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EFFECT OF GUANIDINE ON RELEASE OF NORADRENALINE FROM THE PERFUSED SPLEEN OF THE CAT

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1 Guanidine increased noradrenaline (NA) output at 5 Hz by 3 to 6 fold, and doubled it at 30 Hz. Onset of maximum activity was slow, and reversal was also slow. Output of NA induced by potassium, sodium deprivation, or tyramine was not affected.

2 NA output was doubled at low concentrations (1 to 2 mM) of guanidine, but maximal effect was obtained at 4 mM. At 10 mM, spontaneous release was occasionally observed.

3 The effect of guanidine on NA release was related to the external calcium concentration. Outputs which previously have been shown to be insignificant at 5 Hz in 0.25 and 0.75 mM calcium-Krebs solution were markedly enhanced by guanidine. Guanidine enhanced release at all calcium concentrations up to 7.5 mM, but maximum output was obtained at 2.5 mM.

4 Guanidine had no effect on the recovery of intra-arterially infused NA.

5 The effects of guanidine and tetraethyl-ammonium (TEA) on NA release at 5 Hz were additive.

6 Guanidine reversed the inhibition of NA release by guanethidine during nerve stimulation at 5 and 10 Hz, and the NA output increased nearly 2.5 fold after repeated stimulation of the nerves. Guanidine was less effective in reversing the inhibitory effects of guanethidine on NA release at 30 Hz.

7 Guanidine did not affect release of catecholamines (CA) from the perfused cat adrenal gland by splanchnic nerve stimulation.

8 It is suggested that guanidine enhances NA release partly by increasing the influx of calcium into the neurone during an action potential, and also by interfering with intracellular binding of calcium.

Introduction

Guanidine enhances the release of acetylcholine (ACh) at the neuromuscular junction of the skeletal muscle following stimulation of its motor nerve fibres (Otsuka & Endo, 1960; Kamenskaya, Elmqvist & Thesleff, 1975; Matthews & Wickelgren, 1977). Moreover, it has been reported that guanidine antagonized the neuromuscular block of botulinum toxin (Lundh, Leander & Thesleff, 1977). Guanidine also potentiates the contraction of the vas deferens on stimulation of the hypogastric nerve (Ozawa & Sugawara, 1968) presumably by enhancing the release of NA from sympathetic nerve terminals.

The mechanism of action of guanidine in increasing the transmitter release is probably related to an increase in the intracellular calcium concentrations of the nerve terminal (Lundh *et al.*, 1977). It is known that tetraethylammonium (TEA) and 4 aminopyridine (4-AP) also greatly enhance NA release, presumably by inactivating the potassium current and thus prolonging the duration of the action potential, which would allow calcium channels to remain open longer and more calcium to enter (Thoenen, Haefely, & Staehelin, 1967; Kirpekar, Prat, Puig & Wakade, 1972; Kirpekar, Wakade & Prat, 1976; Kirpekar, Kirpekar & Prat, 1977a). However, guanidine does not appear to have any marked effects on the time course of the muscle or nerve action potential (Lundh *et al.*, 1977; Matthews & Wickelgren, 1977), thereby excluding its effect on potassium inactivation. Thus, it would appear that the mechanism of action of guanidine in enhancing transmitter release might differ from that of TEA or 4-AP. The present investigation was undertaken to study the effects of guanidine on mammalian sympathetic nerve terminals. A preliminary report on some of the findings has been published (Kirpekar, Prat & Hirsch, 1977b).

Methods

All experiments were done on the *in situ* perfused cat spleens, splenic slices or perfused adrenal glands.

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Perfused spleen

Cats (2 to 3 kg) were anaesthetized by ether induction, followed by chloralose (40 to 60 mg/kg, i.v.). The abdomen was opened by a midline incision, and the stomach, adrenals, intestines and colon were removed. The arrangements for spleen perfusion and nerve stimulation, *in situ*, were as previously described (Kirpekar & Misu, 1967). The spleens were perfused with solutions by means of a pump at a rate of about 7 ml/min at 35°C. Venous samples were collected by placing a cannula in the superior mesenteric vein. Control samples were taken 2 min before nerve stimulation, and samples during nerve stimulation were collected for 2 min. In some experiments NA stores were labelled by injecting 200 μCi of [^{3}H]-NA into the femoral vein (specific activity 10.43 Ci/mmol). Perfusion of the spleen was started 30 min after the injection.

Perfusion procedure

The spleens were perfused with Krebs-bicarbonate (Krebs) solution for 30 min before nerve stimulation. They were then perfused with test solutions for at least 15 min before stimulation. In experiments with guanidine and TEA, following stimulation in TEA-Krebs solution, normal Krebs solution was perfused for 15 min and nerves were stimulated once to re-establish outputs of NA comparable to initial control values, before proceeding to perfuse with guanidine. Following perfusion with the last test solution, spleens were reperfused with Krebs solution and nerves stimulated one or more times. NA release was evoked by stimulation of the splenic nerves with supramaximal monophasic rectangular pulses of 1 ms duration at 5, 10 or 30 Hz for a total of 200 stimuli.

Uptake of noradrenaline

Spleens were perfused with Krebs solution containing guanidine and after 30 min (−)-NA infused into the splenic artery at a constant rate of 510 ng/min, by placing a cannula in the hepatic artery, so that the tip of the cannula was at the junction of the hepatic artery with the main coeliac artery (Kirpekar & Wakade, 1968). Samples of venous effluents were collected for 1 min each at 0, 3, 6, 10, 15 and 20 min of NA infusion.

Spleen slices

Cats were anaesthetized with ether, followed by chloralose. Spleens were quickly removed and cut into transverse sections of about 0.5 to 0.7 mm thickness by a tissue slicer. In order to label the endogenous stores of NA with [^{3}H]-NA, slices were incubated with 100 μCi[^{3}H]-NA (specific activity 10.43 Ci/mmol) for 30 min in 20 ml of Krebs solution at 37°C, with continuous shaking in a water bath, followed by 3 washes over a 30 min period. Slices weighing about 1 g each were incubated in Krebs solution to determine background release, followed by incubation in the test solution. Both sets of solutions were analyzed for [^{3}H]-NA and where possible, endogenously released NA.

Perfused adrenal gland

Cats were anaesthetized with ether, followed by chloralose (40 to 60 μg/kg, i.v.). The abdomen was opened by a midline incision, the stomach, intestines, spleen and right adrenal gland were removed, and the left splanchnic nerve was prepared for stimulation. The left adrenal gland was perfused with solutions at 35°C according to the procedure previously described (Dixon, Garcia & Kirpekar, 1975). The perfusate was collected for 2 min through a cannula inserted in the adrenolumbar vein via the renal vein. Nerves were stimulated at 10 Hz, with 300 stimuli given for each stimulation period.

Perfusion and incubation solutions

The composition of normal and other Krebs solutions was as follows: (a) Normal Krebs (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSo_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11 and disodium edetate (EDTA) 0.02. (b) Sodium-substituted solutions contained equivalent amounts of sucrose, choline (as choline chloride), or lithium (as LiCl). Bicarbonate, phosphate, and EDTA were omitted. (c) Potassium-rich solutions were prepared by adding the required amount of K_2SO_4 and omitting the corresponding amount of NaCl. (d) Calcium-rich solutions contained 7.5 mM $CaCl_2$. Bicarbonate and phosphate were omitted. Solutions (a) and (c) were bubbled with 95% O_2 and 5% CO_2 to give final pH of 7.4. Solutions (b) and (d) contained Tris buffer (5 mM), pH was adjusted to 7.4 with HCl (1 N), and equilibration was with 100% O_2.

Assay of noradrenaline

NA contents of the venous samples or incubation media were determined by the trihydroxyindole method described by Anton & Sayre (1962). Standard solutions of NA were analyzed concurrently, with recoveries varying from 70 to 90%, and all values were corrected for recovery. CA contents of perfusates from the adrenals were assayed by the same method, without the purification procedure using alumina. CA values are expressed as NA equivalents.

[^{3}H]-NA content was determined by measuring

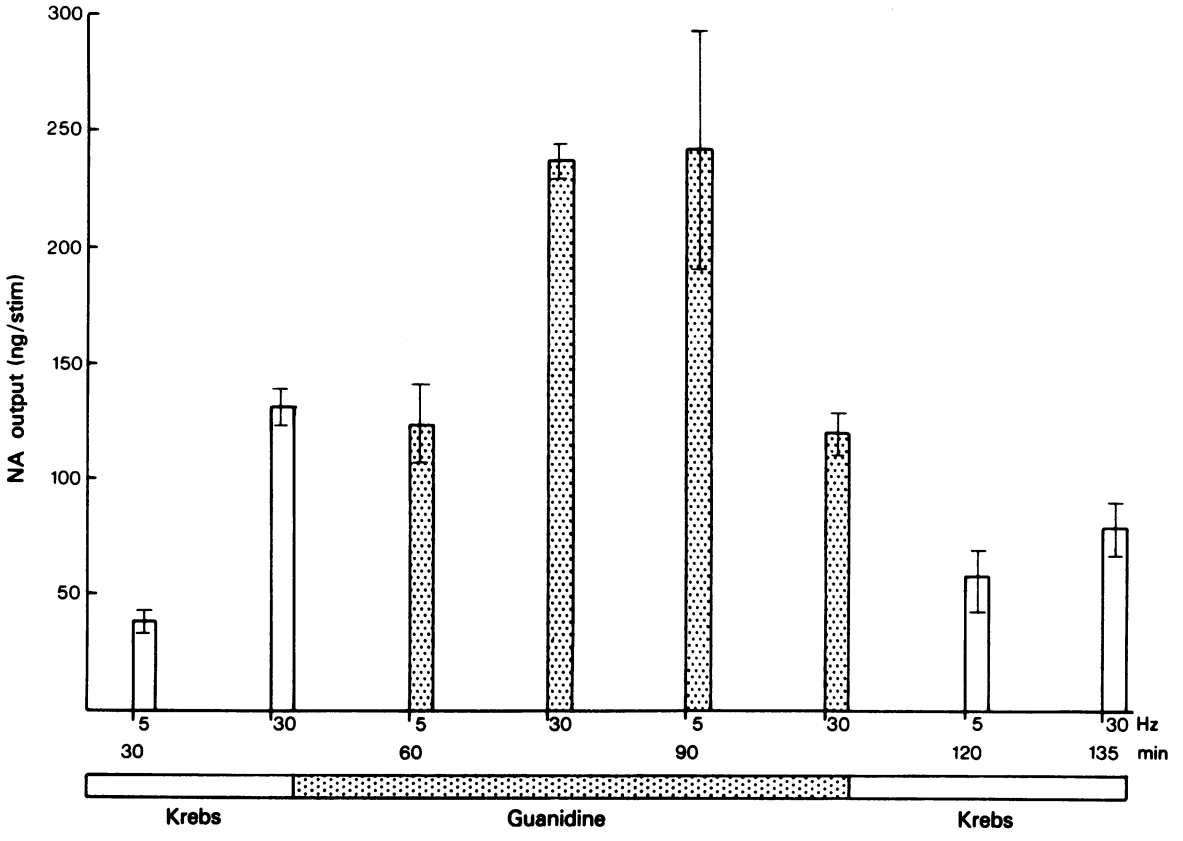

Figure 1 Effect of guanidine on noradrenaline (NA) output from the perfused spleen of the cat. After obtaining control NA outputs in normal Krebs solution, the spleen was perfused with guanidine-Krebs solution, followed by reperfusion with normal Krebs solution. Splenic nerves were stimulated at 5 and 30 Hz; open columns: release in Krebs solution; stippled columns: perfusion with guanidine (4 mM). Vertical lines are s.e. mean of 4 experiments.

radioactivity in a 3-channel Packard Tri-Carb Spectrophotometer after adding 0.5 ml of extracted sample to 10.0 ml scintillation fluid. Background activity of the same volume of solution in the absence of stimulation was deducted from the total output during stimulation (nerve, high K^+, sodium deprivation, etc.) to determine [^{3}H]-NA release due to stimulation. In experiments where percentage release of NA is indicated, each value represents the mean percentage of initial release.

Results

Effect of guanidine on noradrenaline release

The background release of NA without nerve stimulation was usually undetectable. Guanidine (1 to 10 mM) had very little effect on the spontaneous release of NA from the perfused cat spleen. At high concentrations of guanidine (10 mM), occasionally there was some release which was accompanied by a marked contraction of the spleen. Methyl guanidine has been previously reported to enhance efflux of NA (Cubeddu, Mosimann & Weiner, 1974).

In contrast to its lack of direct effect on spontaneous release, guanidine enhanced NA release induced by low-frequency stimulation of splenic sympathetic nerves. Figure 1 shows that at 5 Hz the control output of NA was 0.38 $\pm$ 0.05 ng/stimulus, but in the presence of guanidine (4 mM) NA output increased to 1.23 $\pm$ 0.17 ng/stimulus for the first stimulation ($P < 0.025$). The onset of maximum activity of guanidine was slow, usually requiring 30 to 45 min perfusion with the drug. Thus, after perfusion with guanidine for 30 min more, the output was further

increased to 2.41 ± 0.51 ng/stimulus ($P < 0.025$). Guanidine increased the output at 5 Hz by 321 ± 13 and 623 ± 144% over the control output during perfusion for 15 and 45 min, respectively. At 30 Hz, the effect of guanidine was less pronounced. Control output of 1.31 ± 0.08 ng/stimulus was increased to 2.37 ± 0.06 ng/stimulus ($P < 0.05$; 182 ± 12% of control). When perfusion with guanidine was continued for 30 min more, the output was essentially the same as the control output in normal Krebs solution. The effect of guanidine was sustained for several stimulation periods at 5 Hz and was only slowly washed out. At least 30 to 60 min of reperfusion with normal Krebs solution was required before outputs comparable to initial control values could be obtained.

Effect of guanidine on noradrenaline release induced by high potassium, sodium deprivation and tyramine

NA is released from the sympathetic nerves by a variety of procedures e.g., high potassium, sodium deprivation, tyramine, each causing release by a different mechanism. Therefore, it was of interest to study the effect of guanidine on NA release induced by these procedures.

High potassium To compare the effect of guanidine on NA (or [^{3}H]-NA) release induced by maintained depolarization as against intermittent nerve stimulation, NA was released by incubating the splenic slices in high potassium solution. Table 1 shows that guanidine had no effect on release of NA (or [^{3}H]-NA) from spleen slices by 35, 70 or 140 mM of potassium. Clearly, then, depolarization alone is not sufficient for the enhancement of NA release by guanidine.

Sodium deprivation Unlike nerve stimulation or potassium depolarization, sodium deprivation causes NA release from sympathetic nerves by a calcium-independent process (Garcia & Kirpekar, 1973). Table 1 shows that guanidine had no effect on NA (or [^{3}H]-NA) release from spleen slices when they were incubated in sodium-free (sucrose, lithium or choline) solutions.

Tyramine Release of NA by tyramine is also a calcium-independent process; presumably it acts by displacing NA from storage sites in sympathetic nerves (Lindmar, Löffelholz & Muscholl, 1967). Table 1 shows that guanidine did not affect [^{3}H]-NA release induced by tyramine (0.1 mM). These results would also suggest that guanidine and tyramine do not compete for a common neuronal site, otherwise guanidine should effectively reduce tyramine-induced efflux of [^{3}H]-NA.

Effect of graded concentrations of guanidine on noradrenaline release

Since guanidine was much more effective in enhancing release at 5 Hz than at 30 Hz, these experiments were performed by using the lower stimulation frequency. Figure 2 shows that increasing the guanidine concentration increases the output of NA at 5 Hz. Mean release for control samples without guanidine was 0.28 ± 0.11 ng/stimulus. Release was increased to 0.43 ± 0.15, 0.53 ± 0.39, 0.97 ± 0.45 and

Table 1 Effect of guanidine on noradrenaline (NA) or [^{3}H]-noradrenaline ([^{3}H]-NA) output from spleen slices induced by high potassium, sodium deprivation, or tyramine

Treatment	*n*	*NA output (ng/g tissue)* Control	Guanidine	[^{3}H]-*NA output* ($d\ min^{-1}g^{-1}$ *tissue*) Control	Guanidine
Potassium (35 mM)	12	—	—	8483 ± 4441	11824 ± 4486
Potassium (70 mM)	5	—	—	81162 ± 29493	89245 ± 37521
Potassium (140 mM)	6	159 ± 47	134 ± 67	412920 ± 233076	365641 ± 108107
Sodium-free (sucrose)	5	138 ± 24	122 ± 17	134262 ± 29590	107731 ± 29317
Sodium-free (lithium)	3	261 ± 52	285 ± 5	244421 ± 4714	235314 ± 7517
Sodium-free (choline)	5	118 ± 50	99 ± 55	82666 ± 17934	58141 ± 7159
Tyramine (0.1 mM)	5	—	—	24762 ± 6907	23886 ± 5234

Slices were incubated with [^{3}H]-NA for 30 min and then washed 3 times over a 30 min period. They were then incubated in normal Krebs solution for 5 min (in the potassium or tyramine studies) or 30 min (in the sodium deprivation experiments) to obtain the background release. This was followed by incubation in the appropriate test solutions (5 min in high potassium or tyramine solutions, 30 min in sodium-free solutions). Sodium-free solutions were prepared by replacing sodium with isotonic quantities of sucrose, lithium or choline. Paired experiments are indicated for each treatment. *n* equals number of experiments.

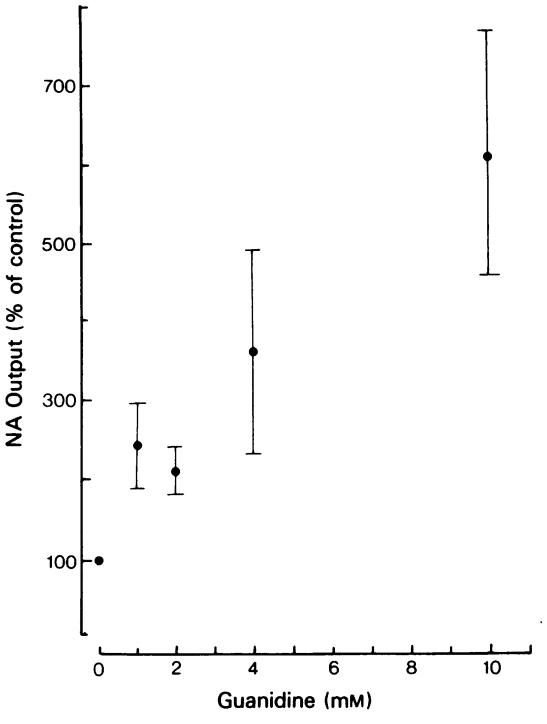

Figure 2 Relationship between guanidine concentration and output of noradrenaline (NA) from the perfused spleen of the cat. After obtaining the initial control output at 5 Hz in normal Krebs solution (0.28 ± 0.11 ng/stimulus), the spleen was perfused with Krebs solution containing 1 mM guanidine for 30 min and then consecutively perfused with 2, 4 and 10 mM guanidine in Krebs solution for 15 min each, followed by reperfusion with normal Krebs solution. Releases in 1, 2 and 10 mM guanidine were obtained from three experiments. Releases in normal Krebs solution and in 4 mM guanidine were obtained from 9 and 7 experiments, respectively. Regression analysis shows that the points lie on a straight line ($r = 0.98$, $P < 0.05$).

1.21 ± 0.61 ng/stimulus at 1, 2, 4 and 10 mM guanidine, respectively. Because of the variation in control outputs in different experiments, a better indication of the effect of graded concentrations of guanidine is a consideration of the mean percentage value taken from individual experiments, as illustrated in Figure 2. The outputs were 242 ± 53, 211 ± 33, 360 ± 129 and 608 ± 156% at 1, 2, 4 and 10 mM guanidine, respectively. The outputs at 4 and 10 mM of guanidine were not significantly different from each other. Again the effect of guanidine was well sustained, since the output upon stimulation still remained high at 0.65 ± 0.25 ng/stimulus or 337 ± 51% of the control

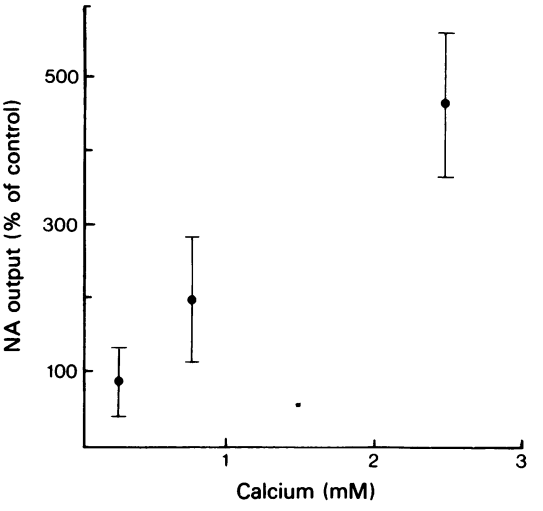

Figure 3 Relationship between guanidine and calcium on the output of noradrenaline (NA) from the perfused spleen of the cat. After obtaining the initial control NA output (0.32 ± 0.15 ng/stimulus) in normal Krebs solution, the spleen was consecutively perfused with guanidine (4 mM)-Krebs solution containing 0.25, 0.75 and 2.5 mM calcium for 15 min each. Splenic nerves were stimulated at 5 Hz during the last 2 min perfusion with normal Krebs solution, or during perfusion with guanidine-Krebs solution containing different calcium concentrations. Releases in 0.25 and 2.5 mM calcium were obtained from 4 experiments. Release in 0.75 mM calcium was obtained from 5 experiments. Regression analysis shows that the points lie on a straight line ($r = 0.99$, $P < 0.005$).

output after the spleen was perfused with Krebs solution for about 15 min.

Mechanical difficulties arose with the use of 10 mM guanidine. Perfusion pressure drastically increased and volumes of venous collection samples were reduced to 1/2 to 1/3 of control values. In some spleens the effect was so strong that the experiments had to be discontinued. Hence, in subsequent experiments 4 mM guanidine was used.

Effect of calcium on the enhancement of noradrenaline release by guanidine

Figure 3 shows an excellent correlation between external calcium concentration (up to 2.5 mM) and percentage of NA release in the presence of guanidine ($P < 0.005$). Previous work has shown that NA release at 5 Hz from spleens perfused with Krebs solution containing 0.25 or 0.75 mM calcium is virtually undetectable (Kirpekar & Misu, 1967; Kirpekar *et al.*, 1977a). However, the NA output from

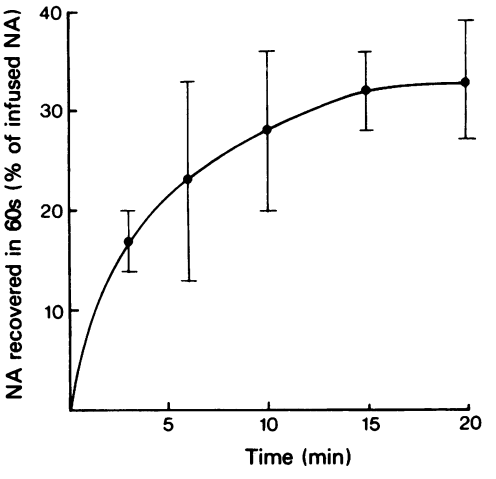

Figure 4 The effect of guanidine on the recovery of noradrenaline (NA) in the splenic venous perfusate during arterial infusion of NA. The spleen was perfused with 4 mM guanidine, and after 30 min NA was infused at a rate of 510 ng/min in the presence of guanidine. Venous samples were collected for 1 min each at 0, 3, 6, 10, 15 and 20 min of infusion, and recovery is expressed as a percentage of the infused NA. Vertical lines are s.e. mean of 4 experiments.

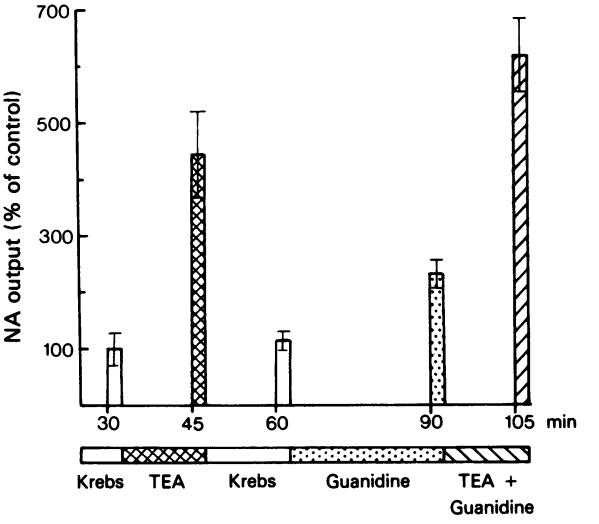

Figure 5 Effect of tetraethylammonium (TEA) on enhancement of release of noradrenaline (NA) by guanidine from the perfused spleen of the cat. Splenic nerves were stimulated at 5 Hz during the last 2 min of perfusion with each solution. NA release is expressed as percentage of the initial release in Krebs solution (=100%). Open columns: release in Krebs solution (0.31 ± 0.09 ng/stimulus); cross-hatched columns: release during perfusion with TEA (1 mM); stippled columns: release during perfusion with guanidine (4 mM); hatched columns: release during perfusion with TEA (1 mM) plus guanidine (4 mM). Vertical lines are s.e. mean of 5 experiments.

spleens perfused with 0.25 mM calcium Krebs solution containing guanidine (4 mM) was almost equal to the control output in 2.5 mM calcium (0.33 ± 0.15 and 0.24 ± 0.13 ng/stimulus for control and 0.25 mM calcium + guanidine, respectively). In 0.75 mM calcium plus guanidine the output was nearly doubled. Even though release was markedly enhanced by guanidine in low calcium solutions, maximum release of 1.64 ± 0.29 ng/stimulus was obtained in 2.5 mM calcium. In three experiments with guanidine in 7.5 mM calcium, a slight decline in NA output as compared to the output in 2.5 mM calcium, was observed (1.11 ± 0.16 ng/stimulus). In addition, mechanical difficulties of the type encountered with high concentrations of guanidine occurred at this high calcium concentration, with volumes of collection samples reduced by 40 to 80%. Sometimes this effect was irreversible, even after prolonged reperfusion with normal Krebs solution, and experiments had to be discontinued.

Effect of guanidine on uptake of infused noradrenaline

Primary inactivation of NA is by uptake into the sympathetic nerves (see Iversen, 1967). Kirpekar & Wakade (1968) showed that of the NA infused into the saline-perfused cat spleen, only 34% was recovered in the venous perfusate. Figure 4 shows that the mean NA recovery during the 10th, 15th and 20th min periods in guanidine perfused spleens was 31 ± 6%, a value comparable to the control recovery. However, it should be pointed out that methyl guanidine blocks uptake of NA into the sympathetic nerves (Cubeddu *et al.*, 1974). Even if guanidine were to block uptake, this property alone would not account for the massive increase in NA efflux seen after nerve stimulation. Cocaine and desmethylimipramine, which are potent neuronal blocking agents, have only minor effects in this regard (Kirpekar & Cervoni, 1963; Geffen, 1965).

Effect of tetraethylammonium on enhancement of noradrenaline release by guanidine

TEA also markedly enhances release of NA from sympathetic nerves by electrical stimulation (Thoenen *et al.*, 1967; Kirpekar *et al.*, 1972; Kirpekar *et al.*, 1976). Moreover, the effect of guanidine on NA release appears comparable to that of TEA. It was of interest, therefore, to see whether guanidine and TEA have a similar mechanism of action in enhancing

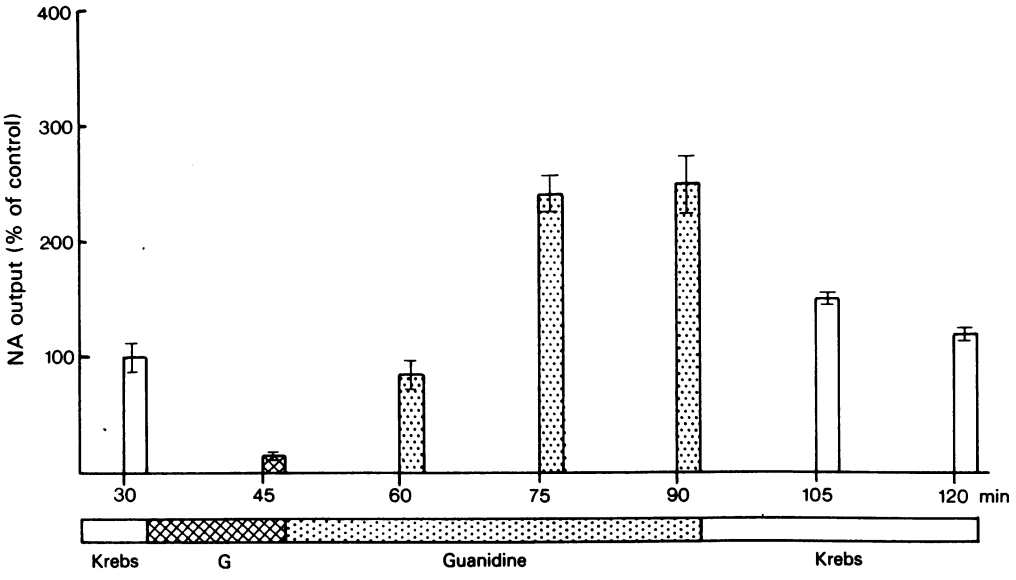

Figure 6 Effect of guanidine on the inhibitory effect of guanethidine (G) on noradrenaline (NA) release from the perfused spleen of the cat. Splenic nerves were stimulated at 10 Hz during the last 2 min of perfusion with each solution. NA release is expressed as percentage of the initial release in Krebs solution (=100%). Open columns: release in Krebs solution (0.62 ± 0.08); cross-hatched columns: release during perfusion with guanethidine (6.1 ± 10^{-7} M); stippled columns: release during perfusion with guanidine (4 mM). Vertical lines are s.e. mean of 4 experiments.

NA release. The following experiments were based on the assumption that if TEA and guanidine act by the same mechanism to enhance NA release, then once TEA had been given in a concentration to produce maximal release, the addition of guanidine should not have produced further enhancement of release. On the other hand, if TEA and guanidine were enhancing release by different mechanisms, then their combined effects should have been additive. Figure 5 shows that the combination of TEA (1 mM) and guanidine (4 mM) gave a greater output of NA at 5 Hz than did guanidine or TEA alone. In these experiments, the effect of guanidine on release was not as marked as reported earlier, and the reason for the smaller effect is not clear to us. Even if guanidine were to have enhanced release as before, the combination of the two drugs is still far more effective than either drug alone. If one compares the effects of TEA or guanidine alone to that of the combination, on the same preparation (i.e., correlated samples), there is a statistically significant difference ($P < 0.05$) between treatment with either drug alone and the combination of drugs.

Effect of guanidine on inhibition of noradrenaline release by guanethidine

Guanethidine blocks release of NA from sympathetic nerves during stimulation. It has been suggested that guanethidine accumulates intraneuronally and acts as a local anaesthetic on the membranes of the sympathetic nerve terminals to block NA release (Häusler, Thoenen, Haefely & Hürlimann, 1968). An alternative suggestion is that guanethidine or bretylium may depress NA release by blocking the increase in calcium permeability associated with evoked release (Kirpekar, Wakade, Dixon & Prat, 1969; Krauss, Carpenter & Kopin, 1970; Kirpekar *et al.*, 1978). Since the effect of guanidine appears to be related in some way to intraneuronal calcium concentration, it was of interest to determine whether guanidine, like TEA and 4-AP (Kirpekar *et al.*, 1978), would reverse the guanethidine blockade of sympathetic nerves.

Figure 6 shows that guanidine reversed the inhibition of NA release by guanethidine during nerve stimulation at 10 Hz. Guanethidine (6.1×10^{-7} M) suppressed NA release by over 90%. After 15 min perfusion with Krebs solution containing guanidine (4 mM), the NA output was restored to the initial control output before guanethidine treatment. As perfusion with guanidine continued, the NA output increased nearly 2.5 fold at each subsequent stimulation. On reperfusion with Krebs solution without guanidine, the output was initially high, but on second stimulation the output was comparable to the initial control value. We are not sure whether the

inhibitory effect of guanethidine reestablishes itself after washout of guanidine, since in two experiments repeating stimulations twice more produced NA outputs which were 70% of the initial control output, whereas in two other experiments the outputs were only 30%. Similar experiments were carried out by using additional stimulation frequencies of 5 and 30 Hz. The mean percentage reversal of guanethidine inhibition of release at 5 and 10 Hz was 298 ± 24 and 249 ± 45%, respectively, after 45 min of perfusion with guanidine. However, at 30 Hz, the reversal of guanethidine was only 32 ± 7% at 30 min and 47% (28, 66%) at 45 min.

In addition, guanidine reverses the inhibitory effect of bretylium on NA release. NA output at 5 Hz was markedly supressed by bretylium (1.2 ± 10^{-5} M). After perfusing with guanidine (4 mM) for 45 min, the output was 267% of the initial control output, a value comparable to that seen in experiments described above with guanethidine first inhibiting release.

Effect of guanidine on release of catecholamines from the cat adrenal medulla during splanchnic nerve stimulation

Early work by Sugawara & Tada (1927) indicated that injection of guanidine into dogs stimulated release of adrenaline from the adrenal medulla, although massive quantities had to be given (150 to 300 mg/kg), and toxic to fatal effects ensued. Guanidine (4 mM) had no effect on CA release from the perfused cat adrenal gland during splanchnic nerve stimulation at 10 Hz. Control output was 4.06 ± 1.76 ng/stimulus. After perfusing with guanidine for 15, 30, 45, and 60 min, CA outputs were 3.50 ± 2.09, 3.31 ± 2.31, 3.44 ± 1.72, and 2.47 ± 1.00 ng/stimulus, respectively. Spontaneous efflux of CA also was unaffected by guanidine treatment. Hence, unlike the perfused spleen, the perfused adrenal gland appears to be refractory to the stimulating effects of guanidine on transmitter release.

Discussion

Guanidine dramatically enhanced the amount of NA which appeared in the venous effluent following stimulation of the splenic nerves at 5 Hz. It also significantly enhanced the amount of NA released at 30 Hz. Since we have shown no inhibition by guanidine of uptake of NA, and since others have demonstrated an increased release of neurotransmitters in other systems (Otsuka & Endo, 1960; Kamenskaya *et al.*, 1975; Lundh, *et al.*, 1977; Matthews & Wickelgren, 1977), we conclude that the increased amounts of NA appearing in the venous effluent during guanidine perfusion are due to facilitation of evoked release of NA from sympathetic nerve endings.

Guanidine is more effective in increasing the release of NA at low frequencies than at high frequencies. The frequency-dependent effect of guanidine has been demonstrated for release of ACh from the neuromuscular junction (Kamenskaya, *et al.*, 1975). TEA and 4-AP also share this property with guanidine (Kirpekar *et al.*, 1976; Kirpekar, Kirpekar & Prat, 1977a). Failure to increase release at 30 Hz in a manner comparable to that at 5 Hz may be related in some way to the proportion of time the nerve membrane remains depolarized since, indeed, release by high potassium is also not affected by guanidine. On the other hand, if facilitation of release with an increase in stimulation frequency reflects a greater accumulation of calcium in the nerve terminals on the time average during a train of pulses at the higher frequency than at the lower frequency (Kirpekar, Prat & Wakade, 1975), then guanidine, like 4-AP and TEA, may be less effective in enhancing NA release at the higher frequency of stimulation.

Enhancement of NA release by guanidine occurred in low and in high extracellular concentrations of calcium, with maximum effect occurring at 2.5 mM calcium. Since guanidine only facilitates release induced by the nerve action potential, it is quite possible that it may mobilize calcium ions which are required for the physiological release of NA. It is not clear from these experiments whether guanidine increases influx of extracellular calcium into the nerve terminals during the course of an action potential and/or it mobilizes an intracellular store of calcium. Guanidine has been shown to inhibit the uptake and binding of divalent cations, e.g., magnesium, in mitochondria and in fact, Lundh *et al.* (1977) have suggested that guanidine may enhance ACh release by allowing calcium to accumulate inside the nerve terminal by interfering with the intracellular binding of calcium which enters during a nerve action potential. The delayed onset for maximum activity and the sustained effect seen after washout with Krebs solution make the concept of an intracellular site of action for guanidine seem even more appealing. Interestingly, guanidine had no effect on calcium-independent release of NA induced by tyramine or sodium deprivation.

The facilitation of transmitter release by TEA appears to be due to a greater influx of calcium into the nerve terminal during the prolonged duration of the action potential (Katz & Miledi, 1969). A similar mechanism for facilitation of NA release was also proposed for 4-AP (Kirpekar *et al.*, 1977a). Guanidine shares many properties with TEA and 4-AP as far as release of NA by nerve stimulation is concerned, and it may be that guanidine somehow enhances calcium entry into the nerve terminals during the course of an action potential. It is possible that guanidine

could bring about enhanced calcium entry by a mechanism different from TEA or 4-AP, since it does not appreciably prolong the duration of the action potential in skeletal muscle fibres (Kamenskaya *et al.*, 1975), or in lamprey reticulospinal fibres (Matthews & Wickelgren, 1977), although an effect similar to TEA or 4-AP on sympathetic nerve terminals cannot be ruled out at present. Since the effects of guanidine and TEA on NA release were additive, it seems likely that guanidine is acting differently from TEA and 4-AP. An alternate possibility is that guanidine could still enhance the effect of TEA, if the latter drug acted as a partial agonist. We would like to conclude, therefore, that guanidine enhances NA release by mobilizing calcium both from intra- and extraneuronal sources partly by enhancing its entry into the neurone, and by interfering with the intracellular binding of calcium which enters the nerve terminals during an action potential.

We have also shown that guanidine reverses the inhibitory effect of guanethidine on NA release from the spleen by nerve stimulation. The restoration of transmission by guanidine can be brought about if guanidine displaces guanethidine from sympathetic nerve terminals. Since we have been unable to determine whether guanidine reverses the guanethidine blockade of NA release permanently, we also cannot decide whether guanidine displaces guanethidine to restore adrenergic transmission. Moreover, guanidine reverses the blockade of NA release by bretylium, which is not structurally related to guanidine or guanethidine. Another possibility is that guanidine restored the guanethidine-induced conduction blockade of the sympathetic nerve endings (Häusler *et al.*, 1968). Guanidine has been shown to increase the excitability of motor nerve fibres since single stimuli result in multiple firing and eventually spontaneous action potentials are produced (Matthews & Wickelgren, 1977). The authors have suggested that guanidine, by reducing the screening effect of divalent cations, produces a covert depolarization of the axon membrane making it more excitable. Even if guanidine were to have similar effects on sympathetic nerve terminals, this property of the drug would not account for the reversal of presumed local anaesthetic action of guanethidine. Local anaesthetics physically block the sodium channels responsible for the action potential and guanidine would not be expected to open the sodium channels which have been blocked by a local anaesthetic. Secondly, if guanidine were simply restoring conduction blockade, then we do not understand why the guanethidine blockade at 30 Hz was only partially restored, while release was not only fully restored at 5 Hz, but was actually potentiated. A third possibility is that guanidine allows greater than normal amounts of calcium to accumulate inside the sympathetic nerve terminals during an action potential to reverse guanethidine blockade of NA release. TEA and 4-AP have also been shown to reverse guanethidine blockade (Kirpekar *et al.*, 1978). Since all three agents are known to enhance the calcium concentration in the nerve terminals (Lundh *et al.*, 1977), and since little is known regarding their ability to open the sodium channels which have been blocked by a local anaesthetic, we would like to suggest that antagonism between guanidine and guanethidine probably involves mobilization by guanidine of calcium within the sympathetic nerve terminals.

Finally, some mention should be made concerning the failure of guanidine to enhance CA release from the adrenal medulla during splanchnic nerve stimulation. It was expected that guanidine would increase CA release during splanchnic nerve stimulation by increasing the release of ACh. We are unable to explain the lack of effect of guanidine on the adrenal gland. However, it should be pointed out that differences in various organs in their responsiveness to guanidine compounds have been previously reported (Cubeddu *et al.*, 1974).

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