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A MAXIMUM CONTRACTION AND SUBSTANTIAL QUANTITIES OF TRITIUM CAN BE OBTAINED FROM TETRAETHYLAMMONIUM-TREATED [^{3}H]-NORADRENALINE PRELOADED, RAT VAS DEFERENS IN RESPONSE TO A SINGLE ELECTRICAL SHOCK

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1 In the normal and phenoxybenzamine-treated vasa deferentia of the rat the overflow of tritium after preloading with [^{3}H]-noradrenaline increased with the increase in frequency of transmural stimulation. Phenozybenzamine enhanced overflow by 5 to 7 fold.

2 After tetraethylammonium (TEA, 10 mM), fractional tritium overflow at 5 Hz increased from a control value of 2.8 to 53 $\times 10^{-4}$, and at 0.5 Hz it reached 154 $\times 10^{-4}$. Enhancement of overflow was inversely related to the frequency of stimulation.

3 Contractor responses of the vas deferens induced by transmural stimulation were markedly potentiated, both in amplitude and duration, by 10 mM TEA. The contraction developed in the presence of 10 mM TEA at 0.5 Hz was comparable to that obtained at 10 Hz in normal Krebs solution.

4 In the presence of 5 mM TEA, contractions of the vas deferens caused by exogenous noradrenaline were potentiated about 5 fold.

5 The overflow and contractor response induced by 45 mM K were enhanced about 5 fold in 10 mM TEA-Krebs solution. Facilitation of K-induced overflow by TEA was reduced over 50% by tetrodotoxin (TTX, 1 μM).

6 Delivery of a single shock (1.0 ms duration) to 10 mM TEA-treated vas deferens resulted in a 63% increase in tritium overflow over background overflow. The fractional overflow amounted to almost 720 $\times 10^{-4}$ after a single shock, as compared to 154 $\times 10^{-4}$ obtained at 0.5 Hz.

7 The vas deferens treated with 10 mM TEA and stimulated by a single shock developed a contraction which was greater in amplitude and duration than that seen in normal tissue at 10 Hz.

8 Five second serial samples collected from superfused 10 mM TEA-treated vas deferens contained maximum quantities of radioactivity in the first 5 s after a single shock. In subsequent samples, radioactivity gradually declined, and reached prestimulation level after about 25 s.

9 The fractional overflow caused by stimulation at 0.1 Hz in 10 mM TEA-treated tissue progressively increased as external Ca was gradually raised from 0.83 to 10 mM.

10 The mechanism of action of TEA in enhancing the overflow of sympathetic transmitter, particularly in response to a single shock, is discussed in relation to Ca.

Introduction

In preliminary studies it was found that tetraethylammonium (TEA) caused a 15 to 20 fold increase in tritium overflow upon transmural stimulation (5 Hz and 60 s) of the rat vas deferens previously incubated with [^{3}H]-noradrenaline. The degree of enhancement was far greater than that reported earlier in the cat spleen (Thoenen, Haefely & Staehelin, 1967; Kirpekar, Prat, Puig & Wakade, 1972), or in the rat salivary gland (Wakade & Wakade, 1978). Further, no other agent, including α-adrenoceptor blocking drugs (which enhance overflow by removing the negative feedback mechanism, see Discussion) exert such a powerful effect on the liberation of the sympathetic transmitter in the vas deferens. It was therefore decided to investigate, in some detail, the effects of TEA on stimulation-induced overflow of tritium in the rat vas deferens. Modulation by TEA of the neurally mediated contractor response of the vas deferens was also investigated.

It will be shown that TEA causes a phenomenal

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increase in the liberation of tritium, together with a marked potentiation of contractions, as the frequency of stimulation is lowered. TEA-induced enhancement of overflow was of such immense magnitude that the amounts of tritium liberated from the vas deferens could be faithfully measured even after stimulation by a single electrical shock. A maximal force of contraction was also developed upon a single shock. A preliminary account of some of these findings has already appeared (Wakade, 1978; Wakade, 1979).

Methods

Male rats weighing 300 to 400 g were killed by a blow on the head and the vasa deferentia were removed. Tissues were kept in oxygenated Krebs solution and were subsequently cleaned.

[^3H]-noradrenaline incubation

Tissues were placed in 10 ml of Krebs solution (gassed with 95% O_2, 5% CO_2) of the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4 \cdot 7H_2O$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2 and glucose 11; and incubated at 37°C. The solution contained 3 μM disodium ethylenediamine tetraacetic acid and 0.56 μM ascorbic acid to prevent oxidation of tritium. All tissues were preincubated for 30 min, and then incubated with 150 ng/ml (±)-[^{3}H]-noradrenaline (specific activity of 6.3 Ci/mmol) (New England Nuclear, Boston, Mass.) for 30 min. After incubation, the tissues were washed three times with Krebs solution during the ensuing 60 min, and were then used to study the release of tritium.

Transmural stimulation and excess K

For the purpose of transmural stimulation, tissues were mounted in a holder between two platinum-plate electrodes, 5 mm apart. Each tissue was secured to the bottom hook of the holder by means of a cotton thread. Another thread was tied to the upper end of the tissue in order to attach it to the transducer. The electrodes, along with the tissue, were placed in a cylindrical muscle chamber containing 20 ml of Krebs solution at 37°C, through which 95% O_2 and 5% CO_2 was continuously bubbled. In order to record the isometric contractions of the vas deferens, the upper end of the tissue was attached to an F-50 transducer connected to an E & M physiograph Type DMP-4A recorder. Each tissue was subjected to a resting tension of 1 g.

Transmural stimulation was carried out by delivering a different number of impulses at different frequencies (80 V, and 1 ms duration) from a Grass stimulator, Model S-88. At appropriate intervals before electrical stimulation the contents of the muscle chamber were removed and replaced with fresh Krebs. The tissue was then stimulated transmurally for a given period of time and the fluid present during stimulation was replaced by fresh Krebs 30 s after termination of stimulation. To obtain net overflow produced by stimulation, the amount of radioactivity spontaneously released within the immediately preceding equivalent time period was subtracted from the total amount released during the stimulation period. K-induced overflow was measured by adding 0.25 ml of 3.6 M KCl solution to the 20 ml of Krebs in the organ bath for 1 min. A sample of Krebs was removed from the organ bath which had been in contact with the tissue for 1 min just before the addition of K, to obtain background activity. The stimulation-induced tritium release is expressed as fractional overflow which represents the ratio of the overflow of tritium to that in the tissue at the onset of stimulation. In some instances fractional overflow is divided by the total pulses given during stimulation, to obtain the overflow per pulse.

Single shock experiments

In one group of experiments the labelled vas deferens was set up in the organ bath for the purpose of recording contractions and to evoke release, as described above. The tissue was stimulated transmurally by delivering a single shock (80 V, 1 ms), using a Grass stimulator, Model S-88. Just before stimulation, fresh Krebs was added for 1 min to determine the overflow of tritium during the nonstimulation period. Then the bath contents were removed and replaced with fresh Krebs; 10 s later one shock was given, and fluid was removed after 50 s to determine overflow of tritium upon a single shock. In another group of experiments the labelled vas deferens was mounted on a holder, as described above, and placed in an organ bath designed for superfusion. The vas deferens was superfused with oxygenated Krebs solution maintained at 37 ± 1.5°C at a rate of about 12 ml/min. To obtain the rate of overflow of tritium during the nonstimulation period, the superfusate was collected in individual tubes every 5 s for about 30 s, at the end of which a single shock was given (see above), and samples were again collected every 5 s for 30 s. Radioactivity in each sample was counted by adding 0.5 ml to 10 ml of Aquasol (New England Nuclear) in scintillation vials.

Measurement of radioactivity

One ml of the tissue-bathing fluid containing [^{3}H]-noradrenaline and ^{3}H-deaminated metabolites was added to 10 ml of Aquasol in scintillation vials. Radioactivity was measured in the Beckman liquid

scintillation system LS 1000C. Appropriate corrections for dilutions and for quenching effects have been made.

Drugs

The drugs used were: tetrodotoxin, noradrenaline bitartrate (Sigma Chemical Corp., St. Louis, MO); phenoxybenzamine hydrochloride (Smith, Kline & French Laboratories, Philadelphia, PA); tetraethylammonium chloride (Eastman Kodak Co., Rochester, NY).

All drug concentrations are expressed in terms of the base (M). Mean values are given with standard errors. Student's *t* test (two-tailed) was used for statistical analysis of differences.

Results

Effect of phenoxybenzamine and tetraethylammonium on tritium overflow

In the case of normal and phenoxybenzamine-treated vasa deferentia, the sympathetic nerves were excited transmurally by giving 300 impulses at different frequencies, as shown in Figure 1. In Krebs solution, a significant overflow of tritium was obtained at 1.0 Hz ($1.2 \pm 0.01 \times 10^{-4}$). Phenoxybenzamine caused a marked enhancement of tritium overflow at all frequencies of stimulation. At 0.5 Hz, the overflow was $7.7 \pm 1.1 \times 10^{-4}$, and it reached a maximum level ($19.7 \pm 2.8 \times 10^{-4}$) at 5 Hz. These findings are consistent with those described earlier (Wakade & Wakade, 1978).

Since in a few initial experiments it was found that TEA caused a profound enhancement of overflow upon stimulation with impulses at 5.0 Hz, it was decided to use only 100 impulses at different frequencies in subsequent experiments. The results of these experiments are shown in Figure 1. Although undetectable amounts of tritium were seen at 0.5 Hz in normal Krebs solution, there was a phenomenal increase in overflow ($154 \pm 17.6 \times 10^{-4}$) at the same frequency of stimulation in the presence of 10 mM TEA. Unlike normal and phenoxybenzamine-treated vasa deferentia, overflow in TEA-treated tissues declined as the frequency of stimulation was raised. Thus, at 1 and 5 Hz the stimulation-induced release was 120 ± 15 and $53 \pm 8 \times 10^{-4}$, respectively. Release at 30 Hz was reduced to $13.7 \pm 3.0 \times 10^{-4}$, which was still over twice the control. The effect of TEA on release was reversible. Spontaneous release was not modified by 15 to 30 min pretreatment with 10 mM TEA.

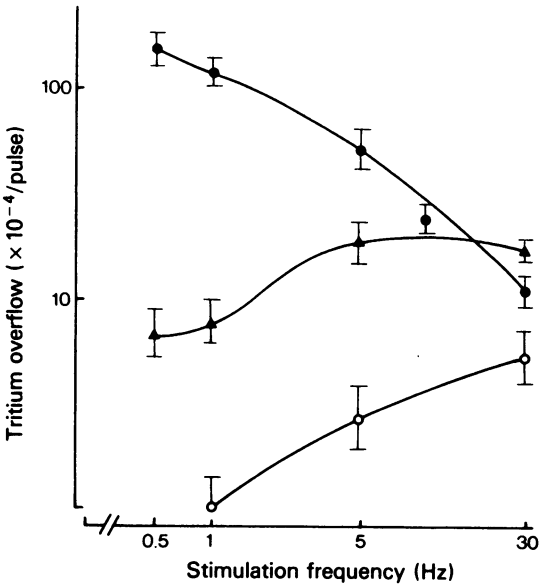

Figure 1 Effects of phenoxybenzamine and tetraethylammonium (TEA) on tritium overflow. Normal (○) and phenoxybenzamine-treated (▲) vasa deferentia were stimulated by delivering 300 pulses, and TEA-treated (●) tissues were stimulated by giving 100 pulses at frequencies shown. Phenoxybenzamine (3 μM) was added for 20 min, and then washed for 20 min. TEA (10 mM) was added for 15 min and remained in Krebs solution for the duration of the experiment. The above-mentioned pulses were given in ascending order, then in descending, and the averaged values from 7 to 18 experiments are represented by each point. Vertical lines show standard errors.

Effect of tetraethylammonium on the contractor response of the vas deferens

Contractions of the vas deferens were recorded after delivering 100 shocks at 0.5, 1.0, 5 and 10 Hz before in normal Krebs solution and in the presence of 10 mM TEA. A typical tracing from one of the nine experiments is shown in Figure 2. In Krebs solution, stimulation resulted in a fast twitch response, followed by a slowly developing contraction of the vas deferens, best seen at 5 and 10 Hz (Figure 2a). The biphasic nature of the contractor response of the rat and guinea-pig vas deferens upon transmural stimulation has been described by Swedin (1971).

In the presence of 10 mM TEA (Figure 2b), the amplitude and duration of the twitch response evoked by 0.5 and 1.0 Hz were greatly enhanced. The more prominently observed secondary response in normal

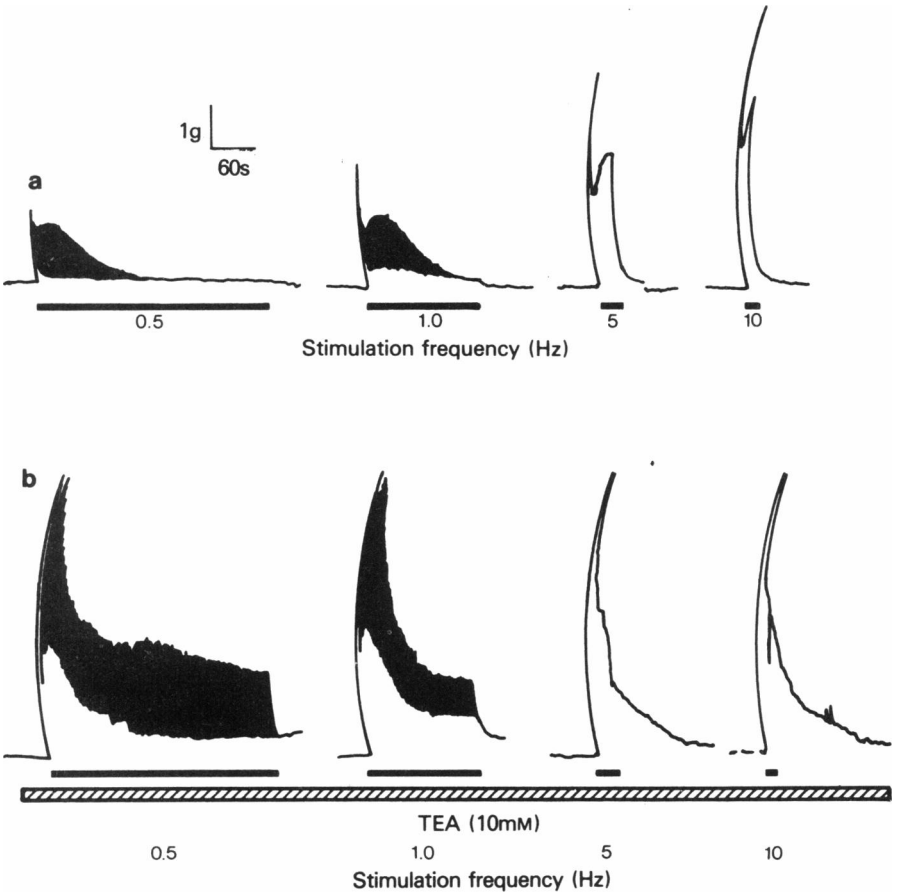

Figure 2 Effect of tetraethylammonium (TEA) on contractor responses of the rat vas deferens. Isometric contractions were recorded from the same vas deferens (a) before and (b) in the presence of 10 mM TEA. A train of 100 pulses was given at frequencies shown.

Krebs solution at 5 and 10 Hz was not seen in TEA-Krebs solution. However, the amplitude and, in particular, the duration of the response at these higher frequencies of stimulation was lengthened by TEA. Thirty min after washout of TEA, responses caused by stimulation returned to almost the normal level.

Relative sensitivity of the vas deferens to exogenous noradrenaline after tetraethylammonium

In seven experiments, sensitivity of the vas deferens to exogenous noradrenaline was tested before and in the presence of TEA. The results of these experiments are shown in Figure 3. After 5 mM TEA pretreatment, the vas deferens was supersensitive to noradrenaline as the entire dose-response curve was shifted to the left by about 0.5 log unit. A minimum concentration of noradrenaline needed to produce a detectable contraction in TEA-treated vasa deferentia was 5.8×10^{-6} M, as compared to 5.8×10^{-5} M in controls. Contractions of the control and TEA-treated vasa deferentia are shown in the inset of Figure 3. A noradrenaline concentration greater than 5.8×10^{-4} M occasionally produced a marked contraction which resembled that observed after transmural stimulation (see below). In the presence of 10 mM TEA, even lower concentrations of noradrenaline produced such maximal contractions, and for that reason 5 mM TEA had to be used in these experiments.

In an attempt to separate the enhancing effect of TEA on the contractions induced by nerve stimulation compared to those induced by exogenous noradrenaline, four experiments were carried out on paired vasa deferentia. In one vas deferens, contrac-

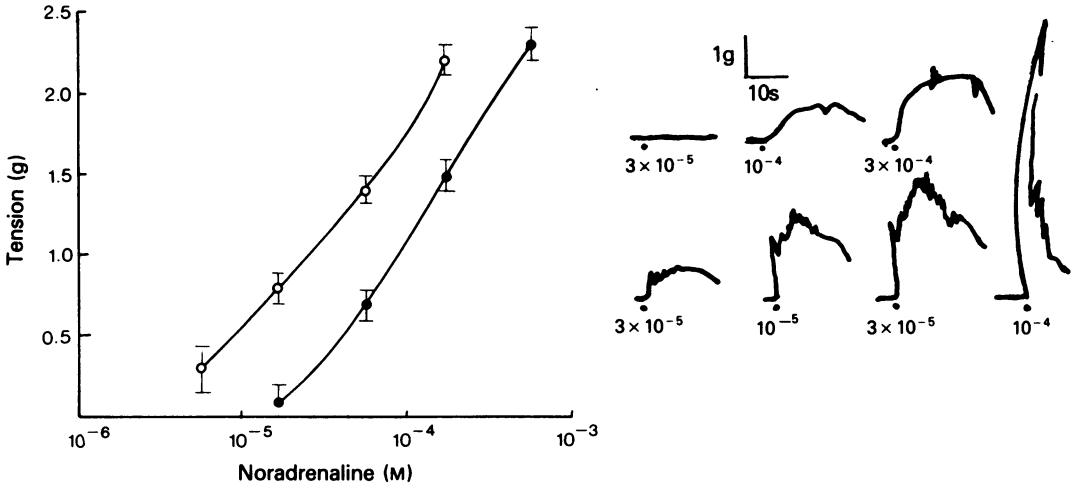

Figure 3 Sensitivity of the isolated vas deferens to exogenous noradrenaline before and after tetraethylammonium (TEA). Dose-response curves to noradrenaline obtained with the normal (●) and TEA-treated (○) vasa deferentia. TEA (5 mM) was added 15 min before, and remained in contact with the tissue during, exposure to noradrenaline. Each point is the average of five to seven observations. Vertical lines show standard errors. Inset shows a tracing of a typical experiment depicting contractions induced by noradrenaline before (top trace) and after (lower trace) TEA.

tions were elicited by exposing the tissue to 5.8×10^{-5} M noradrenaline, while the contralateral vas deferens was excited by delivering a single pulse (see below). It was found that the concentration of TEA needed to obtain approximately 20 to 30% enhancement of both direct and indirect contractile response was 0.2 mM. Further increase in TEA concentrations to 1 and 3 mM enhanced direct and indirect contractions, but the degree of enhancement was much greater in the case of nerve-induced contractions.

Actions and interactions of tetraethylammonium and tetrodotoxin on K-induced overflow of tritium

Since a significant increase in the overflow of tritium over the background overflow can be obtained upon exposure of the vas deferens to 35 to 45 mM K (Wakade & Kirpekar, 1974; Wakade & Wakade, 1978), and since TEA causes a marked enhancement of electrically induced overflow of tritium from the vas deferens, several experiments were carried out to test the effect of TEA on K-induced overflow. The results of these experiments are shown in Figure 4. Exposure to 45 mM for 1 min caused a 46 ± 7% increase in tritium overflow over the background. Fifteen min after exposure to 10 mM TEA, K-induced overflow was enhanced by over 5 fold (Figure 4a). An almost similar degree of enhancement of overflow was reproduced during three subsequent stimulation periods 20 min apart (not shown). However, exposure of TEA-treated vas deferens to 1 μM tetrodotoxin (TTX) for 15 min reduced (65 ± 8%, $P < 0.001$) but did not completely block tritium overflow. Although not shown, the inhibitory effect of TTX was almost completely reversed 30 min after washout of the tissue with TEA-Krebs solution.

Figure 4b shows the effect of TTX on K-induced overflow in the untreated vas deferens. In control experiments it was found that tissues exposed 2 or 3 times to 45 mM K for 1 min every 15 min caused comparable overflows of tritium. After obtaining overflow in normal Krebs solution, in five experiments vasa deferentia were treated with TTX and then stimulated by excess K. The overflow of tritium induced by K in the presence of TTX was reduced by about 30 ± 6%. The inhibitory effect of TTX was significant ($P < 0.05$).

Modification of K-induced contractions of the vas deferens by tetraethylammonium and tetrodotoxin

The actions and interactions of TEA and TTX on the K-induced contractor response of the rat vas deferens were studied in eight experiments. A typical tracing from one such experiment is shown in Figure 5: 45 mM K for 1 min produced a moderate degree of tension, which was potentiated about 6 fold in the presence of TEA. Further, the onset of the response was very rapid compared with the control, and resembled that seen after nerve stimulation (Figure 2b, 5 and 10 Hz). Addition of TTX as well as TEA resulted

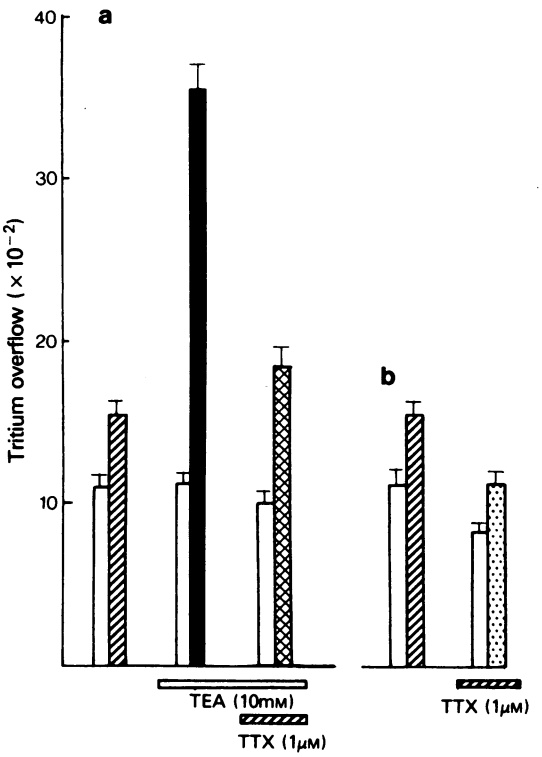

Figure 4 Effects of tetraethylammonium (TEA) and tetrodotoxin (TTX) on K-induced tritium overflow. Overflow was induced by exposing vasa deferentia prelabelled with [^{3}H]-noradrenaline to 45 mM K for 1 min. Open columns represent overflow of tritium during nonstimulation period, and shaded columns show overflow by excess K. TTX was added to TEA-Krebs solution (a) or to Krebs solution (b) 15 min before collection of samples. Each column represents a mean of 4 to 8 experiments. Vertical lines on top of columns show s.e. mean.

in about a 50% reduction in contraction induced by K. Both the inhibitory effect of TTX and the potentiating effect of TEA were reversed after their washout. In normal Krebs solution, TTX had no measurable effect on K-induced contraction.

Overflow of tritium in response to a single electrical shock

As shown in Figure 1, in TEA-treated tissues tritium overflow increased as the frequency of stimulation was decreased up to 0.5 Hz. This observation raised the following question: to what extent can this inverse relationship between frequency of stimulation and overflow be followed? It was discovered that not only did large quantities of tritium appear upon stimulation at 0.01 Hz (lowest frequency setting available on the stimulator), but even a single shock resulted in a significant increase in overflow. This observation was further tested in a series of experiments which are shown in Figures 6 and 7, and Table 1.

In one series of experiments a TEA-treated vas deferens was subjected to stimulation by delivering a single shock at 10 min intervals, to study its effect on the contraction and overflow of tritium. Results of one (of seven) typical experiment are shown in Figure 6. Following a single stimulus (1.0 ms duration), the amounts of tritium liberated were three times as much as found in an immediately previous nonstimulation period (Figure 6b). The increased overflow of tritium in response to a single shock was also accompanied by a contractor response (Figure 6a) which was greater in amplitude and duration than that recorded from a control tissue at 10 Hz (Figure 2). A single stimulus delivered after 10 min (S_3) caused an overflow of tritium comparable to that obtained in the S_2 period. Similarly, the contractor response was also reproducible. The contractor response and the increase in tritium overflow caused by TEA were completely extinguished by a 15 min treatment of the vas deferens with 1 μM TTX. The effect of TTX on both parameters was reversed 30 min after its washout (S_5).

A comparison of the amounts of tritium obtained in this experiment with those shown in Figure 1 reveals that in the presence of TEA the highest amounts are liberated when sympathetic nerves are excited by only a single shock.

Table 1 summarizes the amount of tritium overflow in the vasa deferentia of 17 rats after stimulation with a single shock. The fractional overflow of tritium averaged 720×10^{-4}, which is about six times greater than that obtained after stimulation of the TEA-treated vas deferens at 0.5 Hz (Figure 1). It was found in three experiments that application of a single shock to untreated vas deferens was not accompanied by any increase in the radioactivity over corresponding background counts.

Time-course of the overflow of radioactivity after a single shock

Since it was possible to obtain a substantial overflow of tritium from a single shock, additional experiments were designed to study the time-course of the release of tritium by a single shock. The results of these experiments are shown in Figure 7. A prelabelled vas deferens was superfused with 10 mM TEA-Krebs solution at a rate of about 12 ml/min, and the radioactivity in the superfusate was measured at 5 s intervals for 30 to 35 s (see Methods). In 3 experiments shown (out of 13 carried out), in the absence of stimulation the overflow of radioactivity gradually declined

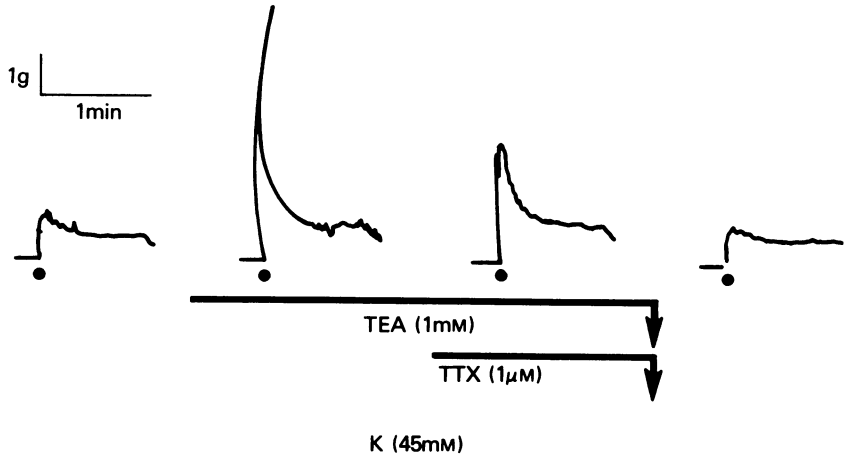

Figure 5 Modification of K-induced contractions of the rat vas deferens by tetraethylammonium (TEA) and tetrodotoxin (TTX). Isometric contractions were recorded from the vas deferens by adding 45 mM K for 1 min (at dots). TEA and TTX were added 15 min before exposure to K.

(broken lines). On the other hand, after giving a single shock the amount of radioactivity found in the effluent was markedly elevated (solid lines). In most cases maximum amounts of radioactivity appeared 5 s after the stimulus. The average increase in radioactivity in the first 5 s was about 60% over the nonstimulation period. Subsequent 5 s samples contained decreasing amounts. By about 30 s after the shock, radioactivity had returned to a level comparable to that found just before stimulation. Similar results were obtained in 7 other experiments.

Effect of low Ca on tritium overflow in the tetraethylammonium-treated vas deferens

Tritium overflow was induced by delivering 60 shocks at 1 and 30 Hz in TEA-treated vasa deferentia bathed in 2.5 and 0.83 mM Ca-Krebs solutions. The results of these experiments are shown in Figure 8. In 2.5 mM Ca-Krebs solution, the overflow of 1 Hz was $95 \pm 12 \times 10^{-4}$, and at 30 Hz it was reduced about 10 fold.

Lowering of external Ca to 0.83 mM resulted in about a 60% reduction in overflow at 1 Hz. However, at 30 Hz overflow was not less. If anything, it was about twice as much in the low Ca medium. The increase was significantly different ($P < 0.025$) compared to the control value. Although not shown in Figure 8, in two experiments it was found that an increase in external Ca to 7.5 mM caused about a 2 fold increase in overflow at 1 Hz, without modifying the overflow at 30 Hz.

Effect of changes in external Ca on tetraethylammonium-induced enhancement of tritium overflow

The results shown in Figure 8 indicate that TEA-induced enhancement of tritium overflow was markedly affected by changes in external Ca if the frequency of stimulation was lowered from 30 to 1 Hz. Therefore, it was decided to examine the influence of changes in external Ca on tritium overflow induced by a still further reduction in stimulation frequency. TEA-treated vasa deferentia were stimulated at 0.1 Hz by delivering a train of six shocks. In 2.5 mM Ca-Krebs solution, stimulated-induced overflow was about 180% over the background overflow, which is represented at $291 \pm 25 \times 10^{-4}$ in Figure 9. Reduction in the Ca concentration to 0.83 mM depressed overflow by 48 ± 8%. On the other hand, an increase in Ca concentrations produced a marked enhancement of overflow induced at 0.1 Hz. Maximum overflow (over 2 fold) occurred in 10 mM Ca-Krebs solution. A further increase in external Ca led to some decline in overflow.

Discussion

The enhancement of tritium overflow by TEA was of such magnitude that it greatly exceeded the value that was reported for α-adrenoceptor blocking agents such as phenoxybenzamine and phentolamine. These agents enhance overflow by blocking presynaptic α-adrenoceptors presumably located on sympathetic

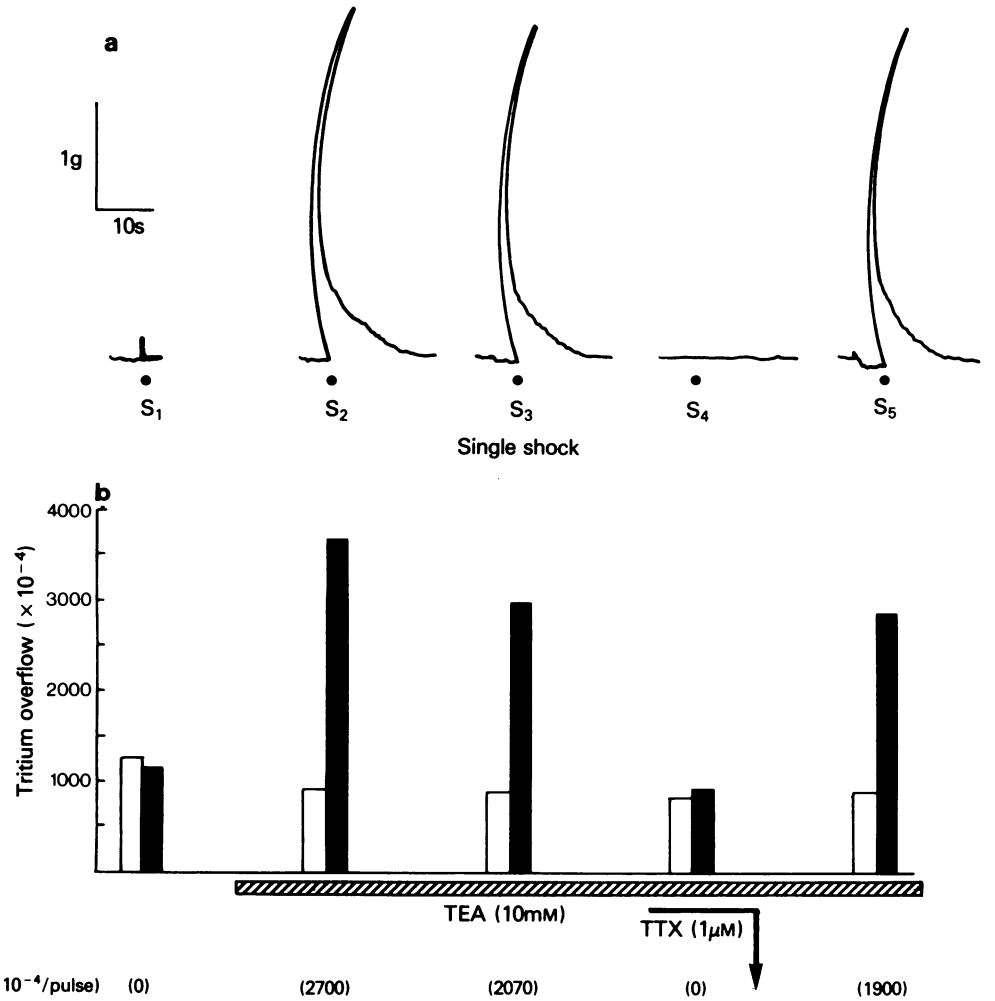

Figure 6 Effect of tetraethylammonium (TEA) on contraction and overflow of tritium induced by a single electrical shock. Isometric contractions of a TEA-treated rat vas deferens are shown in (a), and overflow of tritium during the control period (open columns) and after giving a single shock (solid columns) is shown in (b). A single shock was given at 10 min intervals (S_1, S_2, etc.). TTX was added 15 min before S_4 and washed out 30 min before S_5. Numbers in parentheses represent overflow of tritium per pulse at each stimulation period (see Methods).

nerve endings, and thereby remove negative feedback control on release (see Langer, 1973; Stjärne, 1975; Starke, 1977). In the present study, phenoxybenzamine maximally enhanced overflow from undetectable amounts to 7.7×10^{-4} at 0.5 Hz, whereas 154×10^{-4} was obtained after TEA. The overflow increased even further as the stimulation frequency was lowered (see below). The profound enhancement of transmitter overflow in the presence of TEA must result from a mechanism other than loss of presynaptic control mediated via alpha-receptors and interference with uptake mechanisms.

Kirpekar, Wakade & Prat (1976) proposed that TEA probably lengthens the duration of the nerve action potential by its well-known blocking effect on delayed K current, which allows more Ca to enter and thereby cause greater overflow of transmitter. The present results are consistent with such a proposal, and provide additional evidence in favour of the involvement of Ca in TEA-induced enhancement of tritium overflow.

Both phases of the neurally induced contractions of the vas deferens were markedly potentiated, both in height and duration, by TEA. The potentiation of the

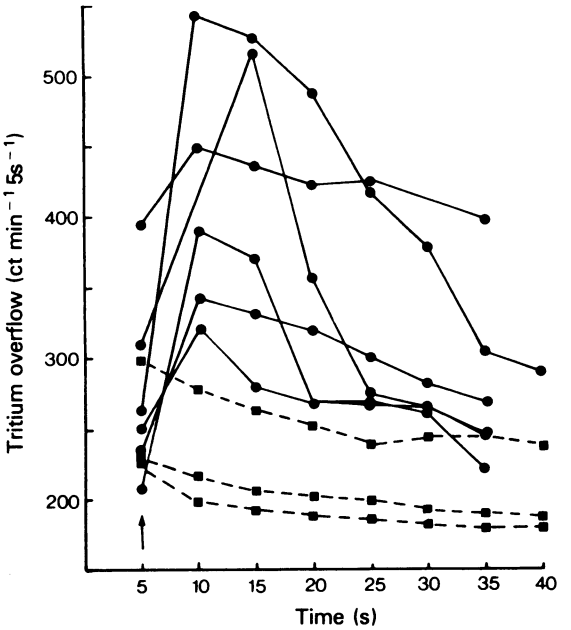

Figure 7 Time-course of the overflow of tritium from tetraethylammonium (TEA)-treated rat vas deferens after a single shock. Prelabelled tissues were superfused with 10 mM TEA-Krebs solution at 12 ml/min. Radioactivity released during nonstimulation period (■) and after a single shock (●) was counted at 5-s intervals. A single shock was given as indicated by the arrow. Each curve represents a single experiment.

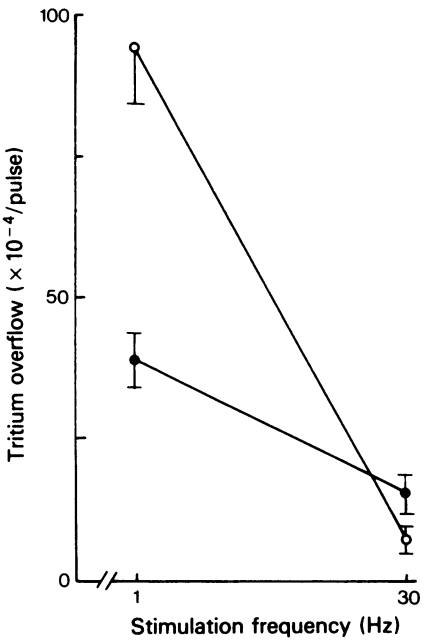

Figure 8 Effect of low Ca on tritium overflow in tetraethylammonium (TEA)-treated rat vas deferens. Overflow was induced by giving a train of 60 pulses, at frequencies shown, in normal Krebs solution (○) and in 0.83 mM Ca-Krebs solution (●). Each point represents a mean of 7 experiments. Vertical lines represent s.e. mean.

indirect contractor response may be due to a combination of two factors. Firstly, in the presence of TEA the amount of noradrenaline liberated in the synaptic region must have greatly increased upon nerve stimulation (Figure 1). Secondly, the sensitivity of the postsynaptic membrane may be greatly enhanced to noradrenaline in TEA-Krebs solution. In favour of such a suggestion, it was found that the contraction induced by exogenous noradrenaline was potentiated about 5 fold by treatment of the vas deferens with 5 mM TEA. Thus, increased amounts of noradrenaline in the vicinity of the adrenoceptors, together with greater reactivity of smooth muscle to noradrenaline, should lead to an exaggerated response to nerve stimulation.

K-induced overflow of tritium was also potentiated by TEA. Although the exact underlying mechanism in the release of transmitter by excess K is not well understood, it is known that release by K is depen-

Table 1 Fractional overflow of tritium in response to a single electrical shock applied to tetraethylammonium (TEA)-treated rat vas deferens

		Induced overflow after a single shock		
No. of expts	*Spontaneous overflow (A)*	*Induced overflow (B)*	*Per pulse (B–A)*	*% Over spontaneous*
17	0.113 ± 0.010	0.185 ± 0.020	0.072 ± 0.018	63 ± 15

Vasa deferentia were treated with 10 mM TEA for 15 min. Collection time for both spontaneous overflow and induced overflow of tritium after a single shock was 60 s. For further details, see Methods.

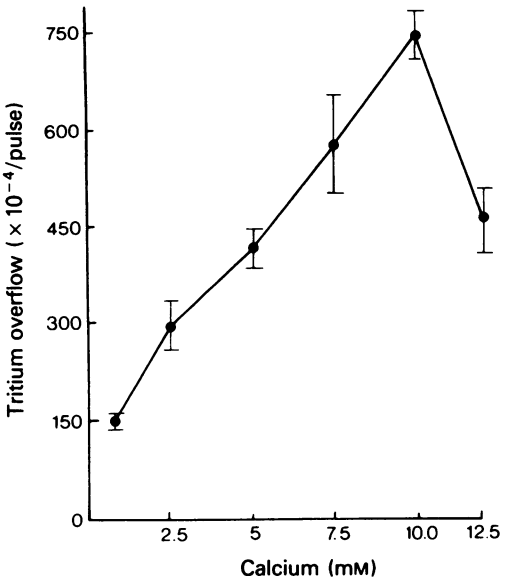

Figure 9 Effect of varying concentrations of Ca on tetraethylammonium (TEA)-induced enhancement of tritium overflow. Overflow was induced by delivering a train of 6 pulses at 0.1 Hz. Ca concentrations of Krebs solution varied as shown. No attempts were made to balance osmolarity of modified Krebs solution. Each point represents a mean of 5 experiments. Vertical lines show s.e. mean.

dent upon the presence of external Ca (Kirpekar & Wakade, 1968; Wakade & Kirpekar, 1974). The fact that TTX reduced K-induced overflow in the normal vas deferens by about 25 to 30% indicated that it is possible that a small fraction of the total overflow is caused by action potential(s) generated in the presence of 45 mM K. Under such circumstances, entry of Ca may be triggered not only by local depolarization produced by 45 mM K, but also by action potential(s). If this is true, then TEA should augment that portion of Ca influx associated with the generated action potential(s) by 45 mM K and thereby increase overflow.

K-induced contractions of the vas deferens were potentiated as much as 5 fold by TEA. As discussed above, the potentiation may be a combination of actions of TEA on the presynaptic (facilitation of release) and postsynaptic (increased responsiveness of smooth muscle to noradrenaline) structures. The same arguments offered for the partial inhibition by TTX of K-induced overflow in the TEA-treated vas deferens may be extended to explain the interactions of TTX and TEA on K-induced contractions.

In contrast to the present findings, noradrenaline output in the perfused cat spleen evoked by injecting 0.2 ml of 3.6 M KCl in the hepatic artery was not enhanced by TEA (Kirpekar *et al.*, 1976). The discrepancy may be due to the concentrations of K used in the two studies. In the presence of a very high K concentration (exceeding 135 mM), the nerve membrane may be completely depolarized so that no action potentials can be generated. If TEA augments that portion of Ca influx triggered by the action potential, it is understandable that the overflow evoked by very high K concentrations should not be enhanced by TEA. Failure of TEA to enhance the overflow may result if stimulation of nerves by very high K is analogous to stimulation at high frequency (García, Kirpekar & Pascual, 1978). As shown in Figure 1, TEA greatly facilitates overflow at low, but not so much at high, frequencies of stimulation.

Unlike normal and phenoxybenzamine-treated tissues, the overflow of tritium in TEA-treated tissues decreased as the frequency of stimulation was raised. The inverse relationship between overflow and frequency of stimulation was seen from 30 Hz to 0.5 Hz (Figure 1), and such a relationship continued up to 0.1 Hz. The reason for such a phenomenon is not known. Stjärne (1975) and Kirpekar, Prat & Wakade (1975) proposed that the increase in liberation of the transmitter per pulse at a higher frequency of stimulation may be due to increased influx of Ca. In the presence of TEA, which is believed to increase Ca influx greatly during excitation of the nerve membrane, the influx of Ca at higher frequencies should be even further exaggerated, causing massive amounts to enter the neurone. In such an event, excess accumulations of Ca within the neurone may desensitize the release process (Kirpekar *et al.*, 1975). In support of such a proposal is the fact that reduction of external Ca to 0.83 mM actually doubled tritium release in TEA-Krebs solution at 30 Hz.

With the use of TEA it was possible to obtain measurable quantities of tritium over spontaneous overflow after exciting the nerves of the vas deferens by a single electrical shock. The most striking feature of this finding was that if net overflow was expressed per pulse the values were in the neighbourhood of 720×10^{-4}. That is, the highest amounts of sympathetic transmitter were liberated following just one impulse.

Time-course studies revealed that although most of the liberated transmitter upon a single shock appeared in the superfusate within the first 5 s, detectable quantities were appearing up to 25 s after the stimulus. To account for the prolonged overflow of transmitter, either the diffusion of released transmitter from biophase into the superfusion medium may be slow, or the release process continues even after cessation of stimulus. Still another possibility is that most of the tritium appearing over a prolonged period may represent the slow leakage of metabolites of the trans-

mitter released immediately after the stimulus. The present data provide no direct evidence for any one of the three possibilities. However, recent electrophysiological studies by Szurszewski (1978) show that 10 mM TEA produced repetitive spikes on the plateau phase of spontaneously-occurring action potential of gastric smooth muscle of the dog. It may be that excitation of sympathetic nerves exposed to 10 mM TEA by a single shock could cause repetitive firing resulting in a greater release and over an extended period of time. Katz & Miledi (1967) have suggested that the amount and the time-course of acetylcholine release are influenced by both the amplitude and the duration of nerve depolarization. Contraction induced by a single shock was greater in amplitude and duration than that recorded after 100 shocks at 10 Hz in normal Krebs solution. Excess and prolonged liberation of endogenous transmitter, plus increased responsiveness of the smooth muscle to noradrenaline, must account for generation of such a large contraction.

Tritium overflow occurred maximally in the TEA-treated vas deferens after a single shock, and declined markedly as the time interval between stimuli was shortened. For example, fractional release after six shocks given at 10 s intervals was 290×10^{-4} compared to 720×10^{-4} after one shock. These findings imply that certain changes induced in the nerve endings by a preceding impulse last for up to 10 s to affect release by the succeeding impulse. One possibility, although unlikely, is that electrical properties of the nerve membrane treated with TEA may be altered after the initial shock, and require more than 10 s for full recovery before the next action potential can be generated by the second shock. If this were the case, then changes in external Ca should have little effect on the overflow, but it was found that release at 0.1 Hz increased about 5 fold when the Ca concentration was increased from 0.83 to 10 mM. An alternate possibility is that following the first impulse, there is a massive influx of Ca in the sympathetic neurone as a result of the well-known blocking effect of TEA on delayed K outward current. If the excess Ca is not rapidly removed from, or redistributed within, the neurone, the influx of Ca triggered by a subsequent impulse will be less, and therefore the release will be less. It appears that even 10 s after the shock, release was depressed by one-third. That is, in the presence of TEA more than 10 s are needed to reduce the intraneuronal levels of Ca to the prestimulation level. By spacing the second impulse at various times after the first shock, one may get some idea as to the time required to 'wash out' the accumulated Ca and restore release.

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Note added in proof

Very recently it was demonstrated that a massive facilitation of sympathetic transmitter release evoked by transmural stimulation in the presence of TEA was accompanied by over 80% depletion of noradrenaline content of the rat vas deferens. As depletion of endogenous noradrenaline affected by TEA and nerve stimulation is a result of exhaustive exocytosis, such a method can be used to study the fate of noradrenergic storage vesicles *in vitro*. (Wakade, A. R. (1979). Recycling of noradrenergic storage vesicles of isolated rat vas deferens. *Nature*, **281**, 374–376).

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