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Guanethidine Effects on the Guinea Pig Vas Deferens Are Antagonized by the Blockers of Calcium-Activated Potassium Conductance, Apamin, Methylene Blue, and Quinine

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SUMMARY

The blocking effects of guanethidine on electrically induced, neurally mediated, contractions of the guinea pig vas deferens *in vitro* could be markedly antagonized by the bee venom polypeptide apamin (20–60 nM), by 0.1 mM methylene blue, and (less regularly) by 0.1–0.15 mM quinine, three substances known to inhibit calcium-activated potassium conductance in a variety of cells. Guanethidine (20 μM) was also found to inhibit (by 88%) the release of [^{3}H]norepinephrine induced by electrical stimulation (20-Hz, 2-msec, biphasic pulses of supramaximal voltage). Such inhibition was decreased to 39% when 20 nM apamin was present together with guanethidine, thus showing that the effect of this polypeptide is presynaptic. On the basis of these findings, we suggest that guanethidine may block adrenergic neurons by activating their calcium-activated potassium conductance, presumably by releasing intracellular calcium.

INTRODUCTION

Guanethidine and other adrenergic neuron-blocking drugs selectively inhibit transmitter release from noradrenergic neurons. However, the mechanism for such an effect remains largely unknown, in spite of much experimental work (1, 2).

On the other hand, the plasma membrane potassium conductance that is specifically activated by cytosolic calcium ion concentration, first described in human red blood cells by Gárdos (3), has now been recognized as an important control mechanism in many cell types, including sympathetic neurons (4–7). As this outwardly directed current leads to plasma membrane hyperpolarization, it represents an inhibitory cell mechanism. If guanethidine were able to activate this potassium conductance, presumably by releasing calcium from reservoirs present in noradrenergic presynaptic regions, neuronal inhibition (i.e., blockade) would result. To test this hypothesis, we have studied whether substances such as apamin, quinine, or methylene blue, known to inhibit calcium-activated potassium conductance, are also able to antagonize the inhibitory effects of guanethidine on adrenergic neurons. The results obtained are compatible with such a hypothesis.

EXPERIMENTAL PROCEDURES

Methods. Male guinea pigs (350–500 g) were killed by cervical dislocation, and their vasa deferentia were removed and carefully cleaned of serosal and fat tissues. Two ligatures were placed in the middle portion of each vas, 2.5–3 cm apart, and the ligated segment was removed and mounted in a conventional jacketed chamber of 12-ml capacity, with connections for fluid removal and replenishment. The chamber was filled with Medium A (8) (composition, millimolar: NaCl, 124; KCl, 5; KH_2PO_4, 1.24; $CaCl_2$, 1; $MgCl_2$, 1.3; $NaHCO_3$, 26; and glucose, 10), and continuously bubbled with 5% CO_2 in O_2. The upper ligature was then fixed to a Statham G10B transducer, and a 1-g resting tension was applied. Contractions were recorded with a Grass 7D polygraph. Electrical field stimulation was with square wave pulses at 20-Hz, 2-msec, supramaximal voltage, applied for 2 sec every 2 min, through parallel platinum electrodes placed 14 mm apart at opposite sides of the preparation. Such stimulation conditions induce exclusively neurally mediated contractions. The chamber was kept at 30°, and all solutions added had been previously equilibrated to the same temperature. In the experiments in which release of [^{3}H]norepinephrine was studied, the ligated vas deferens segments were first incubated at 30° with shaking, at 40 min^{-1}, in 2 ml of Medium A that contained 5 μCi (0.56 μM) of L-[^{3}H]norepinephrine. After 30 min, the vas deferens segments were removed and rapidly rinsed by successive immersion in two beakers containing 50 ml of Medium A at room temperature; they were then placed in the chamber described above, in which the electrodes were of pure (i.e., greater than 99%) silver wire. Electrical stimulation was with 20-Hz, 2-msec biphasic pulses of supramaximal voltage (nominal, 40 V; actual, continuously monitored with an oscilloscope, 6 V). When release was induced by high potassium, the total potassium

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concentration was increased in Medium A to 60 mM by replacing an equivalent amount of NaCl.

Efflux is expressed as a fractional rate constant: [dpm released during the collection period (*cp*)/dpm present in tissue at the beginning of *cpx* duration of *cp*].

Materials. Apamin was purchased from Dr. B. E. C. Banks, University College, London; quinine chloride or quinine sulfate from Sigma; and methylene blue from May and Baker. Phenoxybenzamine HCl (Smith Kline & French) was a gift of Professor H. Miranda, and L-[7-^{3}H]norepinephrine (4.5 Ci/mmole) was obtained from New England Nuclear Corporation.

RESULTS

Guanethidine (20 μM) progressively inhibited electrically induced contractions, until greater than 90% inhibition was found after 24 min of drug application (Fig. 1A). Apamin (20 nM) led to a marked antagonism of the effect of guanethidine, so that only a 28% inhibition was seen following 14 min in apamin. This substance by itself slightly increased contractile force (Fig. 1B), and diminished markedly the inhibition induced by 20 μM guanethidine. Similar results were obtained with 60 nM apamin, whereas a 10 nM concentration was much less effective.

In another preparation, 20 μM guanethidine led to complete neuronal block (Fig. 2A). This was rapidly antagonized by 100 μM methylene blue, which also induced spontaneous contractions of the vas deferens. These spontaneous contractions were suppressed by 0.1–1 μM phenoxybenzamine (data not shown), thus indicating their neural origin. Methylene blue also enhanced contractile force (Fig. 2C), and at 100 μM almost completely antagonized the blocking effect of 20 μM guanethidine. Methylene blue at 1 or 10 μM was an ineffective antagonist.

The effects of 100–150 μM quinine as a guanethidine antagonist, although usually apparent (Fig. 3), were less marked and much more variable than those of apamin or methylene blue. Thus, occasionally quinine even enhanced the blocking effects of guanethidine (Fig. 3D). Spontaneous contractile activity was also frequently seen during quinine-induced recovery of inhibition by guanethidine.

In 3 of a total of about 60 preparations used, insensitivity to guanethidine was found (Figs. 4 and 5). In such cases, spontaneous activity could be observed which on occasion was of a cyclical type (Fig. 5). In the latter case, the number of cycles as well as the duration of the

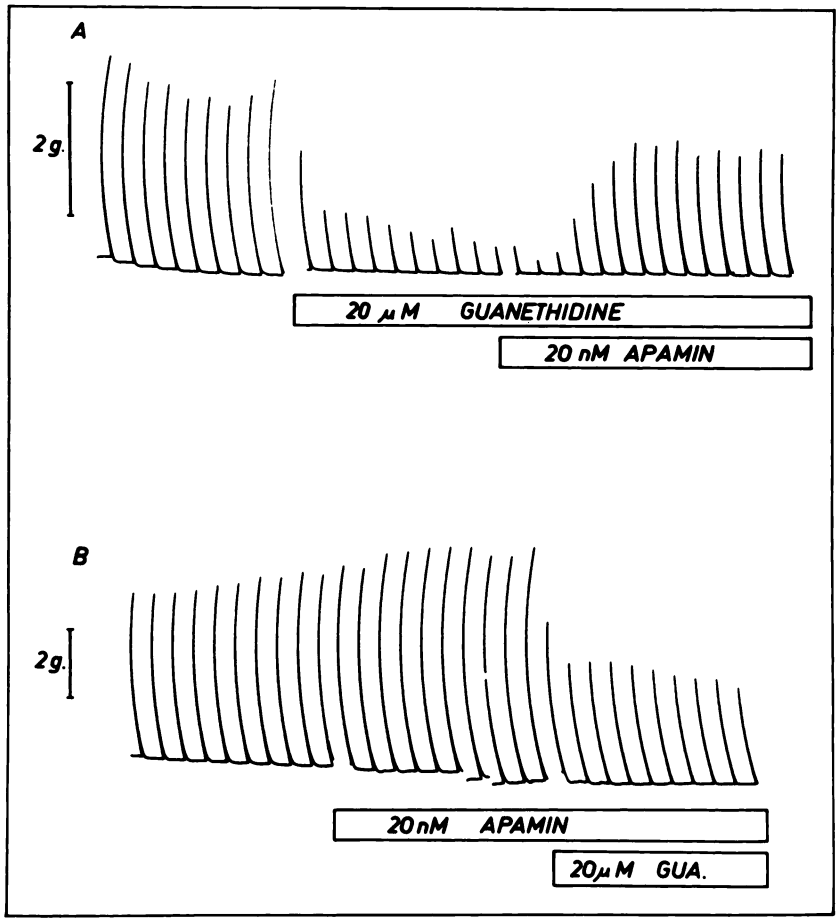

FIG. 1. *Effect of apamin on guanethidine-induced inhibition*

Guinea pig vasa deferentia were field-stimulated with supramaximal, 2-msec, square wave pulses at 20 Hz for 2 sec every 2 min, and the force of contraction was recorded. In A, guanethidine was applied prior to apamin, and the order was reversed in B. Results are representative of 10 experiments.

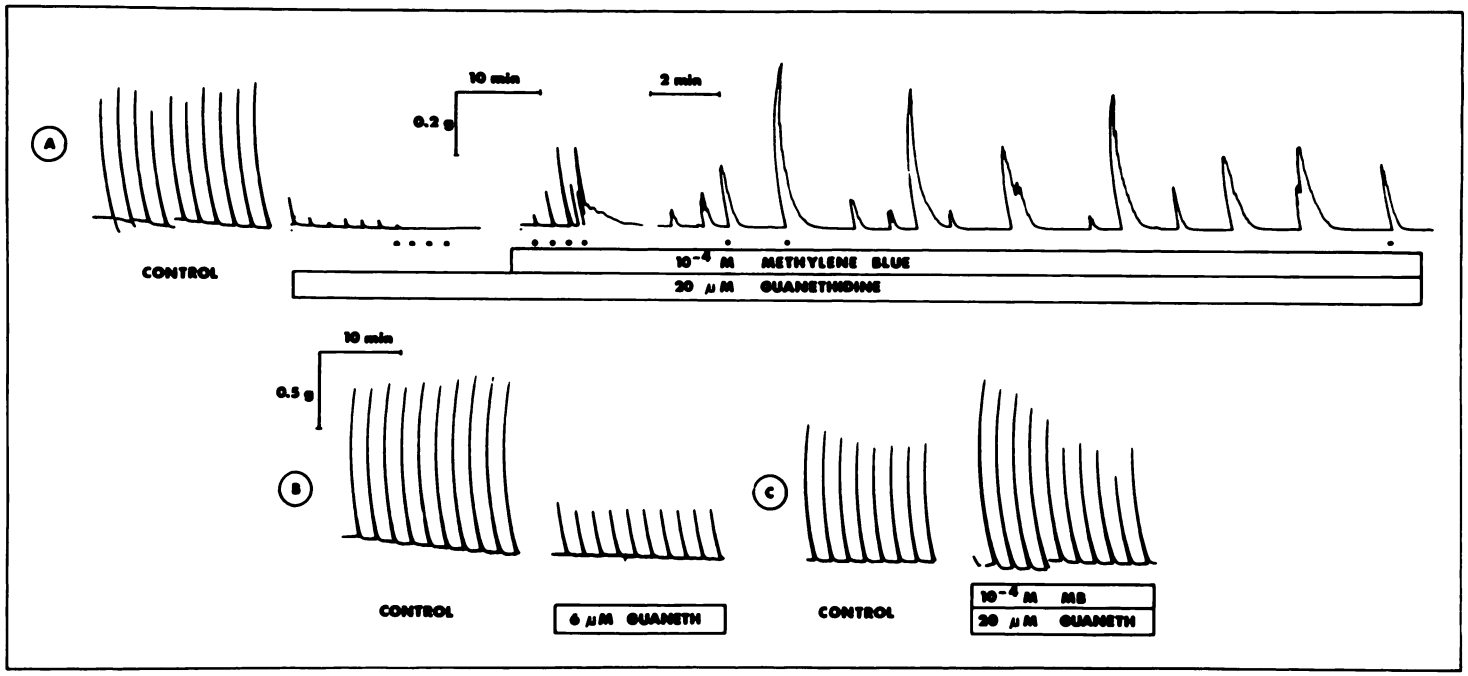

Fig. 2. *Reversal of guanethidine effects by methylene blue*

In A, 20 μM guanethidine leads to complete adrenergic blockade. ●, Electrical stimulation was applied during and after total tissue unresponsiveness. B, Following recovery in methylene blue, spontaneous contractions were frequently seen. Recording speed was increased to observe their more complex configuration. In C, 10^{-4} M methylene blue (*MB*), given together with 20 μM guanethidine, completely antagonizes its effect. These experiments were repeated four times.

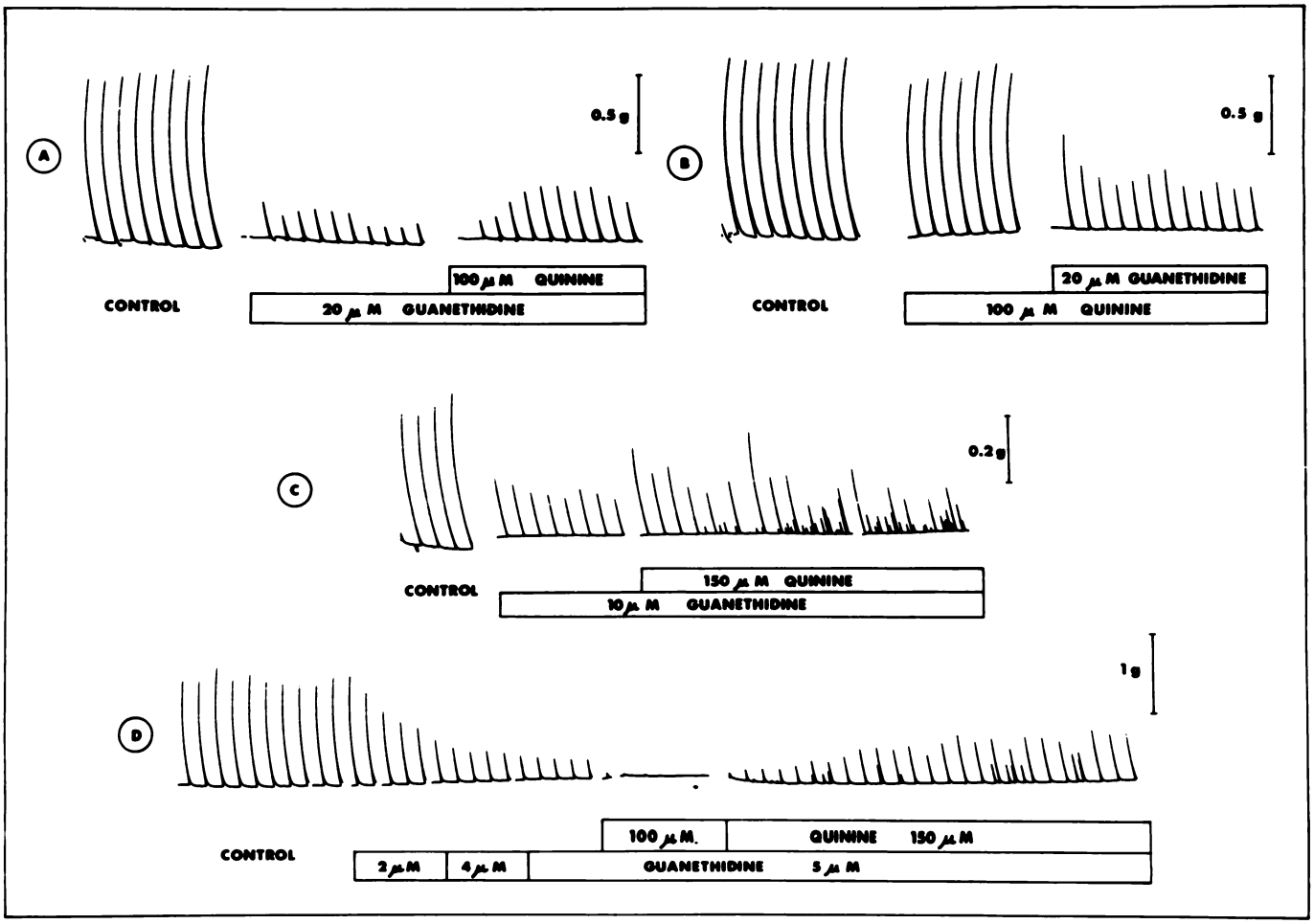

Fig. 3. *Quinine antagonizes the effects of guanethidine*

Inhibition induced by 5–20 μM guanethidine was partially antagonized by 100–150 μM quinine. Spontaneous contractions may be seen in C and D. Stimulation (square wave, 2 msec, 20 Hz) was applied for 2 sec every 2 min. Force calibrations are shown to the *right* of each register. In all, seven experiments with quinine or quinidine were performed.

spontaneous events increased with time of exposure to guanethidine.

The effects of guanethidine and apamin on induced [^{3}H]norepinephrine release were also studied. In 10 experiments, 4 of which are shown in Fig. 6, guanethidine inhibited electrically induced [^{3}H]norepinephrine release by 88%. This inhibition was the same in the second and third stimulation periods (data not shown). When 20 nM apamin was added to 20 μM guanethidine, electrically induced [^{3}H]norepinephrine release increased 2.2-fold, and release inhibition was reduced to 39% (Fig. 6). Apamin (20 nM) by itself increased electrically induced [^{3}H]norepinephrine release by 20% (N = 2, not statistically significant). In control experiments, in which the three successive periods of electrical stimulation were carried out in drug-free Medium A, induced [^{3}H]norepi-

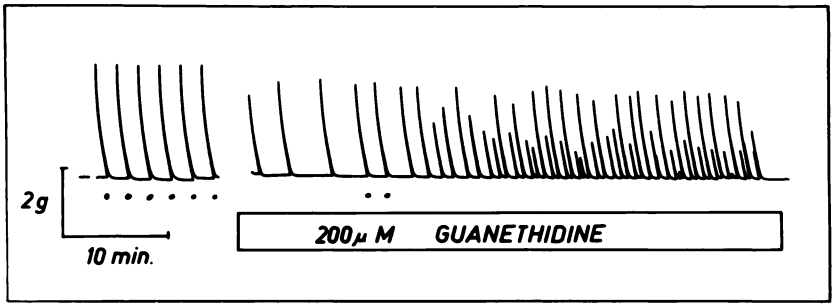

Fig. 4. *Resistance to guanethidine*

Control electrically-induced contractions are shown on the *left*. Guanethidine (60 μM) was then added, and no effect was obtained (data not shown); its concentration was then raised to 200 μM. Induced contractions are those above ●. The other contractions are spontaneous.

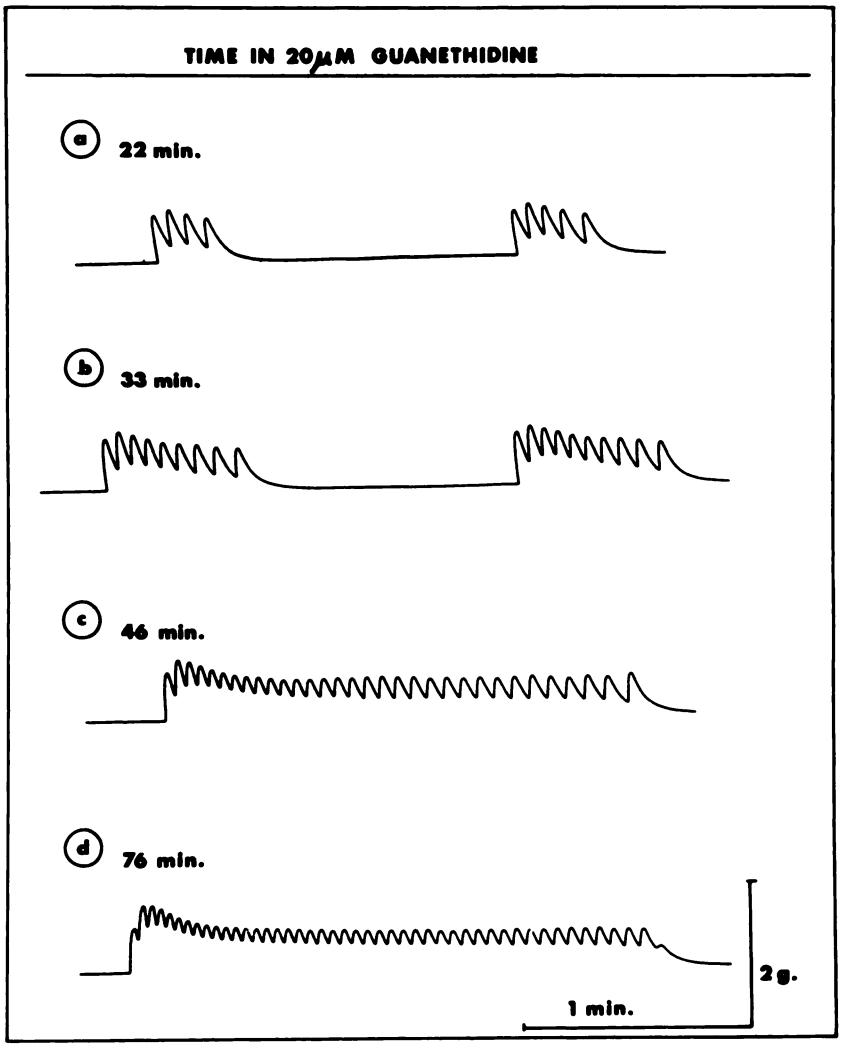

Fig. 5. *Cyclical spontaneous contractions in a guanethidine-resistant vas deferens*
Following 20 μM guanethidine, spontaneous cyclical contractions appeared. The length and number of cycles of the contractions increased with time of exposure to guanethidine.

nephrine release was essentially the same in all three stimulation periods (data not shown). When [^{3}H]norepinephrine release was induced by increasing the potassium concentration in Medium A to 60 mM (Fig. 7), 30 μM guanethidine inhibited such release by only 47%.

DISCUSSION

For an unambiguous interpretation of the results presented, two different questions should be answered. One is related to the specificity as inhibitors of calcium-activated potassium conductance of the drugs used. Thus, quinine and quinidine inhibit such conductance in red blood and other cell types (9–11), but also inhibit catecholamine release (12) and are *alpha*-adrenoceptor (13) and sodium channel (14) blockers. This variety of effects, some of them possibly less specific, may explain the inconsistencies we have found with this drug. Apamin, the basic polypeptide of bee venom (15), has been found to block this type of potassium conductance in smooth muscle and liver cells (11, 16), but not in red blood cells. Although not specifically tested on nerve cells, its convulsive effects (15) suggest that a similar action may also occur in them. No nonspecific membrane effects have been found when apamin is present at nanomolar concentrations. Similarly, methylene blue has recently been found to inhibit calcium-activated potassium conductance in pancreatic β-cells,[1] but not in red blood cells (17). This latter finding suggests that both this dye and apamin do not induce nonspecific membrane perturbations that could lead to multiple drug actions, but may block calcium-activated potassium conductance by acting at a step that is not present in red blood cells. The demonstration that apamin antagonizes the effects of guanethidine by a presynaptic mechanism does not deny that this polypeptide, as well as methylene blue and quinine, may also have postsynaptic effects which can partially contribute to restore contractile force in preparations inhibited by guanethidine.

[1] I. Atwater, personal communication.

Once accepted that the drugs used antagonize guanethidine effects because of their ability to block calcium-activated potassium conductance, a second point should also be addressed: As guanethidine acutely inhibits the adrenergic nerve terminal, possibly by a mechanism that, among other things, involves a change in plasma membrane potential [i.e., the so-called membrane "stabilization" (2)], it is conceivable that any procedure which induces excitation (i.e., depolarization) of the presynaptic region might antagonize guanethidine effects by a mechanism that may be quite unrelated to the one by which guanethidine induces inhibition. This may be the case for the partial reversal of guanethidine effects obtained by blockers of voltage-sensitive potassium conductance, such as tetraethylammonium or 4-aminopyridine (18), and could also hold true for blockers of calcium-activated potassium conductance, which also induce depolarization of the plasma membrane. This question can be answered only when guanethidine is directly shown to release intracellular calcium *in situ* and that this activates potassium conductance, which, in turn, leads to membrane hyperpolarization and thus to inhibition of the adrenergic nerve terminals. Because of the small size of these axons such a demonstration is not technically feasible at present.

The present results, however, added to the known facts that guanethidine and other adrenergic blockers are actively accumulated by the norepinephrine uptake-1 mechanism of adrenergic neurons (19, 20) and that, at drug concentrations that may be attained inside such neurons, guanethidine inhibits mitochondrial electron transport (21)[2] support such a premise. Inhibition of mitochondrial electron transport is known to release mitochondrial calcium, which, in turn, activates plasma membrane calcium-activated potassium conductance. This type of neuronal inhibition also occurs when brain cortex neurons are subjected to hypoxia or to inhibitors of mitochondrial function, such as 2,4-dinitrophenol or oligomycin (22). The blocking effects of nitrophenols, however, may be obscured in nerve terminals because of their intrinsic capacity to induce catecholamine secretion, even in the absence of uncoupling of oxidative phosphorylation (23). The present hypothesis can apparently explain the acute blocking effect of guanethidine,

[2] J. Ferreira, L. Gil, A. Stutzin, and F. Orrego, unpublished results.

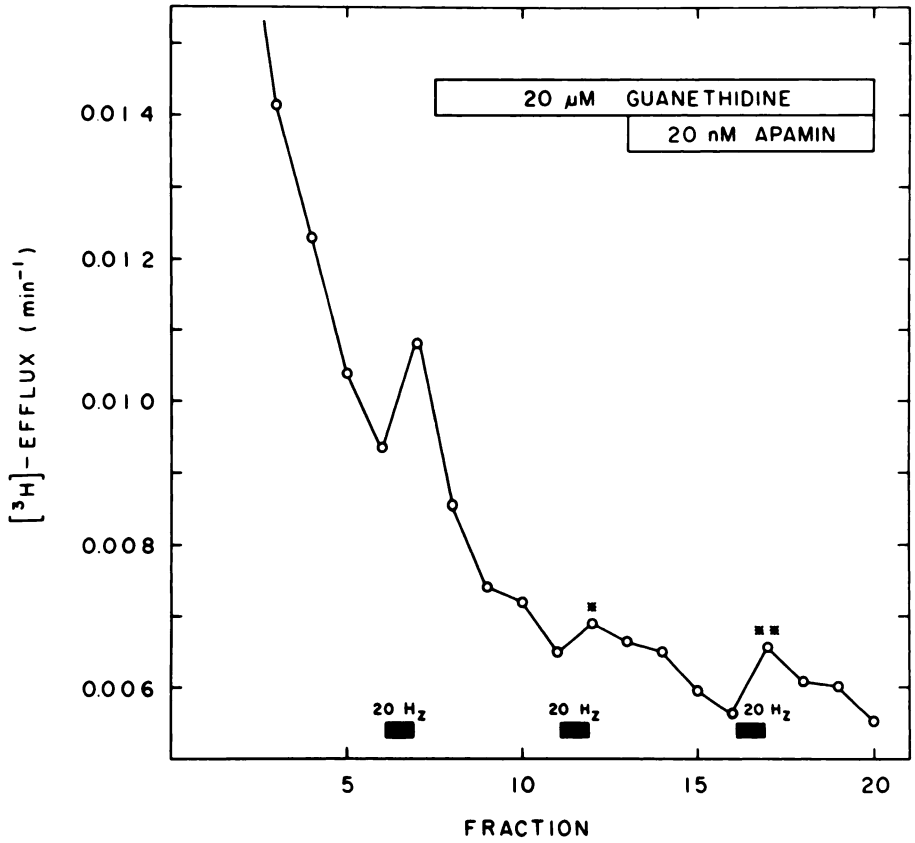

Fig. 6. *Effects of guanethidine and apamin on electrically induced [^{3}H]norepinephrine release*

Ligated vas deferens segments about 3 cm long were first incubated in 2 ml of Medium A with 5 μCi of L-[^{3}H]norepinephrine for 30 min, and then mounted in the incubation chamber that contained 12 ml of nonradioactive Medium A. This was renewed every 5 min, and each aspirated fraction was counted at the end of the experiment. When electrical stimulation was applied, at periods indicated by ▬, it consisted of 20-Hz, 2-msec, biphasic pulses of supramaximal voltage applied for 10-sec periods, alternating with 10-sec periods of rest. The ^{3}H Fractional rate constant (min^{-1}), is plotted against the fraction number. *, $p < 0.01$ relative to control; **, $p < 0.01$ relative to release peak in which guanethidine alone was present. Results of four experiments.

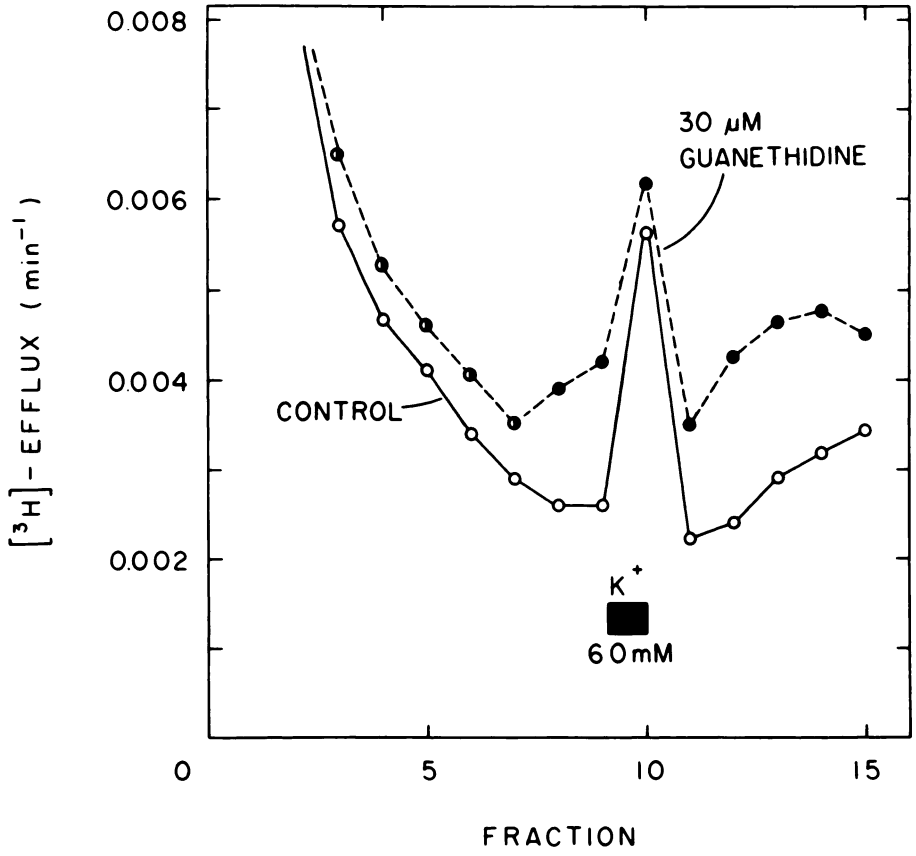

Fig. 7. *Effect of guanethidine on [^{3}H]norepinephrine release by high potassium*

Vasa deferentia were incubated with [^{3}H]norepinephrine and then washed with Medium A in the tissue chamber exactly as described in the legend to Fig. 6. During the 5-min period indicated by ▬, the potassium concentration was increased in Medium A to 60 mM, replacing an equivalent amount of sodium. In the *upper curve* 30 μM guanethidine was present from Fraction 8 onward, as indicated by ●. Each curve represents three experiments. For calculating the inhibition of release by guanethidine, the baseline of release was obtained by extrapolating to Fraction 10 the straight line formed by Fractions 7–9 of the *upper curve*.

as well as why these drugs block presynaptic regions, rich in mitochondria, while leaving unaffected the axonal conduction in non-terminal regions of the adrenergic neuron (24),[3] which also have uptake-1 but where mitochondrial density is very low. This hypothesis can also explain why brief depolarizing stimuli, such as electrical ones, are quite ineffective in inducing norepinephrine release from nerve terminals blocked by guanethidine, while prolonged depolarizations, such as those elicited by high extracellular potassium concentrations, are able to overcome in part such blockade. This discrepancy between electrically induced and potassium-induced norepinephrine release had already been observed in rat salivary glands during recovery following guanethidine administration *in vivo* (25), but remained unexplained.

The finding that 5% of the vasa deferentia are resistant to guanethidine is also consistent with the proposed mechanism, because calcium-activated potassium conductance has been shown in neurons and other cell types to become on occasion unresponsive to calcium (26, 27).

[3] G. Ormeño, A. Stutzin, and F. Orrego, unpublished results obtained with canine splenic nerves *in vivo* and *in vitro*.

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Note added in proof. The recent finding that apamin is a selective inhibitor of calcium-activated potassium conductance in neuroblastoma cells (28) further supports the present work.

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