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FURTHER EVIDENCE FOR THE INVOLVEMENT OF Na^+ CHANNELS IN THE RELEASE OF ADRENAL CATECHOLAMINE: THE EFFECT OF SCORPION VENOM AND GRAYANOTOXIN I

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1 The effects of venom from the scorpion, *Leiurus quinquestriatus*, and grayanotoxin I on catecholamine secretion were studied in the perfused adrenal glands of guinea-pig.

2 Scorpion venom (0.1 to 10 μg/ml) caused a dose-dependent increase in catecholamine output. The response to the venom was partially inhibited by atropine (0.5 mM) plus hexamethonium (1 mM). The dose-response curve was shifted to the right in the presence of these blocking agents.

3 Grayanotoxin I (0.1 to 0.5 mM) caused a dose-dependent increase in catecholamine output which was significantly reduced by atropine (0.5 mM) plus hexamethonium (1 mM). However, when grayanotoxin I (0.1 mM) was applied together with scorpion venom (0.1 μg/ml, a concentration which alone, was almost ineffective) the maximum catecholamine output was reached even in the presence of atropine plus hexamethonium.

4 Tetrodotoxin (0.1 or 0.2 μM) reversibly inhibited the secretory responses induced by scorpion venom (10 μg/ml) and grayanotoxin I (0.1 mM) plus scorpion venom (0.1 μg/ml).

5 Scorpion venom and grayanotoxin I plus scorpion venom did not cause catecholamine secretion in the absence of extracellular Na^+ or Ca^{2+} ions. However, the secretory response was restored by reintroduction of Na^+ or Ca^{2+} ions.

6 It is suggested that both scorpion venom and grayanotoxin I activate Na^+ channels on the chromaffin cell and result in catecholamine secretion.

Introduction

Veratridine caused a tetrodotoxin-sensitive increase in catecholamine secretion from the perfused adrenal glands of guinea-pig (Ito, Nakazato & Ohga, 1978; 1979) and cat (Kirpekar & Prat, 1979). This veratridine-induced catecholamine secretion was considered to be exocytosis because of the accompanying secretion of dopamine-β-hydroxylase and adenine nucleotides (Ito, Nakazato & Ohga, 1980). These facts suggest that the adrenal chromaffin cell membrane possesses Na^+ channels responsible for the catecholamine secretion.

Scorpion venom (Adam, Schmidt, Stämpfli & Weiss, 1966; Narahashi, Shapiro, Deguchi, Scuka & Wang, 1972) and grayanotoxin (Narahashi & Seyama, 1974; Starkus & Narahashi, 1978) like veratridine (Ohta, Narahashi & Keeler, 1973) are known to activate tetrodotoxin-sensitive Na^+ channels in various neuronal tissues. Therefore, both scorpion venom and grayanotoxin may provide further evidence for the involvement of Na^+ channels in adrenal catecholamine secretion. In the present experiments, the effects of scorpion venom and grayanotoxin on the catecholamine output were investigated in perfused adrenal glands isolated from the guinea-pig.

Methods

Guinea-pigs weighing from 500 to 700 g were anaesthetized with sodium pentobarbitone (40 mg/kg) intraperitoneally. Both adrenal glands were perfused and isolated following the general procedure described previously (Ito *et al.*, 1979).

The standard perfusion medium was Locke solution of the following composition (mM): NaCl 154, KCl 5.6, $CaCl_2$ 2.2, Na_2HPO_4-NaH_2PO_4 buffer (pH 7.1) 3 and glucose 10. Pure O_2 was continuously bubbled through this solution and the perfusion was carried out at 25 ± 1°C. In Na^+-free solution, NaCl was replaced by isotonic sucrose (9.25%), equimolar choline chloride or LiCl and Na^+-phosphate buffer was replaced by Tris aminomethane buffer (2.5 mM). In Ca^{2+}-free solution, $CaCl_2$ was omitted and $MgCl_2$ 2 mM was added. Even after Ca^{2+} was reintroduced,

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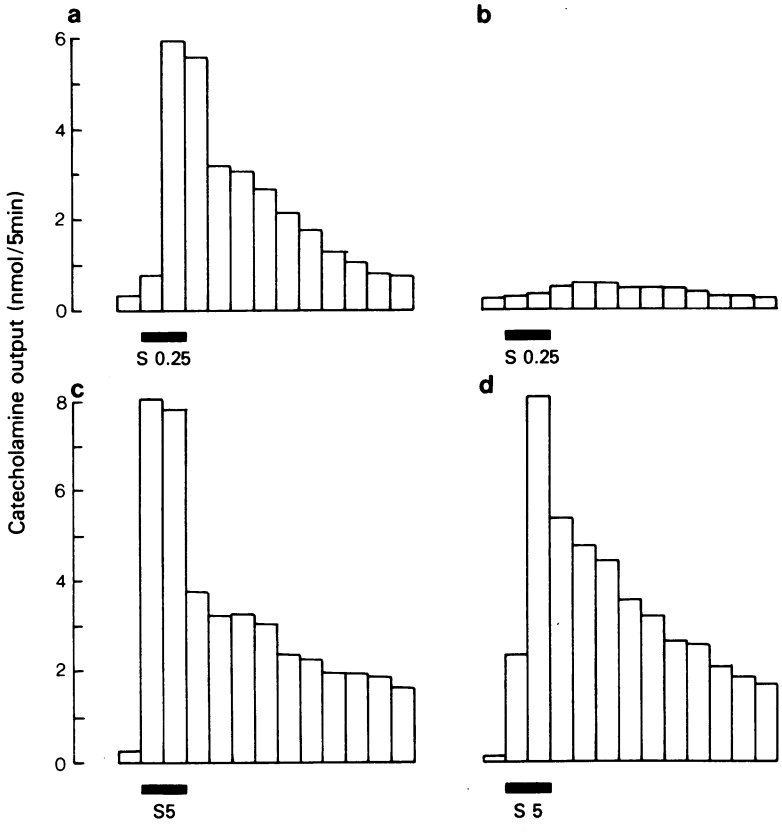

Figure 1 Catecholamine output induced by scorpion venom in the presence or absence of atropine and hexamethonium. Filled bars (■) and numbers below them indicate the length (10 min) of the exposure and the concentration (μg/ml) of scorpion venom (S). Adrenal glands were perfused with Locke solution in the absence (a and c) and presence (b and d) of atropine (0.5 mM) plus hexamethonium (1 mM) throughout the experiments. In this and in all subsequent figures, the columns represent the total amount of catecholamine (nmol) present in the adrenal effluent which was collected for 5 min.

Mg^{2+} 2 mM was not removed from the solution. Scorpion venom from *Leiurus quinquestriatus* was dissolved in 0.01 M Na^+-phosphate buffer (pH 7.5) at a concentration of 1 mg/ml, the insoluble residue was removed by centrifugation for 5 min at 10,000 *g* and the venom was stored at 0°C. Perfusion solution containing grayanotoxin I was prepared by the addition of an appropriate quantity of a stock solution (20 mM) of the drug dissolved in ethanol. The final concentration of this solvent was less than 2.5% which had no effect on catecholamine secretion. The required quantities of scorpion venom and tetrodotoxin (TTX) were added to Locke solution from concentrated stock solutions.

The adrenal effluents were collected over a period of 5 min as described previously (Ito *et al.*, 1979). The assay of catecholamine (adrenaline) was performed by the fluorimetric method of Anton & Sayre (1962).

Results

Catecholamine secretion induced by scorpion venom

Scorpion venom (0.1 to 10 μg/ml) infused for 10 min caused a dose-dependent increase in the catecholamine output from the perfused adrenal glands of guinea-pig. The effect of a low concentration (less than 0.5 μg/ml) of scorpion venom was inhibited by perfusion with Locke solution containing atropine (0.5 mM) and hexamethonium (1 mM) (Figure 1a,b). This indicates that stimulation of cholinergic pre-

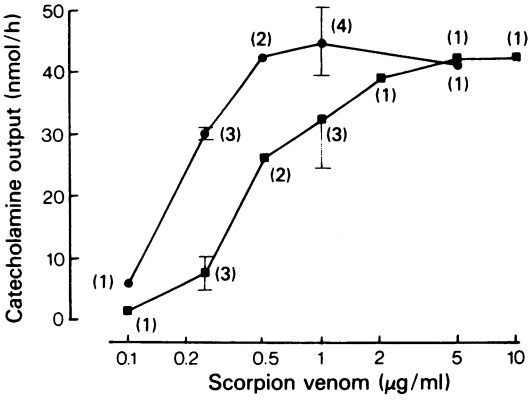

Figure 2 Dose-response curve for catecholamine output induced by scorpion venom in the presence or absence of atropine and hexamethonium. The ordinate scale is the amount of catecholamine (nmol) released during 1 h. The abscissa scale is the concentration (μg/ml) of scorpion venom. Symbols indicate the mean catecholamine output induced by scorpion venom in the presence (■) and absence (●) of atropine (0.5 mM) plus hexamethonium (1 mM); vertical lines show s.e. mean. The numbers in parentheses are the number of experiments.

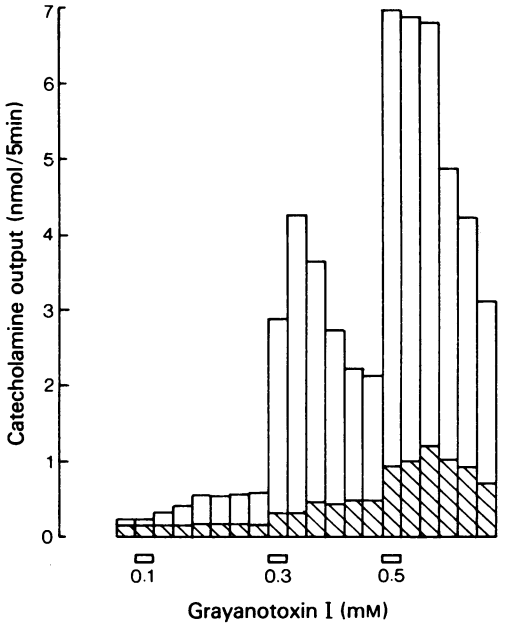

Figure 3 Dose-dependent increase in the catecholamine output induced by grayanotoxin I in the presence or absence of atropine and hexamethonium. Adrenal glands were repeatedly stimulated with grayanotoxin I for a period of 5 min at 25 to 30 min intervals during which the grayanotoxin concentration was progressively increased from 0.1 to 0.5 mM. Open bars (□) and the numbers below them indicate the length (5 min) of the exposure and the concentration (mM) of grayanotoxin I. Hatched columns show the catecholamine output induced by grayanotoxin I in the presence of atropine (0.5 mM) plus hexamethonium (1 mM). Similar results were obtained in two other experiments in the absence and in five other experiments in the presence of the blocking agents.

ganglionic neurones is involved in the venom-induced response as reported by Jacobs, Johanson & Williams (1978). However, when the concentration of scorpion venom was raised, the catecholamine output was increased even in the presence of atropine and hexamethonium (Figure 1 c,d). The response appeared during the first 5 min period of infusion of the venom and usually attained the maximum during the next 5 min period. Then the catecholamine output gradually declined, but some still remained even after 1 h. The amounts of catecholamine released for 1 h during and after the infusion of scorpion venom were plotted against the concentrations of the venom in the presence or absence of atropine and hexamethonium (Figure 2). The dose-response curve was shifted to the right in the presence of these blocking agents. The catecholamine output induced by the venom at concentrations above 1 μg/ml was not significantly different from that in the absence of the blocking agents. These results indicate that the release of catecholamine by scorpion venom at higher concentrations is chiefly by a direct action on adrenal chromaffin cells.

Catecholamine secretion induced by grayanotoxin I

When grayanotoxin I (0.1 to 0.5 mM) was infused for 5 min at about 30 min intervals, the catecholamine output was also increased in dose-dependent manner. The response was significantly reduced by perfusion with Locke solution containing atropine (0.5 mM) and hexamethonium (1 mM), although it was still dose-dependent (Figure 3). This shows that the effect of grayanotoxin I is mediated mainly through stimulation of cholinergic nerves.

Scorpion toxin is reported to act cooperatively with grayanotoxin to activate the action potential Na^+ ionophores in electrically excitable neuroblastoma cells (Catterall, 1977). Therefore, grayanotoxin I was infused for 5 min during perfusion with a low concentration of scorpion venom (0.1 μg/ml) for 10 min (which alone had no effect on the catecholamine secretion in the presence of atropine and hexameth-

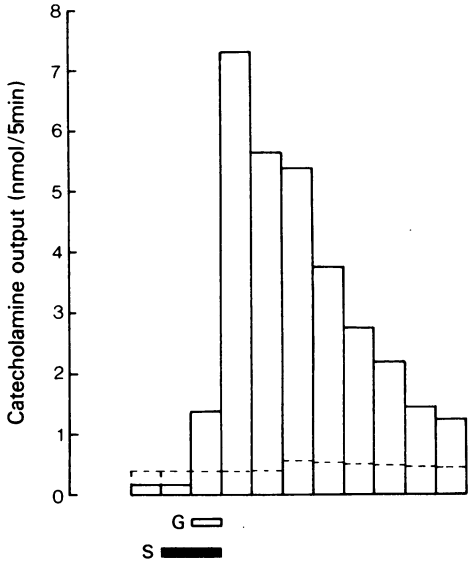

Figure 4 Catecholamine output induced by grayanotoxin I in the presence of a low concentration of scorpion venom. Adrenal glands were perfused with Locke solution containing atropine (0.5 mM) plus hexamethonium (1 mM). Open (□) and filled bars (■) indicate the periods of infusion of grayanotoxin I (G, 0.1 mM) for 5 min and scorpion venom (S, 0.1 μg/ml) for 10 min, respectively. Dashed line indicates the catecholamine output induced by scorpion venom (0.1 μg/ml) alone. Similar results were obtained in three other experiments.

onium). Grayanotoxin I infused with scorpion venom caused an increase in catecholamine output similar to that obtained in the absence of hexamethonium and atropine (Figure 4). The time course of the response was similar to that induced by a higher concentration of scorpion venom. In the following experiments, grayanotoxin I (0.1 mM) was always used with scorpion venom (0.1 μg/ml).

Inhibitory effect of tetrodotoxin on catecholamine secretion induced by scorpion venom and grayanotoxin I

It is reported that either TTX or reduction of extracellular Na^+ ions abolishes the depolarization or the prolongation of Na^+ current induced by grayanotoxin I and/or scorpion venom in various excitable cells (Adam *et al.*, 1966; Narahashi *et al.*, 1972; Narahashi & Seyama 1974; Starkus & Narahashi 1978). Scorpion toxin-induced release of noradrenaline from peripheral adrenergic neurones is also inhibited by TTX or the omission of Ca^{2+} ions (Moss, Thoa & Kopin 1974). Thus, in this and the following sections, the effects of TTX and the deprivation of extracellular Na^+ or Ca^{2+} ions on the catecholamine output induced by scorpion venom and grayanotoxin I were observed in the presence of atropine and hexamethonium.

Scorpion venom (10 μg/ml) infused for 5 min during perfusion with TTX (0.1 μM) for 15 min failed to increase the catecholamine output. However, the response appeared 5 min after withdrawal of TTX and declined gradually. The subsequently infused venom again produced the response (Figure 5a). When TTX was infused throughout the period of perfusion, there was no restoration of the venom-induced response. Grayanotoxin I (0.1 mM) plus scorpion venom (0.1 μg/ml) also failed to enhance the catecholamine output in the presence of TTX (0.2 μM) but was effective after withdrawal of TTX (Figure 5b).

Sodium dependency of the effect of scorpion venom and grayanotoxin I

Scorpion venom (10 μg/ml) or grayanotoxin I (0.1 mM) plus scorpion venom (0.1 μg/ml) was applied 45 min after perfusion with Na^+-free solution, NaCl being replaced by isotonic sucrose. As shown in Figure 6, both neurotoxins failed to increase the catecholamine output during exposure to Na^+-free solutions but the response was restored by the subsequent replacement of the Na^+. Similar results were obtained in four other experiments in which NaCl was replaced by LiCl or choline chloride.

Calcium dependency of the effect of scorpion venom and grayanotoxin I

Adrenal glands were first perfused with Ca^{2+}-free solution containing Mg^{2+} 2 mM for 25 min. Subsequently, scorpion venom (2 μg/ml) was infused for 40 min during which the concentration of Ca^{2+} was increased progressively from 0 to 1.8 mM every 10 min. The result is illustrated in Figure 7a. There was almost no response in the absence of Ca^{2+}. However, perfusion with Ca^{2+}-containing solution induced catecholamine output which increased progressively with increasing concentrations of Ca^{2+}. In the absence of extracellular Ca^{2+}, grayanotoxin I (0.1 mM) plus scorpion venom (0.1 μg/ml) also failed to induce catecholamine output which was restored by the subsequent reintroduction of Ca^{2+} ions (Figure 7b).

Discussion

The present experiments confirm the previous find-

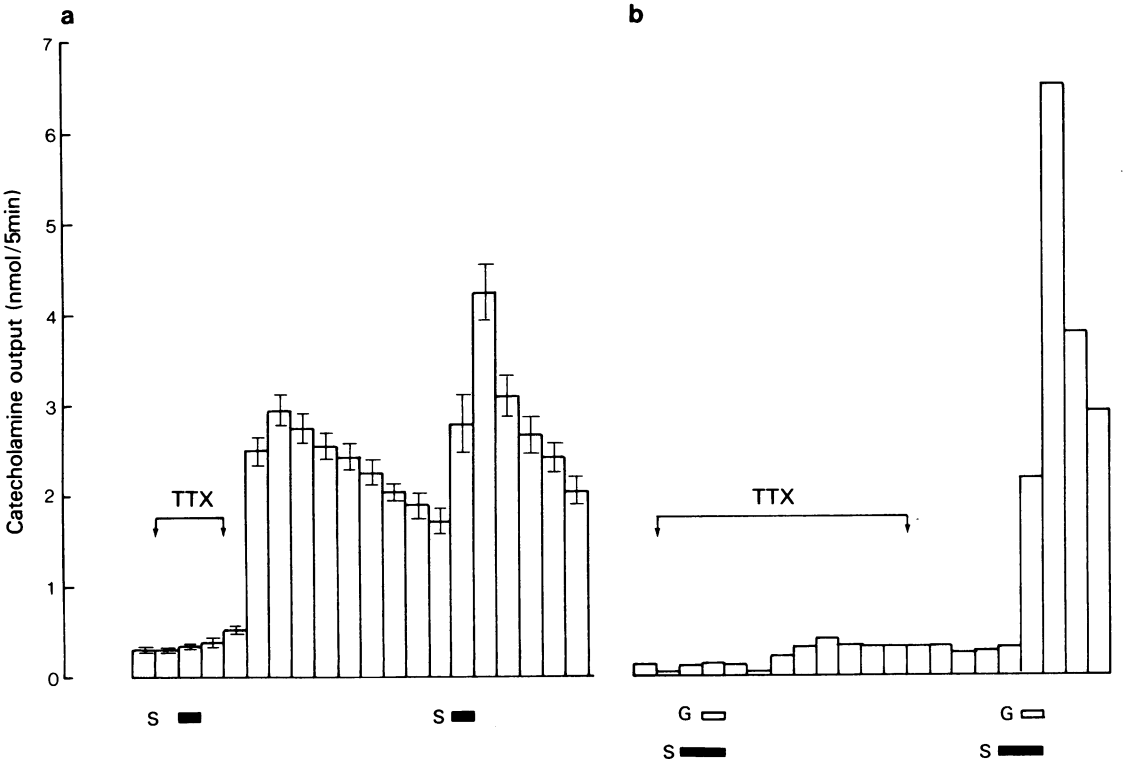

Figure 5 The inhibitory effect of tetrodotoxin on the responses induced by scorpion venom (a) and grayanotoxin I plus scorpion venom (b). Adrenal glands were perfused with Locke solution containing atropine (0.5 mM) plus hexamethonium (1 mM). Filled bars (■) indicate the periods of infusion of scorpion venom (S) 10 μg/ml in (a) for 5 min and 0.1 μg/ml in (b) for 10 min, respectively. Open bars (□) indicate the periods of infusion of grayanotoxin I (G, 0.1 mM) for 5 min. Tetrodotoxin (TTX, 0.1 μM in a, 0.2 μM in b) was infused for the periods indicated by a horizontal lines with arrows. (a): mean of five experiments: vertical lines show s.e. mean. Similar results to those in (b) were obtained in four other experiments.

ings that adrenal chromaffin cell membranes possess TTX-sensitive Na^+ channels responsible for the catecholamine output (Ito *et al.*, 1978; 1979; Kirpekar & Prat 1979). Like veratridine (Ito *et al.*, 1978; 1979), scorpion venom and grayanotoxin I plus scorpion venom caused an increase in the catecholamine output from the perfused adrenal gland in the presence of atropine and hexamethonium and the effects were blocked by TTX or by deprivation of extracellular Na^+ or Ca^{2+} ions. These neurotoxins are known to act on TTX-sensitive Na^+ channels in various excitable cells (Narahashi *et al.*, 1972; Ohta *et al.*, 1973; Narahashi & Seyama 1974; Starkus & Narahashi 1978), although the mechanism of action is somewhat different. In addition, these toxins failed to depolarize the nerve axons in the absence of or in low extracellular Na^+ (Adam *et al.*, 1966; Narahashi & Seyama 1974; Starkus & Narahashi 1978). The release of noradrenaline from peripheral adrenergic neurones induced by scorpion toxin was also blocked by TTX or the omission of Ca^{2+} ions (Moss, Thoa & Kopin, 1974). Taken together, it seems likely that Na^+ channels on chromaffin cell membranes have the same pharmacological properties as those on neurones.

Scorpion venom and other alkaloid neurotoxins are reported to interact cooperatively and stimulate the uptake of ^{22}Na in neuroblastoma cells (Catterall, 1977) and in various cell lines (Stallcup, 1977). Based on an allosteric model, these authors explained that scorpion venom can potentiate the action of the toxins by increasing the affinity of the active site for the toxins and/or by enhancing the maximum effect of the toxins. Scorpion toxin also caused TTX-sensitive insulin release from rat pancreatic islets only in the

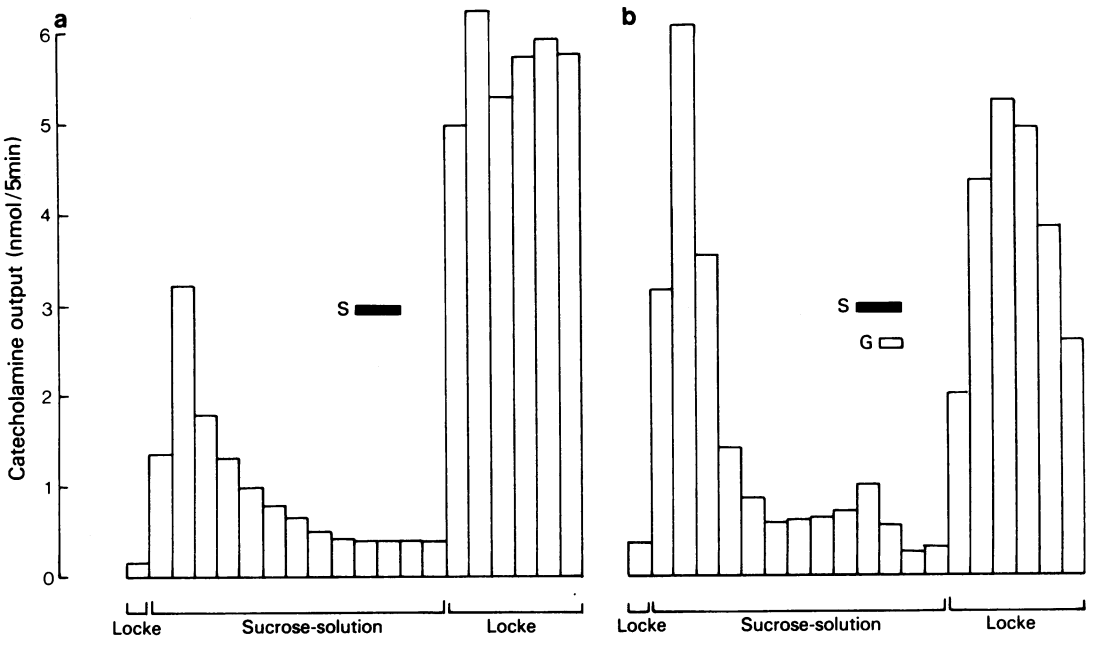

Figure 6 The effects of scorpion venom and grayanotoxin I plus scorpion venom on catecholamine secretion in a Na^+-free medium. Adrenal glands were perfused with Locke solution containing atropine (0.5 mM) plus hexamethonium (1 mM). Tris aminomethane buffer (2.5 mM) was used instead of Na^+-phosphate buffer. After the perfusion with Na^+-free solution for 45 min, scorpion venom (a) or grayanotoxin I plus scorpion venom (b) was infused. Solutions used for each period are indicated below histogram. Filled bars (■) indicate the periods of the infusion of scorpion venom (S) 10 μg/ml in (a) and 0.1 μg/ml in (b) for 10 min, respectively. Open bar (□) indicates the period of infusion of grayanotoxin I (G) for 5 min. Similar results were obtained in four other experiments each for (a) and (b).

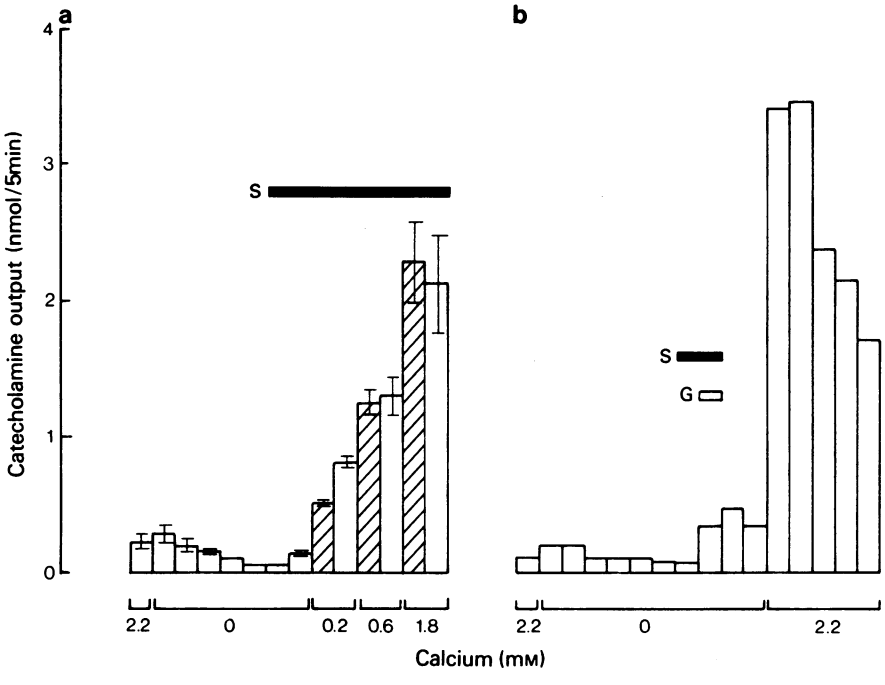

Figure 7 The effect of calcium on the catecholamine output induced by scorpion venom (a) and grayanotoxin I plus scorpion venom (b). Adrenal glands were perfused with Locke solution containing atropine (0.5 mM) plus hexamethonium (1 mM) and Mg^{2+} (2 mM) with or without various concentrations of Ca^{2+}. Numbers below columns indicate Ca^{2+} concentrations present in perfusion solution. Filled bars (■) indicate the periods of the infusion of scorpion venom (S) 2 μg/ml in (a) for 40 min and 0.1 μg/ml in (b) for 10 min, respectively. Open bar (□) indicates the period of infusion of grayanotoxin I (G, 0.1 mM) for 5 min. Hatched columns indicate catecholamine secretion for first 5 min after perfusion with the next high Ca^{2+} concentration. (a): mean of four experiments; vertical lines show s.e. mean. Results similar to those in (b) were obtained in two other experiments.

presence of veratridine (Pace & Blaustein, 1979). We do not know whether the allosteric model is applicable to our experimental results, but at least scorpion venom and grayanotoxin I may also interact cooperatively and activate TTX-sensitive Na^+ channels on the chromaffin cell membranes.

In addition to the direct effect on the chromaffin cell, either scorpion venom or grayanotoxin I may have an indirect action through stimulation of preganglionic cholinergic nerves. This agrees with the results described by Henriques, Gazzinelli, Diniz & Gomez (1968) and Jacobs *et al.* (1978). However, the nerve-mediated response induced by these toxins was replaced by a direct action when the concentration of the venom was raised or grayanotoxin I was used with scorpion venom. It could be due to the different sensitivity of release mechanisms to the increased Na^+ permeability and/or a difference in affinity of Na^+ channels for the neurotoxins between the chromaffin cell and the preganglionic neurone.

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