Effects of nifedipine and ryanodine on adrenergic neurogenic contractions of rat vas deferens: evidence for a pulse-to-pulse change in Ca^{2+} sources

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1 The effects of nifedipine and ryanodine on the adrenergic component of neurogenic contractions of the rat isolated vas deferens were studied in an attempt to identify the sources of Ca^{2+} mediating the contraction. The tissue was electrically stimulated by single pulses or pairs of widely spaced pulses. The purinergic component of contraction was suppressed by the presence of 300 μM suramin.

2 In Mg^{2+} -free medium, nifedipine (0.01–10 μM) reduced the first and, to a greater extent, the second twitch elicited by two pulses 3 s apart. This pattern of inhibition was observed both in the absence of rauwolscline (when twitch 2 was smaller than twitch 1) and in the presence of 0.1 μM rauwolscline (when, due to interruption of prejunctional α_2 -adrenoceptor-mediated autoinhibition, twitch 2 was of similar height to twitch 1). Nifedipine reduced only twitch 2 but not twitch 1 in medium containing 1.2 mM Mg^{2+} .

3 Single pulses of increasing current strength elicited increasing contraction. Nifedipine reduced contractions by about the same absolute extent at all current strengths, so that the relative contribution of the nifedipine-sensitive component decreased with increasing current strength.

4 When the pulse interval in a pair was increased from 5 to 60 s, the inhibition by nifedipine of the second twitch was most marked at an interval of 5 s and declined as the interval increased.

5 In contrast to nifedipine, 20 μM ryanodine reduced the first twitch of a pair to a greater extent than a second twitch 5 s later, so that twitch 2 became greater than twitch 1. The inhibition by ryanodine of twitch 2 increased with increasing pulse interval.

6 In vasa deferentia preincubated with [³H]-noradrenaline, 1 μM nifedipine and 20 μM ryanodine did not change the electrically evoked overflow of tritium, whereas 10 μM nifedipine increased it.

7 It is concluded that, when the sympathetic axons of the vas deferens are stimulated by a single pulse (or the first pulse of a pair) in Mg^{2+} -free medium, both Ca^{2+} mobilization inside the smooth muscle cells and Ca^{2+} entry contribute to the ensuing adrenergic contraction. The relative contribution of Ca^{2+} entry is small at maximal stimulus strength but increases with decreasing stimulus strength. When a second pulse follows the first after an appropriate interval, a switch of Ca^{2+} sources occurs: intracellular Ca^{2+} mobilization is decreased during twitch 2, whereas Ca^{2+} entry is increased.

Keywords: Rat vas deferens; nifedipine; ryanodine; α_1 -adrenoceptors; Ca^{2+} entry; Ca^{2+} mobilization; prejunctional α_2 -autoinhibition

Introduction

The contraction of the rat vas deferens elicited by a single electric pulse, adjusted to stimulate exclusively neural elements, is biphasic, consisting of a rapid and a slow phase (McGrath, 1978). The response to trains of pulses depends on the frequency. When the frequency is low, e.g. 0.5 Hz, each pulse elicits a discrete twitch, with a characteristic development of the fast and slow phases; the fast phase remains relatively constant, whereas the slow phase declines and can become undetectable in the second twitch. When the frequency is high, e.g. 5 Hz, the responses to the individual pulses merge to a tetanic contraction which reaches an initial peak within 1 s (transient contraction), then falls off, and finally rises again (sustained contraction; Swedin, 1971; Brown *et al.*, 1983; Amobi & Smith, 1987).

These various contraction phenomena differ both in the transmitters involved and in the sources of Ca^{2+} used by the smooth muscle cells. The slow phase of single pulse-induced contractions is abolished by α -adrenoceptor antagonists and is therefore mediated by noradrenaline; the fast phase, in contrast, is resistant to α -adrenoceptor antagonists. The tetanus elicited by high-frequency trains contains both an

adrenergic and an α -adrenoceptor antagonist-resistant component throughout its duration (Swedin, 1971; McGrath, 1978; Brown *et al.*, 1983; Amobi & Smith, 1987). Experiments with the P_2 -purinoceptor desensitizing agent α,β -methylene ATP (Meldrum & Burnstock, 1983) and the P_2 -purinoceptor antagonist, suramin (Dunn & Blakeley, 1988) have now shown that the non-adrenergic components are mediated by ATP (Mallard *et al.*, 1989; Bourreau *et al.*, 1991; for review see Burnstock, 1990; von Kügelgen & Starke, 1991). The marked fall of the slow adrenergic but not the fast purinergic phase during low-frequency trains probably reflects a preferential prejunctional α_2 -adrenergic autoinhibition of the adrenergic phase (Brown *et al.*, 1983; cf. for mouse vas deferens von Kügelgen *et al.*, 1989; Bültmann *et al.*, 1991).

Even before the availability of P_2 -purinoceptor antagonists or desensitizing agents, the calcium channel blocker nifedipine was found to suppress selectively the fast phase of the response of the vas deferens to single or low-frequency pulses while leaving the slow phase unaffected (French & Scott, 1981; Brown *et al.*, 1983; Hay & Wadsworth, 1983; see also Amobi & Smith, 1987; Mallard *et al.*, 1992). In the tetanic response to high frequency, nifedipine reduced the transient initial peak but abolished the sustained contraction (Brown *et al.*, 1983; Amobi & Smith, 1987; Mallard *et al.*, 1992). In

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conjunction with present knowledge about cotransmission, these findings have been interpreted to indicate that the purinergic contraction components are nifedipine-sensitive and, therefore, due to Ca^{2+} entry from the extracellular space; that the adrenergic component of the late sustained tetanic contraction is also nifedipine-sensitive and due to Ca^{2+} entry; whereas the adrenergic slow phase of single pulse-evoked twitches is nifedipine-resistant and presumably due to intracellular Ca^{2+} mobilization. The nifedipine sensitivity of the purinergic components is well established (e.g. Bourreau *et al.*, 1991). It has also been shown that the adrenergic component of the late sustained tetanus, when isolated by treatment with α,β -methylene ATP, is in fact abolished by nifedipine (Bourreau *et al.*, 1991). However, when we isolated the adrenergic slow phase of low frequency-induced contractions by treatment with suramin in Mg^{2+} -free medium we observed that, unexpectedly, the first twitch and, to an even greater extent, the subsequent twitches were reduced by nifedipine and thus seemed to contain a component of Ca^{2+} entry, in contrast to previous views (Brown *et al.*, 1983; Amobi & Smith, 1987). These observations are described here, together with the effect of ryanodine, which blocks intracellular Ca^{2+} mobilization, a second possible source of Ca^{2+} for contraction (Iino, 1990). Some results have been reported previously in abstract form (Bültmann & Starke, 1992).

Methods

Male Wistar rats were decapitated and the vasa deferentia removed and cleaned of adherent tissue. Unless stated otherwise, experiments were carried out on tissues from rats 6 to 7 weeks old (150–180 g, 'young' rats). For several later series animals at least 12 weeks old were used (280–340 g; 'mature' rats). The vasa deferentia from mature rats displayed less post-stimulus oscillations in tension (see Results) which, especially in the presence of ryanodine, interfered with the quantification of neurogenic contractions.

Unless stated otherwise, the medium used for incubation and superfusion contained (mM): NaCl 118, KCl 4.8, CaCl_2 2.5, KH_2PO_4 0.9, NaHCO_3 25, glucose 11, ascorbic acid 0.3, and disodium EDTA 0.03. It was saturated with 95% O_2 /5% CO_2 and kept at 37°C.

Contraction

The vasa deferentia were suspended vertically in an organ bath containing 5.7 ml medium. The lower end was fixed and the upper end attached to an isometric force transducer (K30; Hugo Sachs Elektronik, Hugstetten, Germany) under an initial tension of 9.8 mN. The medium was replaced every 15 min. Suramin, 300 μM , was added in all experiments from the beginning in order to suppress the purinergic component of contractions. Solutions of drugs were added to the organ bath in aliquots not exceeding 30 μl .

During a 60 min equilibration period the tissues were allowed to relax to a resting tension of approximately 3 mN. This final resting tension remained constant during the course of the experiment. A Stimulator II (Hugo Sachs Elektronik) operating in the constant current mode was used for electrical stimulation. The platinum electrodes were located at the top and the bottom of the organ bath (0.3 ms pulse width, current strength 100 mA or as indicated). Contractions were recorded on a Graphtec thermal pen recorder (Ettlingen, Germany). In some experiments responses were digitized at a frequency of 30 Hz by means of an analog-digital converter and stored on a microcomputer for later replay.

The IC_{50} value of nifedipine was interpolated as the concentration producing 50% of the inhibition obtained with nifedipine 1 μM , which produced the maximal inhibition and

was the highest concentration that did not alter the evoked release of [^3H]-noradrenaline (see Results).

Drug and solvent experiments were always carried out in parallel. Whenever drug-induced changes were calculated, they were corrected for the mean change occurring in the corresponding solvent group.

Other experimental details are explained in the Results section.

Tritium overflow

The medium did not contain suramin in [^3H]-noradrenaline experiments. Vasa deferentia were split open longitudinally. Four pieces of about 10 mg were cut from the prostatic portions. The eight pieces were incubated for 30 min in medium with reduced Ca^{2+} (CaCl_2 0.2 mM; see Limberger *et al.*, 1992) containing 0.2 μM (-)-[^3H]-noradrenaline, specific activity 43.7 Ci mmol $^{-1}$. They were then rinsed and one piece was transferred to each of six 0.16 ml superfusion chambers (two pieces were discarded). The tissue was held by a polypropylene mesh between platinum wire electrodes 6 mm apart and superfused with [^3H]-noradrenaline-free medium at 2 ml min $^{-1}$ (CaCl_2 2.5 mM). A Stimulator I (Hugo Sachs Elektronik) operating in the constant voltage mode was used for electrical stimulation. An initial stimulation period (180 pulses, 1 Hz, 1 ms pulse width, 24 V cm $^{-1}$ voltage drop between the electrodes of each chamber, yielding a current of 40 mA) was applied after 30 min of superfusion; it was not used for determination of tritium overflow. The tissues were then stimulated six times (S_1 to S_6) after 78, 96, 114, 184, 202 and 220 min of superfusion. Each of the six stimulation periods consisted either of one train of 6 pulses at a frequency of 100 Hz, or of two trains of 6 pulses/100 Hz, train interval 3 s, or of two trains of 6 pulses/100 Hz, train interval 10 s. The superfusate was collected in 2, 10 or 30 min periods from 74 min of superfusion onwards. Drugs and solvents were added to the superfusion medium after S_3 , 64 min before S_4 . At the end of the experiment, the tissue pieces were solubilized in 1 ml Soluene (Packard Instrument, Frankfurt am Main, Germany). Tritium was measured in superfusate samples and solubilized tissues by liquid scintillation spectrometry.

The outflow of tritium was calculated as a fraction of the amount of tritium in the tissue at the start of the respective collection period (fractional rate of outflow, min $^{-1}$). The stimulation-evoked overflow was calculated as the difference between the total outflow of tritium in the 4 min following the start of a stimulation period, and the estimated basal outflow during this time (all in nCi); the basal outflow was assumed to decline linearly from the 2 min interval before, to the interval 4–6 min after the start of stimulation; the difference (total minus basal) was expressed as a percentage of the amount of tritium in the tissue (in nCi) at the start of the stimulation period. For further evaluation of the basal efflux, ratios were calculated of the fractional rate of outflow in the 2 min before S_4 over that in the 2 min before S_3 . For further evaluation of the stimulation-evoked overflow, the sum of the overflow elicited by S_1 to S_3 was formed for each experiment (' S_{1-3} '), as was the sum of the overflow elicited by S_4 to S_6 (' S_{4-6} '), and ratios S_{4-6}/S_{1-3} were then calculated.

Materials

The following drugs were used: suramin hexasodium salt (Bayer, Wuppertal, Germany); ryanodine (Biotrend, Köln, Germany); (-)-[ring-2,5,6- ^3H]-noradrenaline, specific activity 43.7 Ci mmol $^{-1}$ (Du Pont, Dreieich, Germany); prazosin HCl (Pfizer, Karlsruhe, Germany); rauwolfscine HCl (Roth, Karlsruhe, Germany); nifedipine, tetrodotoxin (Sigma, Deisenhofen, Germany). Stock solutions of nifedipine and ryanodine were prepared in absolute ethanol and methanol, respectively; maximal final concentrations of ethanol and methanol were below 0.1 and 0.2%, respectively. Suramin,

prazosin and rauwolscine were dissolved in distilled water. Tetrodotoxin was dissolved in 0.1 M sodium acetate buffer pH 4.85. Experiments with nifedipine were carried out in near darkness or under sodium light.

Statistics

The arithmetic mean and s.e.mean are given throughout, except for IC_{50} values which are geometric means with 95% confidence intervals (c.i.). Differences between means were tested for statistical significance by the Mann-Whitney test. Differences with error probabilities of $P < 0.05$ were taken to be statistically significant. For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to Bonferroni (Wallenstein *et al.*, 1980).

Results

Nature of contractions

The contractions elicited by electric stimulation in medium containing suramin (300 μ M) were monophasic and peaked 460–660 ms after the stimulation pulse. Tetrodotoxin (0.5 μ M) when added at the end of several experiments of the series described below, abolished the contractions. Prazosin was added at two increasing concentrations (10 followed by 100 nM) in 4 experiments using the pulse pair protocol of Figure 1 below, but with 60 min exposure to either concentration. Prazosin 10 nM greatly reduced, and 100 nM abolished, both twitch 1 and twitch 2 of the pair. The responses, hence, were neurogenic and adrenergic and represented the slow phase of the discrete twitches evoked in the vas deferens by single or low-frequency pulses.

Pairs of pulses

Two pulses, 3 s apart, elicited separate contractions; the tension returned to baseline between the twitches (Figure 1). The second twitch was smaller than the first (Figure 1, upper tracings, and Figure 2a). In control experiments in which the pair of pulses was applied every 5 min and solvent was administered, contractions remained approximately constant (left-hand panel of Figure 1, open symbols in Figure 2a). Increasing concentrations of nifedipine (0.01–10 μ M) reduced both twitches, but reduced the second twitch to a much greater extent than the first (right-hand panel of Figure 1, solid symbols in Figure 2a). The IC_{50} values of nifedipine were 42 (95% c.i. 18–98) nM for the first twitch of the pair and 59 (12–288) nM for the second twitch. Nifedipine (1 μ M) reduced the first twitch by 2.5 ± 0.4 mN and the second twitch by 6.2 ± 0.7 mN ($P < 0.05$ for the difference between first and second twitch).

One possible reason for the greater inhibition of twitch 2 than of twitch 1 was the smaller size of twitch 2 which is mainly due to prejunctional α_2 -adrenoceptor-mediated auto-inhibition (see Introduction). In order to examine this possibility, experiments were carried out in the presence of the α_2 -adrenoceptor antagonist rauwolscine which prevents auto-inhibition. In fact, when 0.1 μ M rauwolscine was present in the medium from the beginning, the two contractions of the pair were of similar height (and shape; '0' values in Figure 2b). In control experiments, the contractions remained approximately constant (open symbols in Figure 2b). Nifedipine reduced both twitches, but again reduced the second twitch to a much greater extent than the first, so that the second became smaller than the first (solid symbols in Figure 2b). The IC_{50} values of nifedipine were 54 (20–143) and 82 (57–119) nM for the first and second twitches, respectively.

Nifedipine 1 μ M reduced the first twitch by 1.5 ± 0.3 mN and the second twitch by 4.3 ± 0.7 mN ($P < 0.05$).

The protocol of Figure 1 was used in three additional groups of experiments: on mature rats, in Mg^{2+} -containing medium, and with replacement of the second pulse by two pulses, 10 ms apart.

Responses of vasa deferentia from mature rats differed from those of young rats (Figures 1 and 2) in two ways. Stimulation-evoked twitches were of greater magnitude (see Figure 4 below) and in tissues from young animals, 'spontaneous' contractions always followed the neurogenic contractions proper; nifedipine abolished the spontaneous contractions (Figure 1). No, or much smaller, oscillations developed after the neurogenic twitches in vasa deferentia from mature rats. Despite these differences, nifedipine (0.01–10 μ M) also reduced the first and, to a greater extent, the second of the twitches elicited by two pulses 3 s apart in vasa deferentia from mature rats. Nifedipine (1 μ M) reduced the first twitch by 2.3 ± 0.4 mN and the second twitch by 6.1 ± 0.6 mN ($P < 0.05$; $n = 4$ for solvent and nifedipine).

The experiments described so far were carried out in Mg^{2+} -free medium. In medium containing 1.2 mM Mg^{2+} (young rats), contraction heights were only about 1/3 to 2/3 of those obtained in the absence of Mg^{2+} . Nifedipine (0.01–10 μ M) did not significantly reduce the first twitch but did reduce the second, by 2.1 ± 0.5 mN at a concentration of 1 μ M ($n = 6$ for solvent and nifedipine).

When the second pulse of the pair was replaced by two pulses 10 ms apart, the ensuing response was monophasic and similar in shape and height to twitch No. 1 (so that the pair looked like pairs obtained in the presence of rauwolscine). Nifedipine (0.01–10 μ M) reduced both twitches, but again reduced the second twitch more than the first. Nifedipine 1 μ M reduced the first twitch by 2.0 ± 0.4 mN and the second twitch by 4.8 ± 0.5 mN ($P < 0.05$; $n = 4$ for solvent and nifedipine).

For all subsequent experiments nifedipine was used at a concentration of 1 μ M since this concentration was maximally effective (Figure 2) and since 10 μ M nifedipine enhanced the electrically evoked overflow of tritium in experiments with [3 H]-noradrenaline (see below).

Current strength

Single pulses of increasing current strength elicited twitches of increasing height. A maximum was reached at 80 mA. When the stimulation cycle was repeated under control conditions, all contractions were increased (Figure 3a). Comparison of these controls with the nifedipine group (Figure 3b) shows that 1 μ M nifedipine depressed the entire current strength-response curve. The absolute inhibition caused by nifedipine (mN) was similar for all current strengths (Figure 3c) and averaged 3.2 ± 0.3 mN ($n = 35$; all current strengths pooled).

Similar results were obtained with tissues taken from mature rats ($n = 4$ for solvent and nifedipine).

Pulse interval

The second twitch of a pair of twitches, 3 s apart, was smaller than the first twitch (Figures 1 and 2a). The same was true for a pulse interval of 5 s (Figure 4, upper panel; pulse intervals less than 3 s were not used in order to keep the two twitches well separated). As the interval was prolonged from 5 to 60 s, the height of the second twitch increased progressively, but did not attain the height of twitch 1. This dependence on the pulse interval was observed both before and after (Figure 4, upper panel and Figure 5, open diamonds) the addition of solvent.

Nifedipine reduced the second twitch in a pair, 3 s apart, to a much greater extent than the first twitch (Figures 1 and

Figure 1 Effect of nifedipine on adrenergic twitches elicited by two pulses 3 s apart: representative tracings. The adrenergic component was isolated by the presence of suramin ($300\text{ }\mu\text{M}$). Tissues were stimulated every 5 min. Each stimulation period consisted of two pulses 3 s apart (■). After 30 min of stimulation in nifedipine-free medium, a cumulative concentration-response curve was determined by stepwise increases of the concentration of nifedipine ($0.01\text{--}10\text{ }\mu\text{M}$) every 30 min (right-hand panel). Control experiments with increasing concentrations of solvent were run in parallel (left-hand panel). Shown are the last responses before addition of solvent or nifedipine (top tracings) and the last responses in the presence of each concentration of solvent or nifedipine (i.e. after 30 min of exposure to the respective concentration). The uneven appearance of the tracings is due to the resolution of the computer storage system. Representative tracings from 4 experiments each.

2), and the same was true for an interval of 5 s. When the interval was prolonged, the inhibition caused by $1\text{ }\mu\text{M}$ nifedipine declined (Figures 4 and 5). At the 30 s and 60 s intervals, the inhibition of twitch 2 (by $5.0 \pm 0.7\text{ mN}$ and $3.9 \pm 0.8\text{ mN}$) no longer exceeded that of twitch 1 (by $3.7 \pm 0.7\text{ mN}$ and $3.6 \pm 0.7\text{ mN}$, respectively; $P > 0.05$ for the difference between first and second twitch).

Ryanodine ($20\text{ }\mu\text{M}$) augmented the spontaneous contraction waves following the neurogenic twitches proper (compare top and bottom tracings of Figure 4). Moreover, ryanodine attenuated both neurogenic twitches of the pairs. The first twitch was very markedly reduced (Figure 4). The effect on the second twitch depended on the pulse interval in a manner opposite to that observed with nifedipine: at short intervals, the inhibition was relatively small so that a substantial part of the second twitch persisted and twitch 2 became greater than twitch 1; the inhibition then increased

with increasing pulse interval to become indistinguishable from the inhibition of twitch 1 after 30 s (Figures 4 and 5). There was a significant inverse correlation between the degree of inhibition caused by ryanodine and that caused by nifedipine (Figure 5 inset).

Responses remaining in the presence of $20\text{ }\mu\text{M}$ ryanodine were abolished by subsequent addition of $1\text{ }\mu\text{M}$ nifedipine (nifedipine was added in the ryanodine experiments of Figures 4 and 5 in a third stimulation cycle; $n = 4$).

Tritium overflow

Release of [^3H]-noradrenaline was measured as overflow of total tritiated compounds. Since the overflow elicited by single electric pulses was too low to be measurable, trains of 6 pulses/100 Hz were applied. Nevertheless the evoked overflow of tritium was rather small. For this reason, three stimulation

Figure 2 Effect of nifedipine on adrenergic twitches elicited by two pulses 3 s apart: statistical evaluation. Protocol as in Figure 1. (a) Shows responses in the presence of 300 µM suramin; (b) responses in the presence of 300 µM suramin and 0.1 µM rauwolfscine. Upper panels show responses (mN) to the first, lower panels responses to the second of the two pulses; note difference in ordinate scales. Open symbols show responses in the presence of increasing concentrations of solvent, solid symbols responses in the presence of increasing concentrations (abscissae) of nifedipine. The last responses before addition of solvent or nifedipine ('0' on abscissae) and the last responses in the presence of each concentration of solvent or nifedipine (i.e. after 30 min of exposure to the respective concentration) were evaluated. *Denotes a significant difference between nifedipine and solvent, based on changes from pre-solvent and pre-nifedipine contractions ($P < 0.05$). Means and s.e.mean from 4 (a) or 6 (b) experiments.

periods were applied before and after the addition of drugs, and the sums of the overflow from S_1 to S_3 (before drug) as well as from S_4 to S_6 (in presence of drug) were calculated. In solvent experiments, the overflow of tritium was slightly lower at S_4 to S_6 than at S_1 to S_3 (Table 1).

Figure 3 Effect of nifedipine on adrenergic twitches: influence of current strength. The adrenergic component was isolated by the presence of suramin (300 µM). Tissues were stimulated every 5 min by single pulses of either increasing (35 to 100 mA) or decreasing (100 to 35 mA) current strength. When the first stimulation cycle was completed, either nifedipine (1 µM) or solvent was added and kept in the medium for the remainder of the experiment. The stimulation cycle was repeated 60 min later. Results obtained with increasing and decreasing current strength were pooled. (□) in (a) and (b) represents current strength-response curves before, and (■) represents curves after addition of solvent (a) or nifedipine (b). (c) shows the inhibition by nifedipine; it was calculated as the difference between responses obtained before and those obtained after addition of nifedipine, corrected for the mean change occurring in solvent experiments. *Denotes a significant difference between nifedipine and solvent, based on changes from pre-solvent and pre-nifedipine contractions ($P < 0.05$). Means and s.e.mean from 5 experiments.

Tetrodotoxin (0.5 µM) almost abolished the overflow elicited by any of the three stimulation protocols (Table 1). Rauwolfscine (0.1 µM) changed neither the overflow evoked

Figure 4 Effect of nifedipine and ryanodine on adrenergic twitches elicited by two pulses of varying interval: representative tracings. The adrenergic component was isolated by the presence of suramin ($300 \mu\text{M}$). Tissues from mature rats were stimulated every 5 min. The first stimulation period consisted of a single pulse, the following periods of two pulses of increasing interval: 5, 10, 15, 20, 30 and 60 s (■). When the first stimulation cycle was completed, either $1 \mu\text{M}$ nifedipine, $20 \mu\text{M}$ ryanodine or the respective solvent was added and kept in the medium for the remainder of the experiment. The stimulation cycle was repeated 60 min later. All tracings shown were obtained during the second stimulation cycle. Top tracings show responses in the presence of nifedipine solvent (ethanol), middle tracings responses in the presence of nifedipine, and bottom tracings responses in the presence of ryanodine. Note that ryanodine increased the oscillatory contractions that followed the neurogenic twitches proper; the second neurogenic twitch of the 5 s interval pair is superimposed on an oscillatory contraction. Representative tracings from 4–5 experiments each.

by a single train of 6 pulses nor that evoked by two trains 10 s apart, but increased the overflow evoked by two trains 3 s apart. Nifedipine ($1 \mu\text{M}$) and ryanodine ($20 \mu\text{M}$) caused no change. Nifedipine at the higher concentration of $10 \mu\text{M}$ increased the evoked overflow by more than 60% for all three stimulation protocols (Table 1). The increase in noradrenaline release may explain why $10 \mu\text{M}$ nifedipine depressed stimulation-evoked contractions less than did $1 \mu\text{M}$ nifedipine in several experimental groups (see Figure 2a and b, upper panels).

None of the drugs changed the basal outflow of tritium.

Discussion

Neither nifedipine nor ryanodine reduced the electrically evoked overflow of tritium after preincubation with [^3H]-

Figure 5 Effect of nifedipine and ryanodine on adrenergic twitches elicited by the second of two pulses delivered at varying interval: statistical evaluation. Protocol as in Figure 4. Tissues from mature rats. Shown are contractions obtained in the second stimulation cycle, in the presence of $1 \mu\text{M}$ nifedipine (●), $20 \mu\text{M}$ ryanodine (■) or solvent (◇; nifedipine and ryanodine solvent pooled); these three groups did not differ statistically in contraction heights obtained during the first stimulation cycle. *Denotes a significant difference between nifedipine or ryanodine and solvent, based on changes from pre-solvent and pre-nifedipine contractions ($P < 0.05$). Means and s.e.mean from 5 (nifedipine), 4 (ryanodine) or 9 (solvent) experiments. Inset shows correlation between inhibition by nifedipine (Nif) and inhibition by ryanodine (Rya). The inhibition was calculated as the difference between responses obtained before and those obtained after addition of nifedipine or ryanodine, corrected for the mean change occurring in solvent experiments.

Table 1 Effects of drugs on evoked tritium overflow from rat vasa deferentia preincubated with [³H]-noradrenaline

Drug present from S ₄ to S ₆	S ₄₋₆ /S ₁₋₃ for stimulation periods consisting of:		
	One train of 6 pulses/100 Hz	Two trains of 6 pulses/100 Hz, 3 s interval	Two trains of 6 pulses/100 Hz, 10 s interval
Solvent	0.86 ± 0.04 (5)	0.91 ± 0.06 (5)	0.92 ± 0.07 (6)
Tetrodotoxin 0.5 µM	0.10 ± 0.02* (4)	0.06 ± 0.02* (5)	0.04 ± 0.02* (4)
Rauwolscine 0.1 µM	0.94 ± 0.09 (4)	1.44 ± 0.16* (4)	1.17 ± 0.08 (4)
Solvent	0.89 ± 0.04 (5)	0.98 ± 0.09 (6)	0.98 ± 0.06 (5)
Nifedipine 1 µM	0.98 ± 0.11 (6)	1.13 ± 0.06 (5)	1.05 ± 0.09 (6)
Solvent	0.82 ± 0.09 (5)	0.79 ± 0.03 (6)	0.84 ± 0.07 (5)
Nifedipine 10 µM	1.38 ± 0.10* (4)	1.46 ± 0.09* (6)	1.37 ± 0.11* (4)
Solvent	0.92 ± 0.09 (7)	Not determined	0.83 ± 0.06 (7)
Ryanodine 20 µM	0.81 ± 0.07 (5)	Not determined	0.93 ± 0.05 (5)

Tissues from mature rats. After preincubation, tissues were superfused. There were six periods of electric stimulation (S₁–S₆). Each period consisted either of one train of 6 pulses/100 Hz, or of two trains of 6 pulses/100 Hz, train interval 3 s, or of two trains of 6 pulses/100 Hz, train interval 10 s. Solvent or drug was added 64 min before S₄ and kept for the remainder of the experiment. Values are ratios of the overflow elicited by S₄ to S₆ (S₄₋₆) and the overflow elicited by S₁ to S₃ (S₁₋₃). S₁₋₃ amounted to 0.134 ± 0.007% (one train per stimulation period; *n* = 45), 0.168 ± 0.007% (two trains 3 s apart per stimulation period; *n* = 37) and 0.142 ± 0.004% (two trains 10 s apart per stimulation period; *n* = 46), respectively. Means and s.e.mean from (*n*) tissues.

*Denotes a significant difference from solvent (*P* < 0.05).

noradrenaline, indicating that neither inhibited the release of noradrenaline (see also Hyland *et al.*, 1984; Starke *et al.*, 1984). In reducing contractions, both acted on the smooth muscle rather than the sympathetic nerves, and at the concentrations used, only their major modes of action, i.e. blockade of voltage-dependent Ca²⁺ channels for nifedipine and prevention of the mobilization of intracellular Ca²⁺ for ryanodine, require consideration (see Iino, 1990; Tsien & Tsien, 1990). The main question to be discussed on this basis is: what do the effects of nifedipine and ryanodine tell us about the origins of the Ca²⁺ that mediates the adrenergic component of the smooth muscle response to nerve stimulation?

Single twitches

The adrenergically mediated contractions evoked by single pulses of maximal current strength (in most experiments the first pulses of pulse pairs, after a period of rest of at least 5 min) were very markedly reduced by ryanodine (Figure 4). The decrease shows directly, as far as is possible with pharmacological tools, that the response is largely due to intracellular Ca²⁺ mobilization (Amobi & Smith, 1987; Mallard *et al.*, 1992). However, these adrenergic contractions were also reduced by nifedipine, although only in Mg²⁺-free medium, indicating that Ca²⁺ entry also plays some role. The nifedipine-sensitive, Ca²⁺ entry-mediated, part of the contractions, when expressed in absolute terms (mN), was similar at all stimulus strengths (Figure 3c). On the other hand, the nifedipine-resistant contraction which presumably is due to mobilization of intracellular Ca²⁺ increased with the strength of the neural stimulus (Figure 3b). The relative roles played by Ca²⁺ entry and Ca²⁺ mobilization, hence, depend on the stimulus: Ca²⁺ entry contributes little to adrenergic contractions elicited by the maximal neural stimuli used throughout most of the present study but contributes more to contractions elicited by weak stimuli. Exogenous noradrenaline activates Ca²⁺ entry into smooth muscle cells at lower concentrations than are required for Ca²⁺ mobilization (Casteels *et al.*, 1977; van Breemen *et al.*, 1981; Han *et al.*, 1987). Our observations may indicate that this relationship also holds true for neurally released noradrenaline.

The second twitch

At appropriate pulse intervals, the effects of nifedipine and ryanodine on a second twitch differed quantitatively from

their effects on the first twitch. The inhibition by nifedipine of the second twitch was greater than the inhibition of the first twitch, indicating a greater entry of Ca²⁺ during twitch 2 than during twitch 1 (Figures 1 and 2a, and compare top and middle panels of Figure 4). The greater inhibition by nifedipine was not due to the smaller size of the second twitch under control conditions, as it was also observed when twitch 2 was brought to the height of twitch 1 by interruption of α₂-adrenoceptor-mediated autoinhibition (Figure 2b) or by replacement of the second pulse by two pulses 10 ms apart. Conversely, the inhibition by ryanodine of the second twitch was much smaller than the inhibition of the first twitch, indicating less mobilization of intracellular Ca²⁺ during twitch 2 than during twitch 1 (compare top and bottom panels of Figure 4).

If nifedipine blocks Ca²⁺ entry and ryanodine prevents intracellular Ca²⁺ mobilization, the contraction component abolished by one of the two should behave like the contraction component remaining in the presence of the other. In fact, like the contraction component suppressed by nifedipine, the contraction remaining in the presence of ryanodine was much greater in the case of twitch 2 than of twitch 1 (bottom panel of Figure 4). Conversely, like the contraction component suppressed by ryanodine, the contraction remaining in the presence of nifedipine was smaller in the case of twitch 2 than of twitch 1; again, this was so even when twitch 2 was brought to the height of twitch 1 by interruption of α₂-adrenergic autoinhibition (Figure 2b) or the application of two pulses 10 ms apart.

All of these complementary observations indicate that the first adrenergic neurogenic twitch triggers a change in the Ca²⁺ sources used for contraction. Ca²⁺ entry during a second twitch, evoked after an appropriate interval, is enhanced; conversely, the mobilization of intracellular Ca²⁺ is reduced. As indicated by the effects of nifedipine and ryanodine, the change is pronounced when pulse 2 follows pulse 1 after 3, 5 or 10 s; it then fades within about 30 s (Figures 4 and 5).

The basis for the switch in Ca²⁺ sources is not known. The increase in Ca²⁺ entry and the decrease in intracellular Ca²⁺ mobilization during twitch 2 appeared and faded in a closely parallel manner, yielding a close inverse correlation between inhibition by nifedipine and inhibition by ryanodine and suggesting some connection between the two phenomena (Figure 5 inset). One explanation could be that Ca²⁺ mobilized from intracellular stores during twitch 1 inhibits the entry of Ca²⁺ during twitch 2, that the primary change in

twitch 2 is a decrease in intracellular Ca^{2+} mobilization, and this then leads to disinhibition of Ca^{2+} entry during twitch 2. There is some evidence for this speculation. Inositol-1,4,5-trisphosphate (IP_3) is the second messenger that links α_1 -adrenoceptor activation to intracellular Ca^{2+} mobilization (see Ruffolo *et al.*, 1991). In rabbit intestinal smooth muscle cells, release of IP_3 from its 'caged' precursor by flash photolysis rapidly and markedly inhibited voltage-dependent Ca^{2+} entry, and apparently the inhibition was due to Ca^{2+} mobilized from intracellular stores (Komori & Bolton, 1991; see also Nakazawa *et al.*, 1987). It is also known that the mobilization of Ca^{2+} by IP_3 is subject to rapid tachyphylaxis. In isolated hepatocytes, for example, the second of two applications of IP_3 , a few seconds apart, mobilized much less intracellular Ca^{2+} than the first, a depression that faded with a time course very similar to the depression of Ca^{2+} mobilization in the present experiments (Ogden *et al.*, 1990).

α_2 -Adrenoceptor-mediated autoinhibition

The first pulse of a train can exert an additional influence on subsequent pulses: prejunctional α_2 -adrenergic autoinhibition. The α_2 -adrenoceptor antagonist rauwolscine did not change the overflow of tritium, and presumably the release of [^3H]-noradrenaline, elicited by a single train of 6 pulses/100 Hz (release evoked by such brief trains resembles single pulse-evoked release: Singer, 1988; Limberger *et al.*, 1992) and also did not change the release elicited by two trains 10 s apart; however, rauwolscine significantly increased the release elicited by two trains 3 s apart. The single 50 ms train, hence, was free from autoinhibition, as expected (see Story *et al.*, 1981; Starke, 1987; Singer, 1988). Autoinhibition operated 3 s later, and then declined. This time frame agrees with that found previously with contraction measurements by French & Scott (1983). The shift in Ca^{2+} sources was basically independent of prejunctional autoinhibition: the decrease in Ca^{2+} mobilization from twitch 1 to twitch 2 persisted in the presence of rauwolscine (Figure 2b), and the increase in Ca^{2+} entry cannot of course be due to a decrease in noradrenaline release.

Calcium in pulse trains

In conjunction with reports cited in the Introduction, our results suggest a more complete picture of the contribution of different calcium sources to the adrenergic response of the vas deferens to trains of pulses. As shown by the effect of nifedipine, Ca^{2+} entry can contribute to a single twitch. The contribution of Ca^{2+} entry increases markedly upon the next

stimulus, and simultaneously the contribution of Ca^{2+} mobilization decreases. It is presumably this change in Ca^{2+} sources which continues during subsequent appropriately spaced stimuli and then, late in high-frequency pulse trains, makes the entire adrenergic component susceptible to blockade by nifedipine and resistant to ryanodine (Brown *et al.*, 1983; Bourreau *et al.*, 1991). It should be noted that the switch in Ca^{2+} sources took place despite the fact that the two twitches were separated by an interval during which the tension returned to baseline or, in other words, the concentration of noradrenaline fell to sub-threshold levels. No continuous presence of the agonist is required.

It has been suggested that the nifedipine resistance of the adrenergic response to a single stimulus and the nifedipine-sensitivity of the adrenergic component of a sustained tetanus are due to an effect of noradrenaline on two different α_1 -adrenoceptor populations: a single stimulus would release noradrenaline only onto receptors close to the sites of release and coupled to Ca^{2+} mobilization, whereas a pulse train would also (and mainly) release noradrenaline onto receptors far from the sites of release and coupled to Ca^{2+} entry (Brown *et al.*, 1983; Mallard *et al.*, 1992). Our findings argue against this view: the waves of noradrenaline causing the first and, a few seconds later, the second, separate twitch certainly reached the same receptors. The change from twitch 1 to twitch 2 seems to be a change in the transduction mechanism of one population of receptors.

Most of the present experiments were carried out in Mg^{2+} -free medium. In medium containing a physiological concentration of Mg^{2+} , contractions were smaller (compare Evans & Cunnane, 1992). Moreover, although second twitches were reduced by nifedipine, first twitches were not. There is, therefore, no conflict of evidence between the present findings and previous reports of nifedipine resistance of single pulse-evoked twitches in Mg^{2+} -containing medium (see Introduction). Mg^{2+} , like nifedipine, blocks voltage-dependent Ca^{2+} channels and in this manner may minimize the effect of nifedipine. In Mg^{2+} -containing medium the response of the vas deferens to the first pulse after a period of rest may in fact be practically exclusively due to Ca^{2+} mobilization. The switch to less Ca^{2+} mobilization and more Ca^{2+} entry upon the second pulse, however, also occurs at physiological Mg^{2+} concentrations.

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