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POTENTIATION BY SUBSTANCE P OF CONTRACTIONS OF THE ISOLATED VAS DEFERENS OF THE MOUSE ELICITED BY ELECTRIC FIELD STIMULATION AND BY DRUGS

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1 Isolated vasa deferentia from the mouse were opened longitudinally and suspended in Krebs solution at 37°C in an organ bath. Contractions of the muscle were elicited by electric field stimulation, noradrenaline (10^{-6} M) and acetylcholine (10^{-6} M). Continued transmural stimulation evoked a biphasic response comprising a rapid twitch followed about 10 s later by a smaller, sustained rise in muscle tone.

2 The amplitudes of nerve-mediated and drug-induced responses were considerably potentiated by substance P (SP) in the dose range 10^{-12} to 10^{-7} M. Higher concentrations of SP were directly spasmogenic. The sensitizing property of SP was dose-dependent and was usually well maintained, but always disappeared quickly on washing the preparation. In some experiments SP facilitated the twitch, but not the subsequent phase of the electrically-induced contraction or the response to externally applied noradrenaline.

3 Phentolamine (10^{-6} M) failed to block this effect of SP, but itself potentiated the nerve-mediated twitch, and completely abolished the sustained secondary contraction.

4 Desmethylimipramine (10^{-6} M) enhanced the delayed contraction but not the immediate contraction.

5 The uptake of tritiated noradrenaline (3×10^{-7} M) by vasa was inhibited by desmethylimipramine (10^{-6} M) and increased by nialamide (3×10^{-5} M), but was not modified by SP (10^{-6} M).

6 Nerve-mediated release of accumulated radioactivity was accelerated by phentolamine, but not by SP or desmethylimipramine.

7 These findings suggest that SP sensitizes the muscle cells to depolarizing stimuli but that it has no facilitatory effect on sympathetic neural elements.

Introduction

The sympathetic nerves supplying the vas deferens are uncharacteristic inasmuch as they relay at peripheral synapses (Birmingham & Wilson, 1963; Kuriyama, 1963; Falck, Owman & Sjostrand, 1965; Ferry, 1967). Birmingham & Wilson (1963) devised a simple method of transmural stimulation for triggering this rich intramural network of short postganglionic noradrenergic nerve fibres, thereby contracting the vas, since which time the pharmacology of this preparation has been extensively studied. One feature of the nerve-mediated contractions of guinea-pig vas deferens is their unusual susceptibility to potentiation by exceptionally small concentrations of a wide variety of agonists, among them substance P (SP; Sjostrand & Swedin, 1968; Von Euler & Hedqvist, 1974). The effect of SP was considered intuitively by the latter authors to represent a non-specific postjunctional sensitization of the muscle cells, but the possibility that SP may have augmented neurotransmission was not excluded. Indeed, there is good reason to suppose that SP may enhance transmitter release in a number of cholinergic systems (Beleslin, Radmanović & Varagić, 1960; Hedqvist & Von Euler, 1975).

The purpose of the present study, therefore, was to investigate the possible pre- and/or post-junctional mechanism(s) underlying this phenomenon in the transmurally stimulated mouse vas deferens. Our findings suggest that SP regulates muscle responsiveness, possibly through two separate muscle sites, but

a presynaptic action on sympathetic nerves is not indicated.

Methods

General procedure

Male albino mice weighing 20 to 35 g were used for these experiments. Vasa were removed from freshly killed animals into warm Krebs bicarbonate solution and cut open longitudinally. Lumen contents were removed by thorough washing and the vasa suspended in Krebs solution at 37°C in a 5 ml organ bath. One end of the muscle was fixed in the bath, while the free end was attached by thread to a force displacement transducer. Contractions of the muscle were amplified and displayed on a Beckman Dynograph pen recorder. The reservoir of medium was gassed continuously with 5% CO_2 in O_2. Drugs were injected into the bath dissolved in Krebs solution and in volumes not exceeding 50μl. Washing was by overflow. The composition of the Krebs solution was as follows (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.12, $NaHCO_3$ 25 and glucose 5.6.

Electric field stimulation was delivered via a pair of platinum wire electrodes, 2 cm long, placed on either side of and close to the tissue, and connected to a Grass square wave stimulator. All glass apparatus was siliconized before use.

Uptake experiments

Vasa were opened, cleaned and placed in 5 ml Krebs solution in conical flasks. They were first given a 10 min preincubation with shaking and continuous oxygenation at 37°C, and then incubated for up to 60 min under these conditions with radioactive noradrenaline (3×10^{-7} M, approximately 0.5 μCi/ml). Where used, drugs were present throughout these stages. The tissues were then washed in fresh Krebs solution for 2 min, blotted dry and weighed on a torsion balance. Their radioactive content was determined by dissolving the vasa in 0.2 ml Soluene 350 (Packard), neutralizing with 3 drops of glacial acetic acid and adding 2 ml ethoxyethanol and 10 ml butyl PBD (0.5% in toluene) before counting in a liquid scintillation spectrometer (Packard Model 2003). Counts per min were converted to disintegrations (d/min) after correction for quenching and background radiation. Uptakes were expressed as tissue:medium ratios (d min^{-1} g^{-1} tissue:d min^{-1} ml^{-1} medium).

Release experiments

Vasa were loaded with radioactive noradrenaline for 60 min, washed and suspended in an organ bath. The experimental set-up was essentially the same as that described previously by Swedin (1971). Krebs solution was perfused over the preparation at the rate of 0.8 ml/min with a roller pump and the excess sucked off from above directly into scintillation vials. After a preliminary washing period of 60 min, samples of perfusate were collected every 2 min and counted for radioactivity as above, after the addition of 10 ml Aquasol scintillator (New England Nuclear). Residual tissue radioactivity was extracted and estimated as described earlier.

Drugs were applied either by direct injection into the bath, or the inflow tube was connected to the appropriate reservoir of drugged medium.

Materials

[7-^{3}H(N)]-(−)-noradrenaline (2.20 Ci/mmol) was purchased from New England Nuclear (6072 Dreieichenhain, West Germany), substance P from Protein Research Laboratories (Osaka, Japan) and phentolamine mesylate from Ciba. Butyl-PBD was obtained from Fisons Scientific Apparatus, analytical grade toluene from Koch Light Laboratories Ltd. and scintillation grade 2-ethoxyethanol from BDH Chemicals Ltd. Desmethylimipramine was supplied by Dr. M. J. Neal and nialamide was generously donated by Pfizer Ltd.

Results

Effect of substance P on electrically induced mechanical response

Electric field stimulation of the vas deferens with square wave pulses of 1 ms duration, 10 Hz frequency and supramaximal voltage (40 to 80 V) elicited strong contractions of the muscle. As demonstrated previously by Farnebo & Malmfors (1971), we found that the nature of the mechanical response produced in this way depended on the duration of application of the stimulus. Figure 1a shows typical twitch responses to electrical stimulation (1 ms, 10 Hz, 20 V for 5 s) at 2 min intervals. The addition of SP (10^{-8} M) to the organ bath caused an immediate increase in twitch amplitude, which was frequently sustained for as long as SP was left in the bath. In some experiments this amounted to an hour or more. On washing out the peptide the twitch responses quickly returned to their pre-drug level.

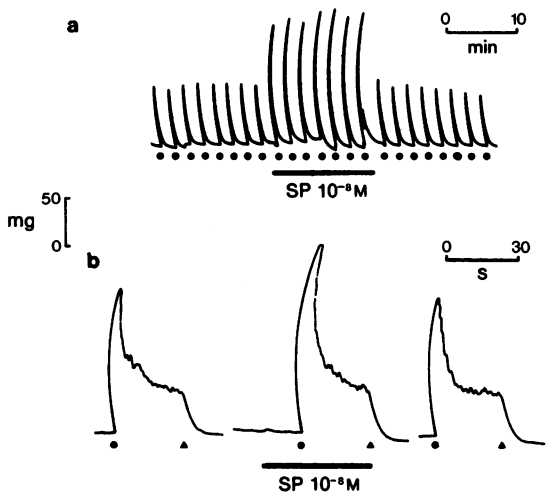

Figure 1 Potentiation of nerve-mediated contractions of mouse vas deferens by substance P(SP). (a) Twitch responses to 5 s electrical pulses (1 ms, 10 Hz, 20 V) delivered regularly at 2 min intervals. (●). During the period depicted by the horizontal bar substance P (10^{-8} M) was present in the bath. (b) Biphasic contractions elicited every 30 min by 30 s field stimulation (1 ms, 10 Hz, 20 V; on at (●), off at (▲). Substance P (10^{-8} M) was present 15 s before and during the middle response, as indicated by the bar.

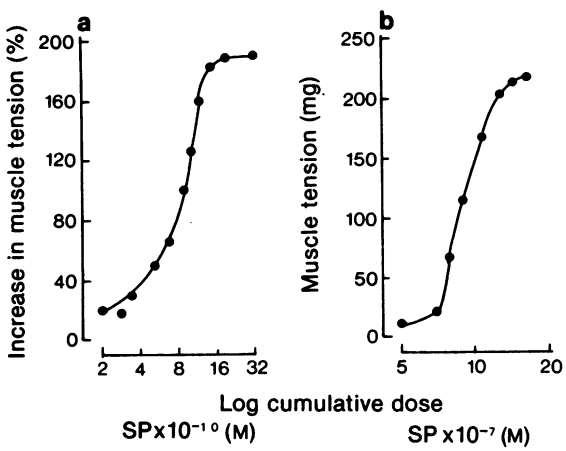

Figure 2 (a) Relationship between cumulative log dose substance P (SP) and potentiation of nerve-mediated twitch response (for stimulus parameters see Figure 1a). (b) Cumulative log dose-response relationship for spasmogenic action of SP on mouse vas deferens. Each point is the mean of three experiments. All s.e. means lay in the range ±6–12%.

More prolonged electrical stimulation (>10 s) evoked an initial twitch contraction followed by a smaller, sustained increase in muscle tension. The effect of SP (10^{-8} M) on this response is illustrated in Figure 1b. In this experiment the early twitch phase alone was potentiated by SP, but in other instances (10/27 experiments) SP was commonly observed to augment both phases of the contraction to an equal extent. This facilitation was found to be dose-dependent with SP generally being effective in threshold concentrations as low as 10^{-10} M, although the efficacy of SP varied widely from one preparation to another. Possibly this variability reflects the different rates at which the SP molecule is bound or degraded by different tissues. A cumulative log dose-response plot of this action of SP is illustrated in Figure 2a. In a few exceptional cases the vas was exquisitely sentitive to the peptide and reacted with an increased twitch height to as little as picomolar SP. In contrast, the spasmogenic action of SP was only recorded at doses of the peptide 1000–10000 times above this threshold level and is depicted by the cumulative log dose-response curve in Figure 2b. Interestingly, when nerve-mediated twitches of the muscle were superimposed on this slower, more prolonged SP contraction, potentiation of the twitch responses was still evident.

Phentolamine (10^{-6} M) added to the bath completely abolished the later stage of the electrically induced contraction, magnifying the twitch amplitude by 88·2 ± 8.4%, $n = 3$; (see also Figure 7). However, this enlarged twitch was still capable of further potentiation by 10^{-8} M SP (48.5 ± 6.7%, $n = 3$), although sometimes with phentolamine present the SP effect only lasted for about 10 minutes. In contrast, desmethylimipramine (DMI, 10^{-6} M), a potent inhibitor of noradrenaline uptake in this tissue (see later), increased the size of the secondary contraction (33.1 ± 5.6%, $n = 3$), but not that of the twitch, and further sustained increases in the magnitudes of both components were still possible with 10^{-8} M SP (26.4 ± 4.2%, $n = 3$).

Effect of substance P on mechanical responses elicited by drugs

In small concentrations (10^{-10} to 10^{-8} M) SP also accentuated the contractile responses of the vas deferens elicited by submaximal doses of acetylcholine (ACh) and noradrenaline (NA). These effects of SP were readily reversed and disappeared completely on washing the preparation. The data for these and some of the above experiments are summarized by the log stimulus intensity- and log dose-response curves plotted in Figure 3. SP invariably shifted these curves to the left, often with a concomitant increase in slope.

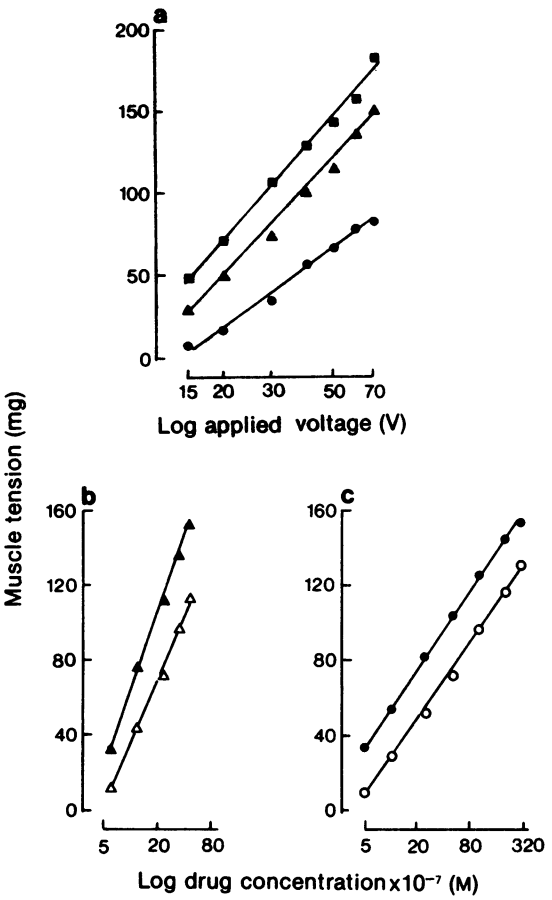

Figure 3 (a) Relationship between log stimulus intensity and twitch height for transmural stimulation (1 ms, 10 Hz) of mouse vas deferens delivered for 5 s every 2 min in the absence (●, controls) or presence of 10^{-8} M (▲, $P < 0.05$ versus controls) and 4×10^{-8} M substance P (■, $P < 0.01$ versus controls, $P < 0.05$ versus 10^{-8} M substance P). (b) Log dose-response curves for spasmogenic action of acetylcholine on mouse vas deferens in the absence (△, controls) and presence (▲, $P < 0.05$ versus controls) of 10^{-8} M substance P. (c) Log dose-response curves for the spasmogenic action of noradrenaline on mouse vas deferens in the absence (○, controls) and presence (●, $P < 0.05$ versus controls) of 10^{-8} M substance P. Each point is the mean of three experiments. All s.e. means lay in the range ±4–8%.

Uptake of [^{3}H]-noradrenaline

When vasa deferentia were incubated with [^{3}H]-NA (3×10^{-7} M) the tissues accumulated radioactivity

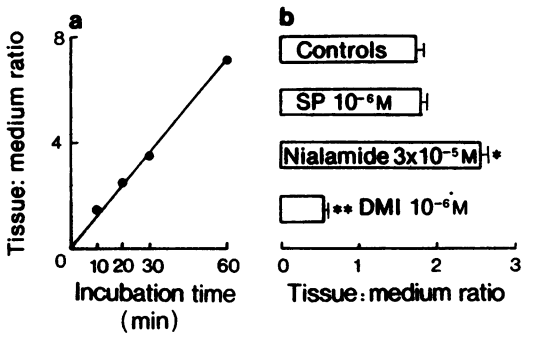

Figure 4 (a) Time course of uptake of [^{3}H]-noradrenaline (3×10^{-7} M, 0.5 μCi/ml) by mouse vasa deferentia. Incubations were conducted at 37°C with shaking in 5 ml Krebs solution (2 vasa per flask) gassed continuously with 5% CO_2 in O_2. (b) Uptakes of [^{3}H]-noradrenaline over 10 min at 37°C in untreated vasa and in the presence of substance P (SP, 10^{-6} M), nialamide (3×10^{-5} M) and desmethylimipramine (DMI, 10^{-6} M). Drugs were present in the bathing fluid throughout. Histograms represent the means of 10 separate experiments. Horizontal lines show s.e. means $^*P < 0.01$; $^{**}P < 0.005$ versus controls.

linearly over a period of 60 min (Figure 4a). The effect of preincubation of the muscles for 15 min with various drugs on the quantities of radiolabelled NA subsequently taken up in 10 min are shown in Figure 4b. In the concentration range 10^{-9} to 10^{-6} M SP had no discernible effect on NA uptake, although this was found to be significantly potentiated by 3×10^{-5} M nialamide, a monoamine oxidase inhibitor, (+47%, $P < 0.01$) and significantly attenuated by 10^{-5} M DMI (−68%, $P < 0.005$). These results exclude any action of SP on NA transport or catabolism.

Release of [^{3}H]-noradrenaline

The aim of this section of the study was to ascertain whether SP could act prejunctionally to modulate transmitter release. Unless stated otherwise in all of the experiments to be described here monoamine oxidase activity was inhibited by nialamide, but it should be stressed that qualitatively similar results were obtained when this drug was omitted from the medium. We made no attempt to eliminate *O*-methylation, although methylated metabolites of NA can account for as much as one fifth of the total radioactivity liberated when the sympathetic nerves are stimulated (Farnebo & Malmfors, 1971).

With a total radioactivity present in the tissue of the order of 0.761×10^6 d/min, the basal release per min after 60 min preliminary washout represented

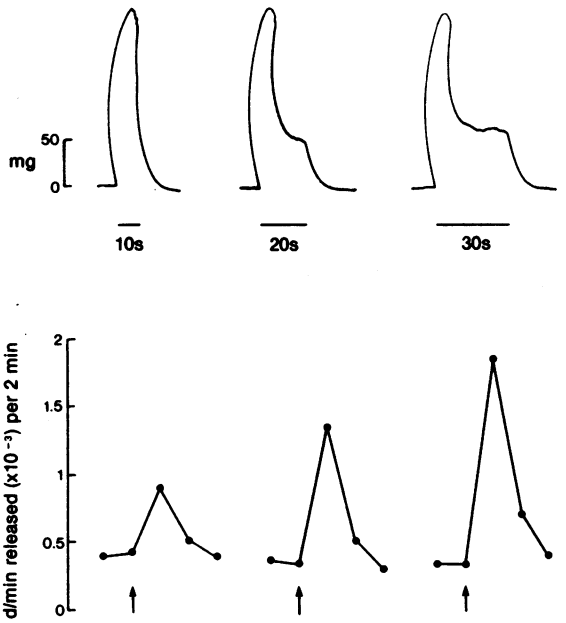

Figure 5 Mechanical responses (a) and corresponding overflow of tritium (b) from mouse vas deferens stimulated electrically (1 ms, 10 Hz, 80 V) for different time periods as indicated by the horizontal bars. Nialamide (3×10^{-5} M) was present in the superfusion medium only.

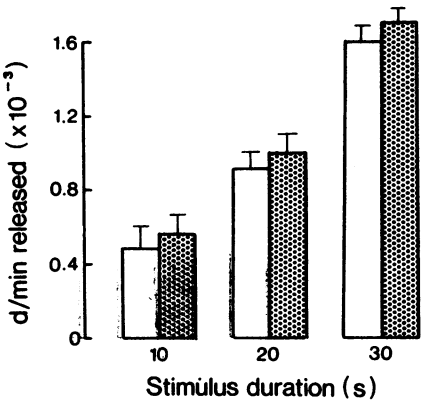

Figure 6 Electrically stimulated release of radioactivity from control (open columns) and substance P-treated (10^{-7} M, stippled columns) mouse vasa deferentia preloaded with [^{3}H]-noradrenaline. Results are the means of nine experiments. Vertical lines show s.e. means. Nialamide (3×10^{-5} M) was included in the superfusion medium only.

$0.033 \pm 0.004\%$ of tissue stores ($n = 18$). Electric field stimulation (1 ms pulse width, 10 Hz, 80 V) was used routinely to evoke measurable and reproducible releases of tritium. The increase in d/min recovered 2 min after initiating the stimulus was found to be directly proportional to the number of electrical pulses applied (Figure 5). The fraction of the tritium content overflowing per impulse at a stimulus frequency of 10 Hz was determined for time periods of 10, 20 and 30 s and calculated to be 7.33×10^{-5} (s.e. mean $\pm 0.16 \times 10^{-5}$, $n = 9$; Figure 6). Similar values were obtained at other stimulus frequencies (5 to 20 Hz). Superfusion of the vasa with Krebs solution containing 10^{-7} M SP for 20 min before and during stimulation raised this value to 8.14×10^{-5} (s.e. mean 0.31×10^{-5}; Figure 6), but this difference was not statistically significant ($P > 0.05$ by paired t test).

In nialamide-free Krebs DMI (10^{-6} M) likewise failed, somewhat surprisingly, to modify either the spontaneous or the electrically stimulated output of label (Figure 7), whilst micromolar phentolamine elevated the evoked release by $54.5 \pm 7.7\%$ ($P < 0.01$, $n = 3$; Figure 7).

Discussion

Earlier workers have stated that low levels of a variety of agonists, among them SP, are capable of potentiating the nerve-mediated contractions of guinea-pig vas deferens (Sjostrand & Swedin, 1968; Von Euler & Hedqvist, 1974). The exquisite sensitivity of this preparation to small doses of SP prompted Von Euler & Hedqvist (1974) to conjecture that subspasmogenic doses of the peptide may depolarize the muscle membrane subliminally and thereby raise its senstivity to all subsequent forms of depolarizing stimuli (cf. Sjostrand, 1973). Unfortunately these authors offered no supporting evidence to show that SP similarly enhances the contractile responses to exogenously applied spasmogens, as would be expected from their prediction. Nor did they exclude the possibility that SP may possess a prejunctional action and could conceivably potentiate the electrically stimulated contractions of the vas by augmenting NA release from, or by blocking its reuptake into sympathetic nerve terminals. We undertook the present study, therefore, with the aim of resolving these specific points.

Firstly, we established that SP was similarly capable of magnifying the mechanical responses of the mouse vas deferens to electric field stimulation of the postganglionic sympathetic nerves. Von Euler & Hedqvist (1974) have remarked on the practically all-or-nothing nature of the sensitization phenomenon with SP in the guinea-pig, whereas in our experience with mouse vasa, this effect of SP was more clearly

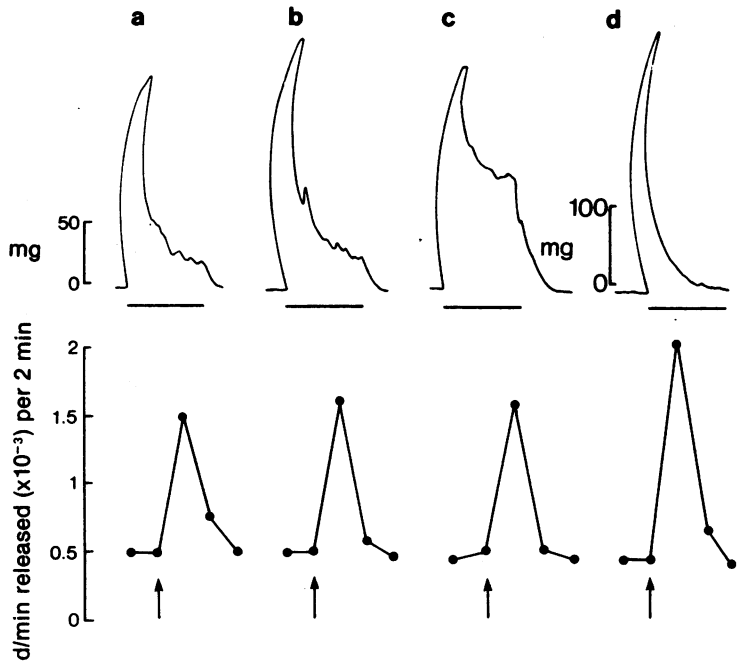

Figure 7 Mechanical responses (top trace) and concomitant [^{3}H]-noradrenaline release (bottom trace) from mouse vasa stimulated electrically (1 ms, 10 Hz, 80 V for 20 s) in the presence of: (a) normal Krebs solution (control); (b) substance P (10^{-7} M); (c) desmethylimipramine (10^{-6} M); (d) phentolamine (10^{-6} M). Note that owing to the expectancy of a considerably bigger contraction in (d), the amplification of the mechanical response was attenuated by 35%.

dose-related (see Figure 2a). In both species, however, SP has proved effective at concentrations far below those required to contract the muscle directly.

Secondly, we observed that SP also enhanced the contractions elicited by externally administered NA and ACh, which we believe clearly indicates that SP exerted a non-specific postsynaptic sensitizing effect on the muscle cells. However, facilitation of the twitch responses was not always accompanied by a greater secondary contraction (see Figure 1b), or by an enhanced response to exogenous NA. The reasons for these differences are not clear at present.

In order to determine whether the facilitatory action of SP might include a presynaptic component, its effect on the uptake and re-release of [^{3}H]-NA by the vas was investigated. The experiments with DMI indicated that it was possible to increase the amplitude of the second phase of the electrically-evoked mechanical response by blocking the neuronal uptake of NA, but without concomitantly increasing [^{3}H]-NA output. One might expect that inhibition of transmitter reuptake should be reflected in an increased transmitter output, but the fact that this did not occur with DMI in the mouse vas deferens was interpreted by Farnebo & Malmfors (1971) as evidence for the presence of a sensitive trans-synaptic regulatory mechanism for controlling NA release. Thus raising the NA concentration in the synaptic cleft (e.g. with DMI) is considered to activate presynaptic autoreceptors, which in turn suppress further transmitter release, thereby maintaining a steady level of NA within the neuromuscular junction. However, SP showed no signs of inhibiting NA uptake, nor did it increase the overflow of tritium during electrical stimulation of tissues prelabelled with [^{3}H]-NA. Although our d/min values were uncorrected for tritiated metabolites, we consider they should nevertheless be capable of registering changes in [^{3}H]-NA flux (see phentolamine results in Figure 7), owing to the preponderance of unchanged amine in the total radioactivity released by nerve stimulation (~73%; Farnebo & Malmfors, 1971). In our opinion, therefore, SP does not potentiate the twitch response by enhancing transmitter release.

It is also possible to discount the thesis that SP could exaggerate muscular contraction of the vas by liberating endogenous NA, which might in turn sensitize the tissue via extrajunctional NA receptors (Sjos-

trand & Swedin, 1968), since the facilitatory effects of SP were not antagonized by phentolamine. Also, the fact that phentolamine potentiated electrically-stimulated twitches of the vas, but, unlike SP, abolished the secondary phase of the contraction and accelerated tritium overflow, rules out the likelihood of any interference between SP and prejunctional adrenergic autoreceptors. Our data, therefore, offer no evidence for SP having a presynaptic action on sympathetic neural elements.

In conclusion, the present findings conform to the view that SP potentiates the muscular contractions of the mouse vas deferens to nerve stimulation and to drugs by influencing the responsiveness of the muscle cells rather than the nerves. That is, SP acts solely postjunctionally, probably somewhere along the final common pathway peripheral to receptor activation. Owing to SP's exceptionally high efficacy in this and other smooth muscle preparations, and its apparent widespread occurrence in subcellular particles in peripheral nerves (Von Euler, 1963), we would endorse the comment made earlier by Von Euler & Hedqvist (1974) that this peptide is worthy of consideration as a physiological modulator of smooth muscle activity.

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