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Contractions induced by grayanotoxin I in the guinea-pig vas deferens

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1 In the guinea-pig vas deferens, grayanotoxin I (G-I), a diterpenic toxin isolated from certain ericaceous plants caused rhythmic contractions which were dose-dependent (5×10^{-5} M – 10^{-3} M); these followed an initial transient contraction.

2 The G-I (3×10^{-4} M)-induced contraction was markedly inhibited or abolished by reserpine (2 mg/kg on 2 days), phentolamine (2×10^{-7} M and 10^{-6} M) or tetrodotoxin (TTX, 5×10^{-7} M), but remained almost unaffected by atropine (10^{-6} M) or mecamylamine (3×10^{-5} M). This contraction was also abolished after storage at 4°C for 7 days or incubation in Na-deficient or Ca-free medium.

3 After treatment with G-I (3×10^{-5} M), which did not alter the tension of the preparation, transmural stimulation (10–50 Hz, 0.5 ms, supramaximal voltage, for 3 s) induced a slower contraction (second contraction) following the first rapid contraction caused by stimulation.

4 The second contraction was inhibited or abolished by reserpine (2 mg/kg on 2 days), phentolamine (2×10^{-7} M and 10^{-6} M) and TTX (2×10^{-8} M), but was not affected by atropine (10^{-6} M) and mecamylamine (3×10^{-5} M).

5 G-I (3×10^{-5} M) shifted the dose-response curves for noradrenaline (NA), acetylcholine and high-K contractions to the left in a parallel manner and slightly increased the maximal response to these agonists.

6 G-I (3×10^{-4} M) caused a release of endogenous NA from the vas deferens which was approximately 120 times that of control preparations. This response was inhibited or abolished by TTX (5×10^{-7} M) or incubation in Ca-free medium.

7 These results suggest that the G-I-induced contraction of the vas deferens and the G-I-induced second contraction on electrical stimulation are the result of an indirect action mediated through the release of NA from the adrenergic nerve endings.

Introduction

It is well known that certain ericaceous plants contain a number of toxic diterpenic substances. Studies on these toxins have been carried out by a number of investigators revealing their chemical structures and their acute toxicities (Hikino, Ohta, Ogura, Ohizumi, Konno & Takemoto, 1976).

These toxins have been studied extensively since they have biologically characteristic activities (Narahashi, 1974). One of these toxins, grayanotoxin I (G-I) has been found to depolarize skeletal muscle membrane (Deguchi & Sakai, 1967; Seyama, 1970) and squid axon membrane (Narahashi & Seyama, 1974; Hironaka & Narahashi, 1977). In the guinea-pig atria, G-I produces a membrane depolarization, positive inotropic effects and arrhythmias (Akera, Ku, Frank, Brody & Iwasa, 1976). Also, one of its derivatives, α-dihydrograyanotoxin II, causes similar effects on squid axons (Seyama & Narahashi, 1973) and heart muscle (Ku, Akera, Frank, Brody & Iwasa, 1977).

In the present work, the mechanism of the excitatory effects of G-I on the smooth muscle of the guinea-pig vas deferens has been studied.

Methods

Mechanical response

Male guinea-pig (Dunkin-Hartley) weighing 250–350 g were stunned and bled to death. The procedure for preparing the vas deferens and the technique for measurement of contractions were described previously (Ohizumi & Shibata, 1980). Tis-

sues were suspended in a 20 ml organ bath containing Krebs-Ringer-bicarbonate solution of the following composition (mM): NaCl 120, KCl 4.8, $CaCl_2$ 1.2, $MgSO_4 \cdot 7H_2O$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 25.2 and glucose, 5.8, pH 7.4. Transmural stimulation (2–50 Hz, 0.5 ms, supramaximal voltage) was applied for 3 s at 4 min intervals. For the Na^+-deficient Krebs solution, 60 mM NaCl was replaced with 120 mM sucrose.

Reserpine treatment

Guinea-pigs received an injection of reserpine (2 mg/kg i.p.) on each of 2 days. The experiment was performed 24 h after the second injection.

Cold storage

The vas deferens was stored at 4°C for 7 days as described by Varma & McCullough (1969). The preparations were equilibrated in Krebs-Ringer solution at 32°C for 2 h before drugs were applied.

Assay of endogeneous noradrenaline (NA)

The method of incubation of vas deferens and the determination of released endogenous NA were carried out as previously described (Ohizumi & Shibata, 1980). The vas deferens was equilibrated for 45 min during which the solution was changed at 15 min intervals. Then, for the treated group, the tissue was pretreated with tetrodotoxin (TTX) or Ca-free medium. Finally the tissues were transferred to the test solution containing grayanotoxin in the presence or absence of TTX or Ca^{2+} for 30 min. The bath medium containing released NA was chromatographed over P-cellulose (Brown Co., Kalamazoo, U.S.A.) equilibrated with 0.01 M phosphate buffer (pH 6.2). After passing 0.01 M phosphate buffer (30 ml), NA was eluted with 21 ml of 0.06 M phosphate buffer collected from the columns in 3 ml samples. The amount of NA in each fraction was determined by the trihydroxindole reaction (Haggendal, 1963). After 0.1 ml of 0.0025% $CuCl_2 \cdot 2H_2O$ solution was added to 3 ml of each sample, the oxidation was performed by addition of 0.1 ml of 0.25% $K_3Fe(CN)_6$ solution. After 2 min, the oxidation was stopped with 0.4 ml of 1% mercaptoethanol in a sodium sulphate solution (5 g $Na_2SO_3 \cdot 7H_2O$/10 ml H_2O). After 2 min, 0.4 ml of 2 N NaOH was added to the solution. The fluorescence was measured at 330/500 nm (activating/fluorescent wavelengths) by means of an Aminco-Bowman spectrophotofluorometer (Aminco SPF 500). Sensitivity of assays for NA was 0.5 ng/3 ml. Mean recovery was about 95% through column chromatography.

Statistical analysis

Three to six preparations from different animals were used for each experiment. Differences between mean values were tested for significance by Student's *t* test.

Drugs

The following drugs were used: grayanotoxin I (G-I)

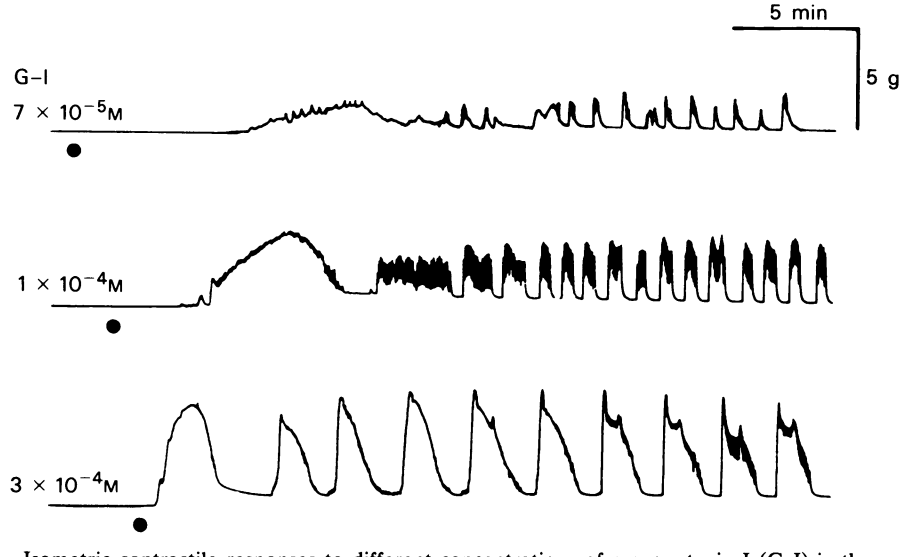

Figure 1 Isometric contractile responses to different concentrations of grayanotoxin I (G-I) in the guinea-pig isolated vas deferens. G-I was added at ●.

isolated from *Leucothoe grayana* L., tetrodotoxin (TTX, Sankyo), reserpine (Apoplone; Daiichi Seiyaku), phentolamine methansulphanate (Regitine; Ciba-Gaigy), phenoxybenzamine hydrochloride (Smith Kline and French Laboratories), atropine sulphate (Tokyo Kasei), mecamylamine hydrochloride (Meiji Seika), noradrenaline bitartrate (Sigma), chlorpheniramine maleate (Sankyo), indomethacin (Merk), methysergide (Sandoz). Indomethacin was dissolved in ethanol at 10^{-2} M and 6.7 μl of the solution was added to the 20 ml organ bath medium. Noradrenaline bitartrate was freshly dissolved in distilled water before experiments. All other drugs were dissolved in distilled water at a high concentration and kept frozen as stock solution.

Results

Mechanical response

Grayanotoxin I (G-I) (5×10^{-5} M – 10^{-3} M) induced concentration-dependent contractions in the guinea-pig isolated vas deferens. The maximal response to G-I was obtained with a concentration of 10^{-3} M. The G-I-induced contraction consisted of an initial, transient contraction followed by rhythmic contractions. An example of the contractile responses to different concentrations of G-I is shown in Figure 1. Washing with fresh medium 3 times for 1 min removed the contractile effect of G-I (10^{-4} M). The response to G-I (10^{-4} M) gradually decreased during repeated applications for 15 min every 45 min and the responses to the second and the third application of G-I were reduced by approximately 30% and 60% respectively. Therefore, in determining the contractile response to G-I only the response to the first administration of G-I to each preparation was used. Figure 2 shows the effects of phentolamine and TTX on the concentration-effect curves for G-I in the initial, transient contraction and the following rhythmic contractions. Both concentration-effect curves for G-I were shifted to the right by phentolamine 2×10^{-7} M and the maximal responses were decreased to some extent. Increasing the concentration of phentolamine to 10^{-6} M profoundly depressed the maximum responses. Both contractions induced by G-I (5×10^{-5}–10^{-3} M) were abolished in the presence of TTX (5×10^{-7} M), but were little or not affected by treatment with atropine (10^{-6} M), mecamylamine (3×10^{-5} M), methysergide (10^{-6} M), chlorpheniramine (10^{-6} M) and indomethacin (3×10^{-6} M). Reserpine pretreatment (2 mg/kg for 2 days) inhibited both initial and rhythmic contractions induced by G-I (3×10^{-4} M) by approximately 70% and 98%, respectively. Both contractions were inhibited or abolished following cold storage treatment and incubation in Ca-free or low Na (85.2 mM)-high sucrose medium (Table 1).

Transmural stimulation (2–50 Hz, 0.5 ms, supramaximal voltage, for 3 s) caused a rapid contraction (twitch) in the vas deferens. G-I 3×10^{-5} M did not alter the resting tension of the vas deferens nor did it affect the contraction induced by stimulation at 2 and

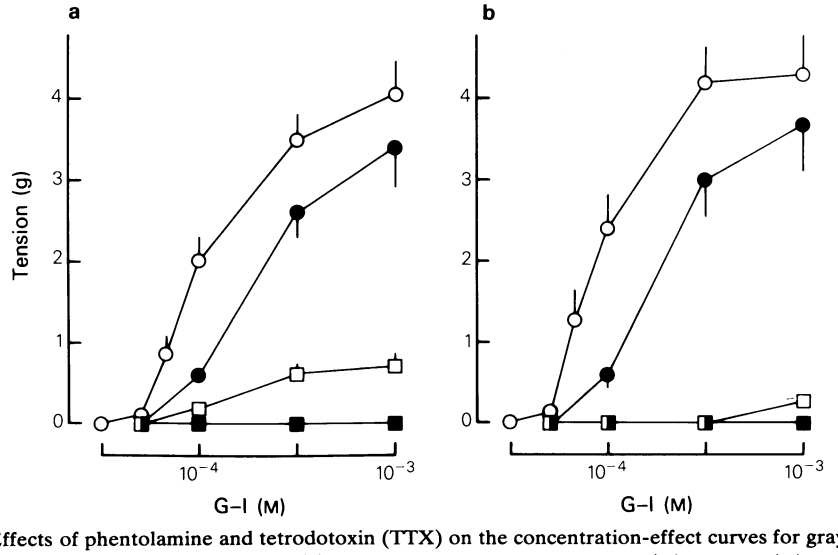

Figure 2 Effects of phentolamine and tetrodotoxin (TTX) on the concentration-effect curves for grayanotoxin I (G-I): (a) the initial, transient contraction; (b) the largest rhythmic contraction; (○) control; (●) phentolamine 2×10^{-7} M; (□) phentolamine 10^{-6} M; (■) TTX 5×10^{-7} M. Phentolamine and TTX were added 15 min before application of G-I. Each point and bar represents the mean and s.e.mean of 3 to 6 experiments.

Table 1 Effects of incubation in low Na- and Ca-free medium or cold storage treatment on the grayanotoxin I (G-I)- and noradrenaline (NA)-induced contraction in the guinea-pig vas deferens

Drugs (M)	*Component*	*Tension development* (g) *Fresh preparation*			*Cold-stored preparation*
		Normal medium (Control)	Low Na medium	Ca-free medium	Normal medium
G-I (3×10^{-4})	Initial contraction	3.5 ± 0.3	0.1 ± 0.03*	0	0
	Rhythmic contraction	4.3 ± 0.5	0	0	0
NA (10^{-5})		4.0 ± 0.4	3.7 ± 0.5	0	3.8 ± 0.3

G-I and NA were added after 20 min incubation in the low Na^+ (85.2 mM)-sucrose and Ca^{2+}-free medium. For cold-stored preparations (4°C, for 7 days), the drugs were added after 2 h equilibration.
*Significantly different from control ($P<0.01$).
Results are given as mean ± s.e.mean of 5–6 preparations.

5 Hz. But 2 min after application of G-I, stimulation at 10 Hz produced a second slower contraction which followed the first rapid contraction (Figure 3). Both responses to stimulation were smaller 10 min after application of G-I. When the frequency of stimulation was increased to 20 or 50 Hz, the second slower contraction induced by G-I was bigger than the initial response and both contractions were almost completely inhibited at and remained so after 10 min (Figure 3). Twenty minutes after application of G-I, washing with the normal medium almost completely removed this inhibitory effect on the first rapid contraction. Each tissue was exposed only once to a single concentration of G-I because tachyphylaxis developed during repeated applications. As shown in Figure 4, G-I (10^{-5}–3×10^{-5} M) induced the second slower contraction in a concentration-dependent manner when stimulated at 20 Hz. The concentration-effect curves for G-I were markedly shifted to the right in the presence of phentolamine 2×10^{-7} M. The slower contraction induced by G-I (10^{-5}–$3-10^{-5}$ M) was almost abolished by treatment with phentolamine (1×10^{-6} M) and TTX (2×10^{-8} M) (Figure 4), whereas the first rapid contraction was left almost unaffected; atropine (10^{-6} M) and mecamylamine (3×10^{-5} M) had no effect on both contractions. Furthermore, reserpine (2 mg/kg on 2 days) inhibited the first rapid contraction by approximately 65%, whereas the second slower contraction was completely inhibited.

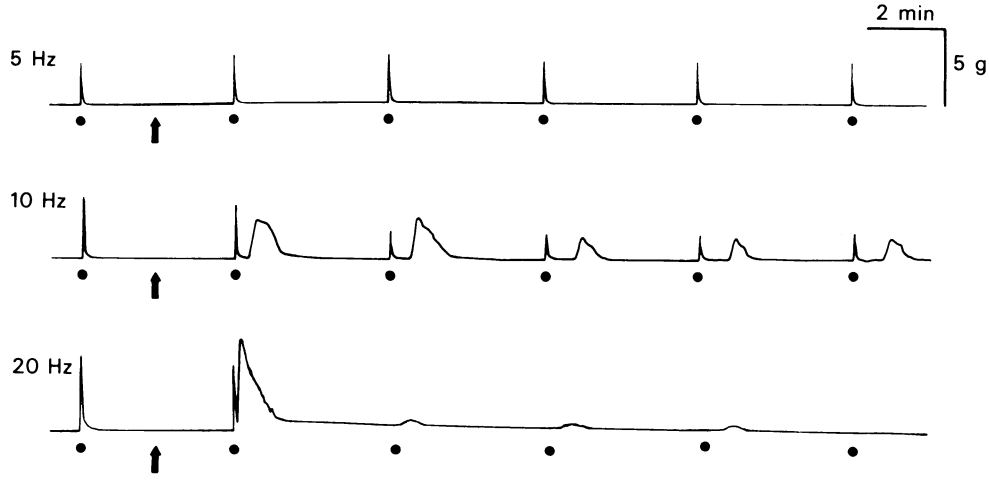

Figure 3 Effect of grayanotoxin I (G-I) on the contractile response to transmural stimulation (TS) at different frequencies in the guinea-pig isolated vas defeerens. TS (0.5 ms, 40–60 V) was applied at ● for 3 s every 4 min. G-I (3×10^{-5} M) was added at the arrow.

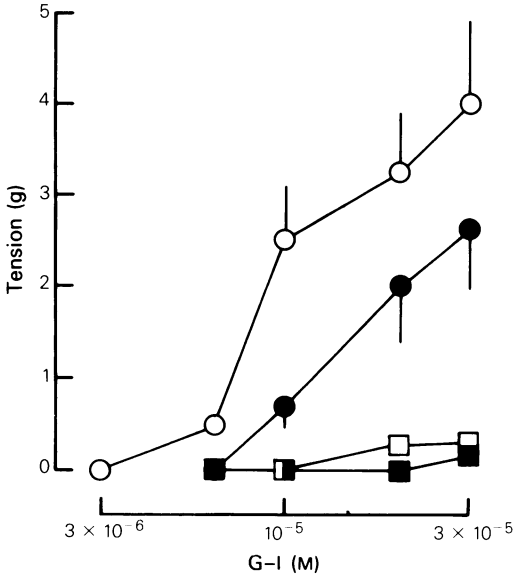

Figure 4 Effects of phentolamine and tetrodotoxin (TTX) on the concentration-effect curves for grayanotoxin I (G-I) in the second slower contractile response of the guinea-pig vas deferens to transmural stimulation (TS): (○) control; (●) phentolamine 2×10^{-7} M; (□) phentolamine 1×10^{-6} M; (■) TTX 2×10^{-8} M. TS (0.5 ms, 20 Hz, supramaximal voltage) was applied for 3 s every 4 min. Phentolamine and TTX were added 12 min before G-I. The contractile response to TS was measured 2, 6 and 10 min after application of G-I. Each point and bar represents the mean and s.e.mean of 5 experiments.

The effects of G-I (3×10^{-5} M) on the dose-response curves to NA-, ACh- and high-K-induced contractions of the vas deferens are shown in Figure 5. G-I shifted these dose-response curves to the left in a parallel manner. On the basis of ED_{50}, the potencies of NA, ACh, and high-K were increased approximately 3 fold 30 min after treatment with G-I. G-I also increased the maximal response to NA and ACh by approximately 15%.

Assay of endogenous noradrenaline

The release of endogenous NA from the vas deferens to the bath medium after 30 min treatment with G-I (3×10^{-4} M) was increased by approximately 120 times (4696.0 ± 627 ng/g tissue) that of control (untreated) (40.0 ± 5.3 ng/g tissue). The release of NA induced by G-I was almost abolished by treatment with TTX (5×10^{-7} M) (71.7 ± 10.3 ng/g tissue), the amount approaching that produced in the control. This NA releasing action of G-I was markedly inhibited after incubation in Ca^{2+}-free solution (277.3 ± 24.1 ng/g). The spontaneous release of NA was unaffected in Ca^{2+}-free solution or in a solution containing TTX.

Discussion

In the guinea-pig isolated vas deferens, G-I caused rhythmic contractions after the initial, transient contraction. The G-I-induced contraction was markedly inhibited following treatment with an α-adrenoceptor blocking drug (phentolamine), but not with cholinoceptor blocking drugs (atropine and mecamylamine), a 5-hydroxytryptamine receptor blocking drug (methysergide) or a histamine receptor blocking drug (chlorpheniramine). The G-I-induced contraction was markedly inhibited or abolished after treatment with a catecholamine depleting drug (reserpine) or cold storage treatment. Furthermore, it was found that G-I actually caused an increase in the amount of endogenous noradrenaline (NA) released from the vas deferens. On the basis of these results, it is suggested that this contraction is mediated by a release of endogenous NA from the adrenergic nerve terminals.

In the present experiments, the G-I-induced contraction was inhibited or abolished by the specific Na channel blocker, tetrodotoxin (TTX). This contraction was also markedly inhibited by incubation in low Na-high sucrose medium. On the other hand, electrophysiological studies on G-I by other investigators have revealed that G-I causes a TTX-sensitive depolarization of skeletal muscle membrane (Deguchi & Sakai, 1967; Seyama, 1970), squid axon membrane (Narahashi & Seyama, 1974; Hironaka & Narahashi, 1977) and heart muscle membrane (Akera *et al.*, 1976). These observations suggest that G-I may have caused nerve cell membrane depolarization resulting in increased membrane permeability to Na, which may play an important role in the excitation of the adrenergic nerve fibres, resulting in the release of endogenous NA.

G-I induced an intense slower contraction (second contraction) following the first rapid contraction (twitch) caused by electrical stimulation. The second contraction was inhibited or abolished by phentolamine, reserpine or TTX, suggesting that this contraction is due to endogenous NA released from the adrenergic nerve endings in the tissue. Asebotoxin III, a derivative of G-I, caused similar effects in the guinea-pig vas deferens (Ohizumi & Hikino, 1980). However, other depolarizing drugs such as aconitine (Herzog, Feibel & Bryant, 1964; Ellis & Bryant, 1973), veratridine (Ohta, Narahashi & Keeler, 1973) and ciguatoxin (Boyarsky & Rayner, 1970; Rayner, 1972) do not produce this effect in the vas deferens (unpublished data) which suggests that this effect may be specific for G-I and its derivatives.

In the vas deferens the release of NA induced by

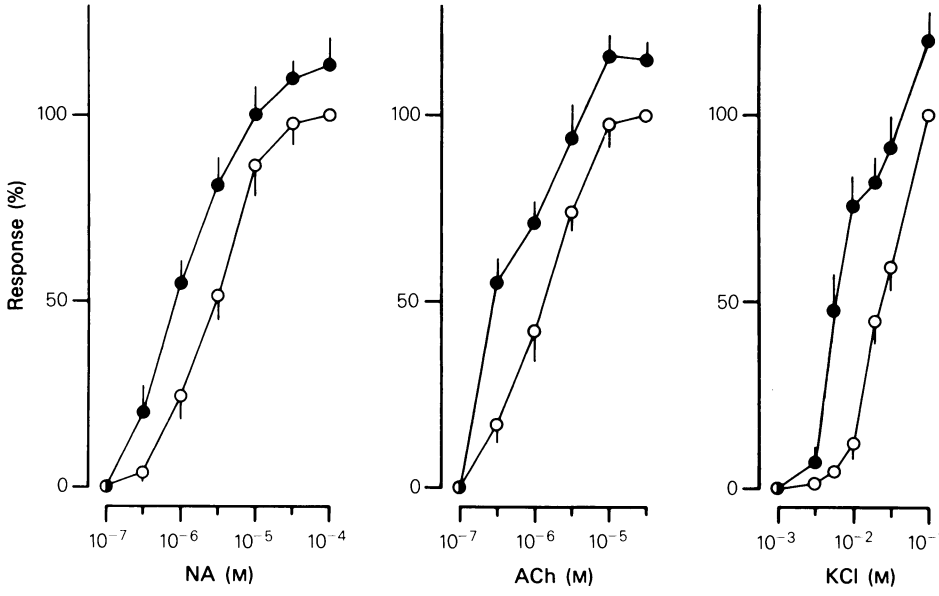

Figure 5 Effects of grayanotoxin I (G-I) on the dose-response curves for noradrenaline (NA), acetylcholine (ACH) and high-K (KCl) in the guinea-pig isolated vas deferens: (○) control; (●) treatment with G-I (3×10^{-5} M). NA, ACh and KCl was added 30 min after administration of G-I. The response to NA (10^{-4} M), ACh (3×10^{-5} M) or KCl (100 mM) is expressed as 100%. Each point and bar represents the mean and s.e.mean of 6 experiments.

depolarizing drugs such as veratridine and aconitine was abolished by TTX and was dependent upon extracellular Ca^{2+} (Thoa, Wooten, Axelrod & Kopin, 1975; Sato, Ohizumi & Hikino, 1979; Ohizumi, Shibata & Tachibana, 1981). On the other hand, the NA releasing action of indirect sympathetic amines such as tyramine was not affected by TTX or by a reduction in extracellular Ca^{2+} (Thoenen, Huerlimann & Haefely, 1969; Sato, *et al.*, 1979). In the present experiments, the NA releasing action of G-I in the vas deferens was almost abolished by TTX or incubation in Ca-free medium. Thus, the mechanism of NA release by G-I may be similar to that involved in the effect of veratridine or aconitine, but not of tyramine.

G-I brought about a marked leftward shift of the dose-response curves for the contractile effect of NA, ACh and high-K, indicating non-specific supersensitivity. It is generally assumed that postjunctional supersensitivity of smooth muscle innervated by adrenergic nerve fibres is not specific for NA. Further, in the cold stored guinea-pig vas deferens preparation (4°C, for 7 days), G-I still markedly potentiated the response to these agonists (unpublished data). It is well known that degeneration of the nerve tissue after cold storage treatment leads to loss of physiological nerve function, (Ambache, 1946; Lum, Hermani & Heilman, 1966; Varma & McCullough, 1969; Shibata, Hattori, Sakurai, Mori & Fujiwara, 1971; Hattori, Kurahashi, Mori & Shibata, 1972; Kuchii, Miyahara & Shibata, 1973). Therefore, it is possible that the G-I-induced supersensitivity of responses to agonists may be a postsynaptic effect.

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