline, which was maximum during the first 2 min and then progressively declined during the remaining period of stimulation. Outputs of noradrenaline during the initial 2-min period of stimulation at 2, 10 and 30 Hz were 34 ± 4, 242 ± 4, and 527 ± 102 ng, respectively. In the final 2-min stimulation period the outputs at these frequencies were 19 ± 2, 32 ± 7, and 31 ± 15 ng. If a second stimulation at 30 Hz is repeated after about 60 min, noradrenaline output is still substantially reduced (Fig. 1*A*). At 30 Hz, the initial 2-min output during the second stimulation was only 22 ± 4% of the corresponding

output during the first stimulation, and it declined to a low level as stimulation continued, in a manner similar to the first stimulation period. In order to compare the rates of transmitter release at different frequencies, the outputs were normalized by expressing them as percentages of the maximal initial output. Fig. 1*B* shows that the rate of decline in noradrenaline output during prolonged stimulation was related to the stimulation frequency. Thus, during the last 2-min stimulation period the

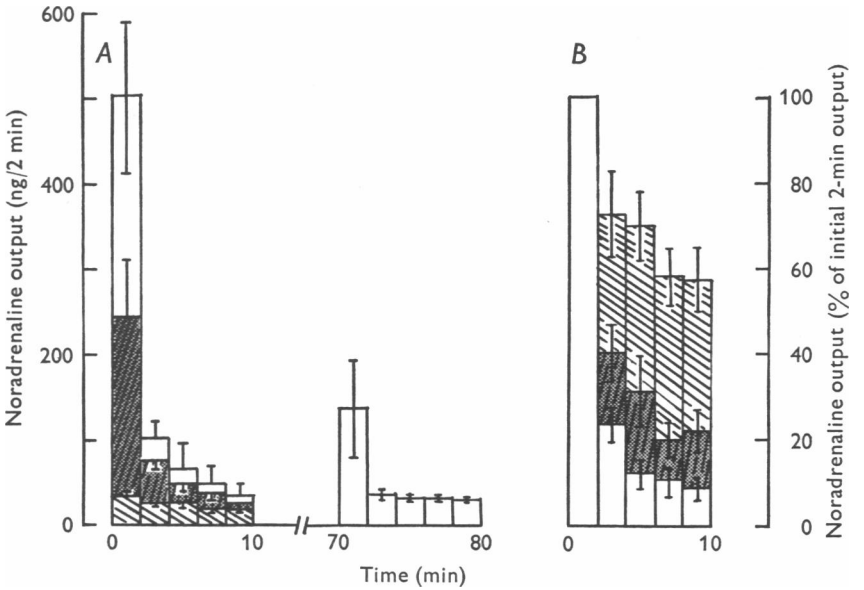

Fig. 1. A, release of noradrenaline by repetitive nerve stimulation. Splenic nerves were stimulated at 2 (▨), 10 (▦), and 30 Hz (□) for 10 min. Nerves were stimulated for the second time at 30 Hz for 10 min, after a rest period of about 60 min. Vertical bars represent S.E. of the mean of five, eight and fifteen experiments at 2, 10 and 30 Hz respectively. *B*, same as *A*, except that outputs are expressed as percentages of the initial 2-min output. The first column represents 100% output for all three frequencies.

transmitter output at 2, 10 and 30 Hz amounted to 58 ± 7, 22 ± 5, and 6 ± 1% of the initial output for each stimulation frequency. In 0·5 mM calcium the rate of decline of noradrenaline release at 30 Hz was not much different than that in 2·5 mM calcium. In low calcium the noradrenaline release is markedly reduced (Kirpekar & Misu, 1961), and it becomes increasingly difficult to measure the noradrenaline outputs during the last half of the stimulation period.

The release of noradrenaline during prolonged nerve stimulation after treatment with phenoxybenzamine

Since the original observation by Brown & Gillespie (1957) that phenoxybenzamine markedly enhanced the transmitter output from the spleen following nerve stimulation at low frequencies, other investigators have confirmed this observation in saline-perfused spleens (Haefely, Hurlimann & Thoenen, 1965; Kirpekar & Misu, 1967). It was therefore of interest to

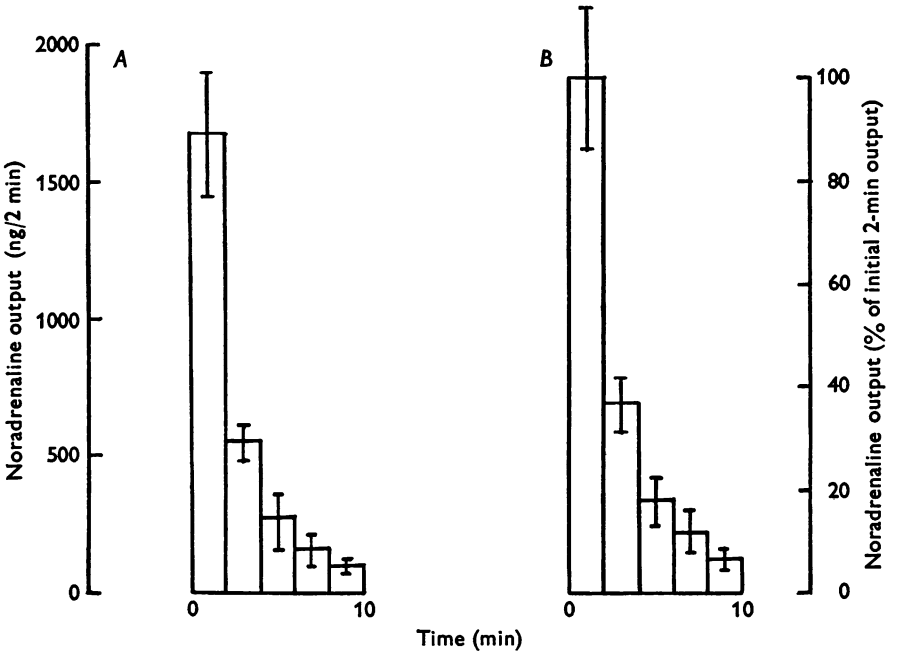

Fig. 2. Release of noradrenaline from phenoxybenzamine-treated spleens by nerve stimulation. *A*, splenic nerves were stimulated at 30 Hz for 10 min. *B*, outputs are expressed as percentages of the initial 2-min output. Vertical bars are the S.E. of the mean of twelve experiments.

study the release of noradrenaline during repetitive electric stimulation from spleens treated with phenoxybenzamine. Fig. 2*A* shows that stimulation of the splenic nerves at 30 Hz for 10 min resulted in a very marked release of noradrenaline. The peak output during the first 2 min of stimulation was 1739 ± 575 ng, as compared to 527 ± 102 ng from control spleens and the rate of decline in output was also similar to or slightly slower than that from the control spleen (Fig. 2*B*). Thus, during the last 2 min of stimulation the output was only 6–10% of the initial 2-min output. The similarity of decline in transmitter release is even more remarkable in

view of the fact that the transmitter output after phenoxybenzamine was nearly three- to fourfold greater than that from an untreated control spleen and amounted to roughly a third to a quarter of the total noradrenaline content of the spleen. Therefore, these experiments probably suggest that the failure to maintain release from the untreated spleen during prolonged stimulation probably is not due to exhaustion of a readily releasable pool of noradrenaline.

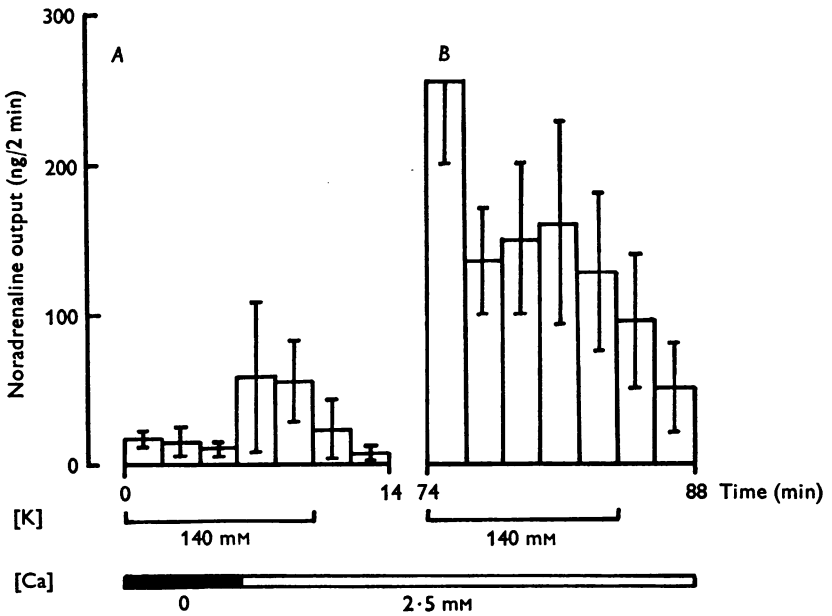

Fig. 3. Release of noradrenaline from the perfused spleen by potassium. *A*, the effect of calcium on release during perfusion with calcium-free, 140-mM potassium solution. Calcium was added at the 6th min of perfusion with high potassium containing no calcium, as shown. *B*, shows the effect of simultaneous exposure to high potassium and calcium on release. Each column in this and the following Figures represents a 2-min collection period. Vertical bars are the S.E. of the mean of four experiments.

The release of noradrenaline by high potassium

Perfused spleen. Kirpekar & Wakade (1968) showed that noradrenaline was readily released on injecting potassium into the saline-perfused spleen. The potassium-induced noradrenaline release required calcium in the perfusion medium and high magnesium prevented it. In preliminary experiments, noradrenaline was released by high potassium as K_2SO_2 or KCl, but the release appeared more reproducible with K_2SO_4 than KCl. Since anions do not contribute to the transmitter release process, all

effects of the high-potassium solution on release are mainly attributed to potassium ions. Figure 3*B* shows that perfusion of the spleen with high potassium for 10 min resulted in the output of noradrenaline which was maximum during the first 2 min and then declined rather slowly as the perfusion with high potassium was continued. There was a considerable amount of variation in noradrenaline outputs from perfused spleens in response to potassium infusion and the rate of decline also varied considerably.

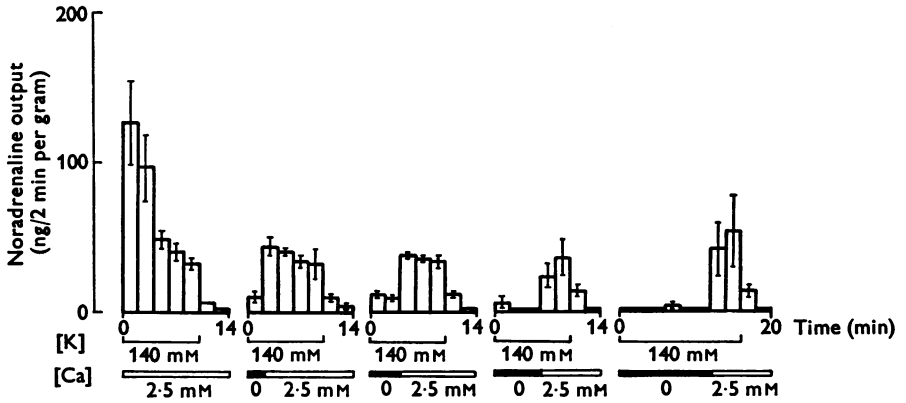

Fig. 4. The effect of addition of calcium at various times during incubation of isolated spleen slices in calcium-free, 140 mM potassium solution on release. Control data are obtained from twelve experiments, while the effect of calcium was studied in three experiments at each time interval. Vertical lines are S.E. of the mean.

These experiments were technically much more difficult to perform than those with electric stimulation, since the spleen contracted markedly in response to potassium infusion and the flow rate of the perfusion solution was also markedly diminished. The spleen remained contracted and had a blotchy appearance even after its perfusion with Krebs solution for a long time. This would artificially reduce the noradrenaline ouput and make its diffusion from the site of release much more difficult. A part of the reduction in total output and its slow wash-out during potassium infusion may be attributed to the intense splenic contraction.

Isolated spleen slices. In order to overcome some of the difficulties mentioned above, most of the experiments with potassium were performed on isolated slices. Fig. 4 shows that incubation of the splenic slices in high-potassium (140 mM) solution caused release of noradrenaline which was, as with repetitive electric nerve stimulation, maximum during the first 2 min and then progressively declined during the remaining period of incubation. Thus the mean outputs during the first and the last 2-min

incubation periods were 127 ± 28 and 33 ± 5 ng/2 min, respectively. When release was induced by 70 and 35 mM of potassium, the rate of decline in noradrenaline output was slower compared to 140 mM potassium.

The effect of calcium on release by-nerve stimulation during perfusion with low-calcium solution

Since transmitter release is dependent on calcium entry into the nerve terminal, reduction in release during maintained depolarization or repetitive nerve stimulation could be due to calcium entry being reduced, for instance, by inactivation. It is conceivable that during depolarization,

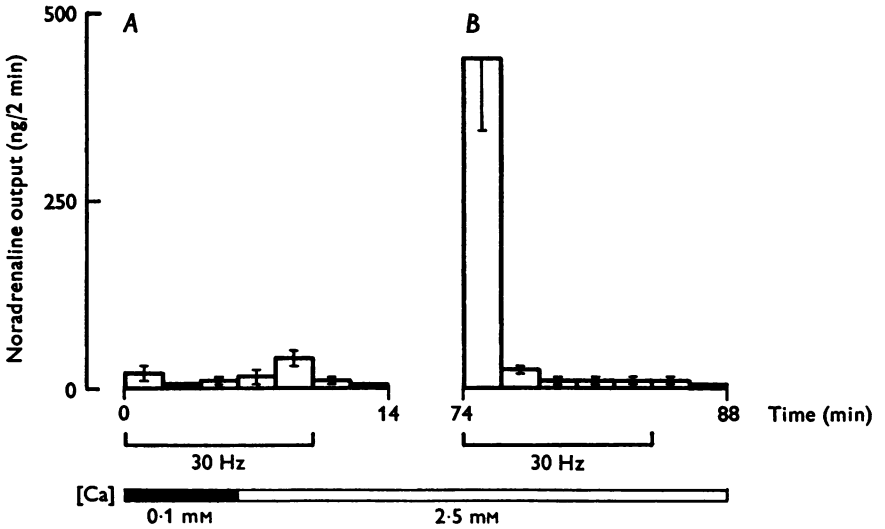

Fig. 5. Release of noradrenaline from the perfused spleen by nerve stimulation. *A*, the effect of calcium on release during perfusion with low-calcium solution. Calcium was added at the 6th min of nerve stimulation. *B*, shows the effect of nerve stimulation on release during perfusion with normal Krebs solution after a 60-min rest period. Vertical bars are S.E. of mean of four experiments.

inactivation of calcium channels could go on even in the absence of external calcium. The following experiments were performed to test this suggestion. Fig. 5*A* shows that during the first 6-min stimulation of the sympathetic nerves at 30 Hz in low-calcium Krebs solution there was virtually no release of noradrenaline. On adding calcium to the perfusion solution at the end of the 6th min of stimulation, the release in the first 2 min (6–8 min period) was markedly suppressed as compared to the release obtained in normal Krebs solution at the start of the experiment. Thus, stimulation

of the nerves in the presence of calcium produces a much larger secretory response than addition of calcium at a later stage during stimulation. At the end of the first stimulation period, the spleen was further perfused with normal Krebs solution for 1 hr and nerves were stimulated for the second time. The release in the first 2-min stimulation period was now much greater than that obtained after addition of calcium at the 6th min of nerve stimulation (Fig. 5*B*). It should be mentioned that perfusion of the spleen with calcium-free solution first (without nerve stimulation) and then stimulation of the nerves in the presence of calcium caused release of normal amounts of noradrenaline. This experiment indicates that: nerve impulses were conducted in low-calcium solution, since reduction in transmitter release was evident at the 6th min, when calcium was introduced; the sympathetic nerves were not damaged by prolonged stimulation, since the release of noradrenaline during the second stimulation was partially restored; and the reduction of transmitter output at the 6th min of stimulation was not due to depletion of releasable stores of noradrenaline, since noradrenaline was not released during the first 6 min of stimulation when calcium-free solution was perfused and, secondly, the release was at least partially restored on subsequent stimulation.

The effect of calcium on release during perfusion with calcium-free, high-potassium solution

Experiments using high potassium were done in a manner similar to nerve stimulation both in the perfused spleen and the isolated slices.

Perfused spleen. Fig. 3*A* shows that during the first 6 min of perfusion of the spleen with high-potassium solution containing no calcium, release of noradrenaline was only minimal. Addition of calcium to the perfusion medium at the end of the 6th minute evoked release which was only 20% of that released by simultaneous perfusion with high potassium plus calcium solution (Fig. 3*B*).

Isolated slices. A more detailed study of the effect of calcium on release, after different times of its application during exposure to high potassium, was done in isolated slices (Fig. 4). When the outputs after addition of calcium to high-potassium solution at 2, 4 and 6 min are expressed as percentages of the initial output during simultaneous exposure of slices to high potassium, they were 43, 37 and 22% of their respective control outputs. When calcium was added 12 min after exposure to high potassium, the reduction in output was comparable to that seen after 6 min. Thus, prolongation of exposure time to calcium-free, high-potassium solution did not completely stop release on subsequent addition of calcium.

The effect of calcium on release from phenoxybenzamine- and phentolamine-treated spleens during perfusion with low-calcium solution

Nerve stimulation. Alpha-adrenergic blocking agents, phenoxybenzamine and phentolamine, are known to potentiate the release of noradrenaline from the cat spleen by nerve stimulation (see Kirpekar, 1975). We have already presented results which showed that after treatment with phenoxybenzamine the rate of decline in noradrenaline output

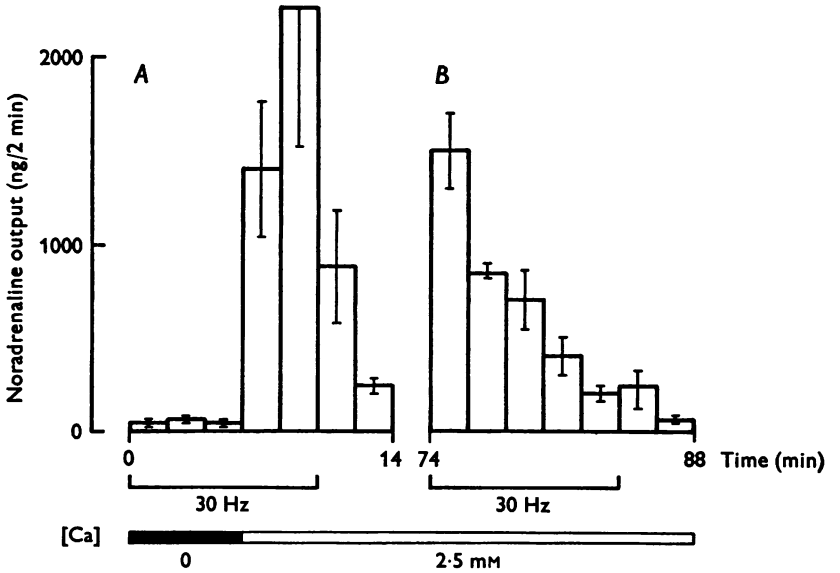

Fig. 6. Same as Fig. 5, except that the spleens were pre-treated with phenoxybenzamine. Vertical bars are S.E. of three experiments.

during repetitive nerve stimulation at 30 Hz was comparable to the control release. It was therefore of interest to study the secretory response after addition of calcium at different times during nerve stimulation. Figure 6*A* shows that in phenoxybenzamine-treated spleens addition of calcium to the perfusion medium at the 6th min of stimulation resulted in a rather brisk release of noradrenaline which was roughly comparable to the peak release obtained during stimulation of the splenic nerves in the presence of calcium. In two experiments we extended the period of nerve stimulation in calcium-free solution from 6 to 12 min, and no inhibition of release was obtained when calcium was re-introduced.

Since phenoxybenzamine has a number of other actions besides its effect on alpha-receptors, phentolamine was used. Phentolamine gave essentially similar results to phenoxybenzamine, and transmitter output

on addition of calcium occurred normally, as if the pre-conditioning stimuli were not applied.

High potassium. Kirpekar & Wakade (1968) were unable to show potentiation of noradrenaline release by high potassium from phenoxybenzamine-treated spleens. Preliminary experiments done with phenoxybenzamine-treated slices also showed only slight potentiation of release by high potassium. Fig. 7 shows that phenoxyzbenzamine does not interfere

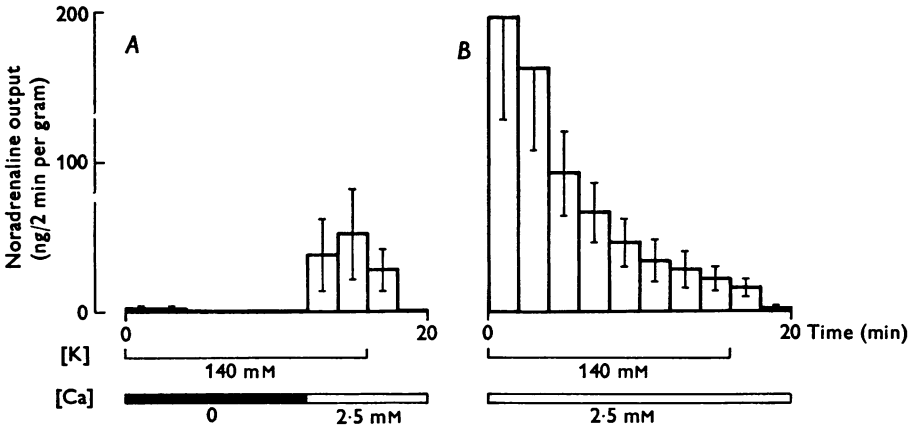

Fig. 7. The effect of addition of calcium on release during incubation of phenoxybenzamine-treated slices in calcium-free, 140 mM potassium solution. *A*, calcium was added at the 12th min of incubation with calcium-free high-potassium solution. Vertical bars represent S.E. of four experiments.

with the inactivation process when noradrenaline release is evoked by high potassium. Upon addition of calcium at the 12th minute of perfusion with high potassium, the output was only 20% of that obtained during simultaneous exposure to calcium and high potassium. Inhibition of release was also similar when calcium was added at the 6th minute of high-potassium exposure.

The release of noradrenaline by tyramine

Since noradrenaline release from adrenergic nerves by tyramine occurs in the absence of external calcium (Lindmar, Löffelholz & Muscholl, 1967), the presumed depolarization-dependent activation and inactivation of calcium permeability should not interfere in the release of noradrenaline by this indirectly acting sympathomimetic amine. Fig. 8*A* shows that when tyramine (0·1 mM) was added at the 6th minute to the calcium-free, high-potassium medium, there was no desensitization of release. This

experiment shows, therefore, that inactivation of release by maintained depolarization does not occur if the release is induced by a calcium-independent process.

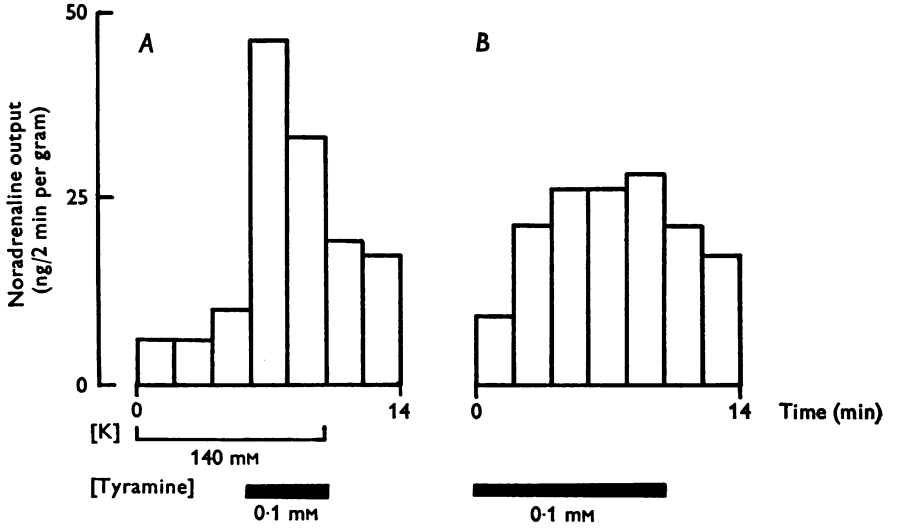

Fig. 8. Effect of tyramine on release from isolated spleen slices. *A*, slices were exposed to calcium-free, 140 mM potassium solution for 10 min and tyramine was added at the 6th min. *B*, slices were exposed to tyramine in a calcium-free Krebs solution.

DISCUSSION

The aim of the present study was to determine the factors responsible for the inhibition of noradrenaline release from the cat spleen during prolonged excitation of its sympathetic nerves. The output of noradrenaline was evoked either by repetitive electrical stimulation at different frequencies or by an intra-arterial infusion of high-potassium solution. Irrespective of the nature of the stimulus, the initial high output declined rapidly as the electrical stimulation or infusion of potassium was continued.

An obvious explanation for the decline in output during continuous stimulation could be the exhaustion of releasable transmitter stores. This suggestion, however, cannot account for the present results, since we have already shown that the rate of decline in noradrenaline release was similar, or even slightly slower, when spleens were treated with phenoxybenzamine, despite the ability of this agent to markedly enhance the transmitter output. Secondly, in experiments in which the spleen was perfused with calcium-free solution during the initial period of electrical stimulation or potassium infusion, the output still remained depressed on reintroducing calcium, even though no noradrenaline was released initially. Finally, the

rate of decline in output in low calcium solution was comparable with that in 2·5 mM calcium. It appears, therefore, that the reduction in output during prolonged nerve stimulation (but see below), or at least with high potassium, is mainly owing to failure of transmitter mobilization.

Since the importance of calcium ions has been firmly established in the transmitter release process (Katz, 1969), it is reasonable to suggest that the phasic release of noradrenaline during prolonged electrical stimulation or potassium infusion is perhaps related to alterations in calcium permeability. Nerve terminals appear to possess a specialized calcium-entry mechanism as compared with the parent axon. Katz & Miledi (1969) showed in experiments with giant synapses of the squid stellate ganglion, pre-treated with tetrodotoxin externally and tetraethylammonium (TEA) internally, that a local regenerative response confined to the presynaptic terminal was obtained upon depolarization, provided the external calcium concentration was sufficiently high. Strontium and barium substituted for calcium, while manganese and magnesium reduced the regenerative response. Miledi (1971) also showed that lanthanum blocked the 'calcium response'. These results offer direct evidence for the existence in the nerve terminals of a late calcium channel for transmitter release. It is also known that strontium and barium substitute for calcium in sustaining release of noradrenaline from post-ganglionic sympathetic nerves, while magnesium, manganese and lanthanum block release (Kirpekar & Misu, 1967; Kirpekar, Dixon & Prat, 1970; Kirpekar *et al.* 1972). If a similar late calcium channel exists in the sympathetic nerve endings, then it can be argued that perhaps the enhancement of noradrenaline release in the early stages of excitation is due to activation, and the subsequent reduction is due to inactivation, of calcium channels. The observation, that during maintained depolarization with high potassium in low-calcium solution the release was markedly attenuated when calcium was re-introduced at a later period during depolarization, may offer some support to this suggestion. Assuming that inactivation of calcium permeability occurs during maintained depolarization, it should be pointed out that this process is very slow and in no way comparable to the inactivation of sodium permeability in axons. It is not clear at present whether inactivation of calcium permeability plays a role in the regulation of physiological release of neurotransmitters.

It should be noted, however, that Katz & Miledi (1971) have shown in the giant synapse of the stellate ganglion of the squid that the effect of conditioning pulses on transmitter release by a test depolarization was removed by TEA and, they suggested, that the effect of conditioning pulses on transmitter release was due to changes in space constant of the presynaptic nerve terminal. When they applied large depolarizing pulses of

several seconds duration to the nerve terminal, release of transmitter was blocked during the pulse but it still occurred when the pulse was turned off. Martin & Ringham (1975) showed in the lamprey synapse that conditioning pulses to the presynaptic fibre did not influence subsequent transmitter release, provided the presynaptic recording electrode was located in very close proximity to the synaptic contact. These observations would suggest that calcium inactivation does not occur during brief depolarization pulses to the nerve terminal. However, Katz & Miledi (1971) pointed out that inactivation of calcium permeability may develop slowly when a large depolarization is maintained over a much longer period. In this study, the output of noradrenaline by potassium was studied over several minutes and it is conceivable that activation and inactivation of calcium permeability probably would explain the phasic release of the transmitter. A more direct support for this suggestion comes from the work of Baker *et al.* (1973*b*), who described in squid axons a late calcium channel which shows voltage and time-dependent inactivation. It is interesting to note that the time course of inactivation of calcium permeability in squid axons appears comparable, though shorter, to that of the phasic release of noradrenaline from post-ganglionic sympathetic nerve endings.

We also made a curious observation that in the presence of alpha-adrenergic blocking agents, phenoxybenzamine and phentolamine, electrical stimulation of the sympathetic nerves in the presence of calcium produced outputs of noradrenaline which were similar to those obtained when calcium was added 6 min after the stimulation began, a finding completely different when the experiments were carried out in the absence of the blocking agents. This observation was surprising, since the rate of decline in noradrenaline output from phenoxybenzamine-treated spleen was comparable to that of the untreated spleen. The fact that these adrenergic blocking agents remove negative feed-back inhibition, and thereby enhance noradrenaline output (see Kirpekar, 1975), is also insufficient to explain this finding, since in the untreated spleen there was no release of noradrenaline during the initial period of stimulation, yet on introduction of calcium the response remained markedly attenuated. Adrenergic blocking agents do not appreciably affect the amplitude of the preterminal compound action potential of sympathetic nerves (Bennett & Middleton, 1975) and their actions on terminals are largely unknown. Moreover, release of noradrenaline by high potassium from normal or phenoxybenzamine-treated slices was comparable. These findings probably reflect on the nature of the two stimulation procedures used in this study. Electrical stimulation was transient, and hence discontinuous, whereas potassium-induced depolarization probably was maintained throughout

the exposure period. Irrespective of the mechanism by which these blocking agents antagonize the desensitization of release, these results raise some doubts whether failure to maintain release during prolonged repetitive electric stimulation is, in fact, owing to inactivation of calcium permeability.

As this work was in progress, Baker & Rink (1975) published similar results on the transient release of catecholamines from the perfused bovine adrenal gland in response to maintained depolarization by potassium. They showed that perfusion of the gland with high-potassium solutions containing calcium produced a much larger secretion of catecholamines than introduction of calcium after a few minutes of potassium depolarization. Their interpretation of the results was that potassium depolarization first activated, and then slowly inactivated, potential-dependent calcium permeability of the adrenal chromaffin cells.

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