Br. J. Pharmacol. (1989), **96**, 333–340

Interactions of calcium antagonists and the calcium channel agonist Bay K 8644 on neurotransmission of the mouse isolated vas deferens

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1 The present study compares the effects of verapamil and Bay K 8644 on twitches of the mouse vas deferens induced by field stimulation at 0.1 Hz. The influence of interactions between these drugs and nifedipine on neurotransmission was also investigated.

2 Bay K 8644 (0.1 nM–3 μM) and verapamil (1–100 μM) potentiated twitches maximally by about 1000% (EC_{50} 17.3 nM) and 300% (EC_{50} 17.5 μM), respectively. Nifedipine (0.1 nM–1 μM) only reduced twitch magnitude (IC_{50} 7.7 nM). All effects were reversed following washout.

3 Yohimbine (1–100 μM) reversed twitch potentiation caused by verapamil but not by Bay K 8644. Prazosin (1 μM) did not reduce basal twitch tension nor antagonize twitch potentiation by verapamil.

4 Twitch inhibition by nifedipine was unaltered by previous incubation with verapamil (30 μM), but Bay K 8644 (1 μM) shifted the curve to nifedipine 120 fold to the right. Previous incubation with nifedipine (1 μM) blocked potentiation induced by verapamil but did not modify responsiveness to Bay K 8644.

5 Previous addition of verapamil (30 μM) markedly enhanced twitch potentiation caused by Bay K 8644 in a supra-additive fashion. In experiments conducted in the reversed condition, Bay K 8644 (1 nM but not 10 nM) potentiated the effect of verapamil in a similar manner but to a lesser extent.

6 It is concluded that verapamil, in contrast to nifedipine, markedly enhances neurally-evoked twitches of the mouse vas deferens. Bay K 8644 produces essentially the same effect as verapamil, but its potency is 1000 fold and its maximal effect about 3 fold greater than that observed for verapamil. It is suggested that the mechanism of twitch potentiation by verapamil is different from that of Bay K 8644 and may involve an increased release of non-adrenergic co-transmitter(s).

Introduction

A vast number of pharmacological, electrophysiological and binding studies have characterized the inhibitory effects of different subclasses of organic calcium antagonists at voltage- and receptor-operated calcium channels on cardiac and smooth muscles (for recent reviews see Spedding, 1985; Cauvin & Van Breemen, 1987; Kamp & Miller, 1987). However, much less is known about the influence of such compounds on neuronal tissues, which posess a high density of calcium channels (Murphy & Snyder, 1982). The number of studies is limited and these have provided controversial results. Although some studies have shown that calcium antagonists are inactive or only weakly active on neurones (Daniell *et al.*, 1983; Freedman & Miller, 1984; Rampe *et al.*, 1984; Taube & Schwarzkroin, 1986), others demonstrated that such compounds can effectively inhibit calcium influx and/or transmitter release under certain circumstances (Middlemiss & Spedding, 1985; Turner & Goldin, 1985; Nowycky *et al.*, 1985; Woodward & Leslie, 1986; Kingsbury & Balazs, 1987).

It is becoming increasingly evident that the population of calcium channels in neuronal and muscular cells are not identical (for review see Hoffman *et al.*, 1987). Such differences may account for the differential sensitivity of autonomically innervated tissues to inhibition, by calcium antagonists, of responses induced by agonists and those evoked by nerve stimulation. Studies have shown that verapamil increases electrically-induced release of [^{3}H]-noradrenaline from sympathetic nerves innervating rat

caudal artery, guinea-pig vas deferens (Zsotér *et al.*, 1984) and rabbit aorta (Karaki *et al.*, 1984) and urethra (Larsson *et al.*, 1984). Moreover, verapamil enhances the magnitude of field stimulation-evoked twitches of the prostatic portion of the rat vas deferens (French & Scott, 1981; 1983; Hay & Wadsworth, 1983). Recently, Moritoki *et al.* (1987) reported that verapamil potentiates non-adrenergic nerve-mediated contractions of the rat vas deferens, as does diltiazem and the calcium-channel activator dihydropyridine Bay K 8644. However, verapamil was found only to inhibit twitches of the field-stimulated mouse vas deferens (Zetler & Kaschube, 1985). This discrepant finding, which is surprising in view of the very dense and apparently exclusive motor sympathetic innervation of the mouse vas deferens (Stjärne & Lundberg, 1986), prompted us to reinvestigate the effects of verapamil on neurotransmission in this preparation. Also, we have investigated the influence of interactions between verapamil, nifedipine and Bay K 8644 on nerve-induced twitches.

Methods

Animals

Male Swiss albino mice (25–35 g), raised in temperature-controlled (22 ± 1°C) ambience with 12 h light/dark cycle and allowed free access to water and Purina lab chow, were used throughout the study.

Mouse isolated vas deferens

The general set up and procedure employed has been described by Rae & De Moraes (1983). Mice were killed by cervical dislocation and both vasa deferentia were carefully excised, united by a small portion of the prostate gland, and placed in a Petri dish containing physiological salt solution (see composition below). After removing the excess of adherent connective and adipose tissues, each preparation (consisting of two vasa deferentia) was transfered to a double-jacketed organ bath containing 5 ml of a modified Krebs-Henseleit solution at 30°C bubbled with 5% CO_2 in O_2. The composition of the salt solution was (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 0.9 and glucose 11 (pH = 7.3). Preparations were submitted to a basal tension of 0.15 g and allowed a 60 min equilibration period, during which the bathing solution was renewed every 15 min, before initiating electrical field stimulation. Rectangular wave electrical pulses of 1 ms duration, delivered at 0.1 Hz and of supramaximal strength, were applied via a pair of platinum electrodes consisting of a hook below and a ring above each preparation. Isometric contractions were registered by means of strain gauge transducers coupled to a pen recorder (Narco Biosystems).

Once the field stimulation-evoked twitches became stable, preparations were exposed to increasing and cumulative concentrations of the calcium-channel activator Bay K 8644 (0.1 nM to 3 μM), or of the calcium-channel antagonists verapamil (1 to 100 μM) or nifedipine (0.1 nM to 1 μM). In some experiments, a second cumulative concentration-response curve to one of these drugs was obtained in the presence of a maximally- or submaximally-effective concentration of another.

Other sets of experiments examined the influence of prazosin (1 μM) or yohimbine (0.3 to 300 μM) on the magnitude of field stimulation-evoked twitches and on the responsiveness to verapamil. In the latter experiments prazosin was added to the bathing solution at least 10 min before exposure to verapamil (1 to 100 μM), whereas yohimbine (0.3 to 300 μM) was given in the presence of a maximally effective concentration of verapamil (30 μM).

In some experiments, single vas deferens were challenged repeatedly with noradrenaline (100 μM) or acetylcholine (1 mM) at 15 min intervals. The mean value of the first three control exposures to the agonist was taken as the 100% response. Subsequent challenges with the agonist were conducted in the presence of increasing concentrations of verapamil (1 to 30 μM), added 10 min prior to each challenge.

Drugs

Drugs used were acetylcholine iodide, nifedipine, noradrenaline bitartrate, tetrodotoxin, verapamil hydrochloride, yohimbine hydrochloride (all from Sigma Chemical Company), Bay K 8644 (methyl 1,4-dihydro - 2,6 - dimethyl - 3 - nitro - 4 - (2 - tri - fluoromethylphenyl)-pyridine-5-carboxylate, Bayer A.G.) and prazosin hydrochloride (Pfizer, São Paulo, Brazil). All drugs were stored as 10 mM stock solutions for up to a week at −4°C and diluted to the desired concentrations in distilled and deionized water just before use. Stock solutions of Bay K 8644, nifedipine, prazosin and yohimbine were made up in 100% ethanol before dilution with distilled water. Other stock solutions were made up in water and that of noradrenaline also contained 0.1 N HCl. At lower concentrations ethanol (0.01%) failed to modify twitch tension, so no corrections were necessary. In highest concentration (0.1 and 0.3%), ethanol caused a discrete inhibition in field stimulation-evoked twitches. Control experiments were performed in order to correct for the inhibition caused by ethanol alone. Solutions containing the dihydropyridines Bay K 8644 and nifedipine were protected from light and, when experiments were

conducted with these compounds, the organ baths were covered with aluminium foil and the laboratory lights were switched off, leaving only enough illumination to enable the experimenter to make drug additions.

Statistics

The EC_{50}'s and IC_{50}'s are presented with 95% confidence limits. All other values are presented as the means ± s.e.mean. The significance of drug-induced effects was assessed by Student's t test for unpaired samples. Differences between groups were considered to be significant at $P < 0.05$.

Results

Characterization of field stimulation-evoked twitches

Responses to field stimulation were entirely of neurogenic origin as they were completely blocked by tetrodotoxin (0.1 μM, $n = 3$, not shown). Basal field stimulation-evoked twitches were unaffected by yohimbine (up to 300 μM, twitch tension 104.3 ± 8.7% of basal value at 300 μM, $n = 3$) or prazosin (1 μM, twitch tension 99.2 ± 6.1% of basal value, $n = 7$) indicating that they derived mainly from the release of a non-adrenergic transmitter.

Effects of verapamil, Bay K 8644 and nifedipine on twitch tension

As shown in Figure 1, verapamil (1 to 100 μM) enhanced twitches evoked by supramaximal field stimulation. These effects were concentration-dependent and reversed upon washout. Twitch potentiation induced by verapamil usually peaked at concentrations between 30 and 100 μM. In half of the experiments, potentiation followed by depression of twitches was observed upon exposure to 100 μM verapamil (Figure 1). The depressant effect of verapamil possibly derives from a local anaesthetic action (Hay & Wadsworth, 1982; Beattie *et al.*, 1986). The

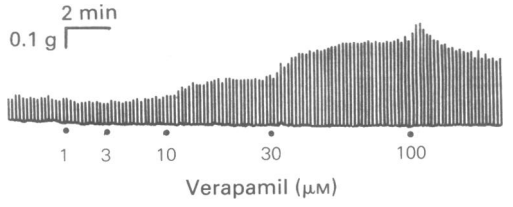

Figure 1 Representative isometric recording showing the changes of twitch tension of the isolated field-stimulated vas deferens of the mouse caused by cumulative additions of verapamil (1 to 100 μM).

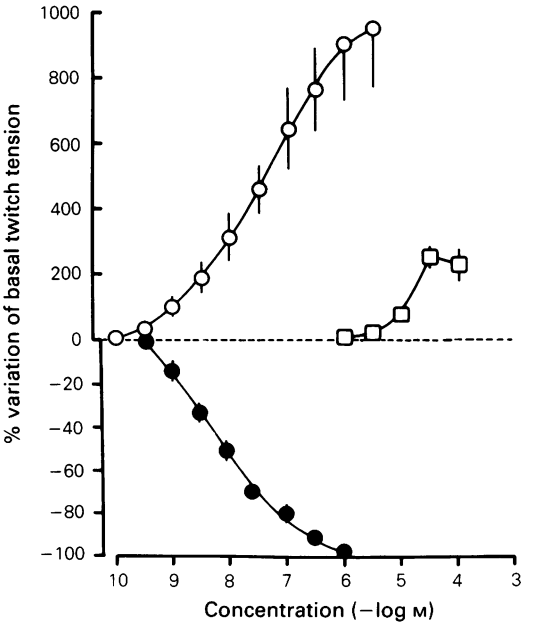

Figure 2 Mean concentration-response curves for Bay K 8644 (○) and for the calcium antagonists verapamil (□) and nifedipine (●) in altering basal twitch tension in the isolated field-stimulated vas deferens of the mouse. Each point represents the mean of 8 to 12 experiments and the vertical lines indicate s.e.mean. Note that scales used to illustrate potentiation and inhibition are different.

α_1-adrenoceptor antagonist prazosin (1 μM) failed to modify basal twitch tension or the twitch enhancing effect of verapamil (EC_{50} 13.0 μM, 8.8–19.2; maximal potentiation 299.6 ± 56.0%, $P > 0.05$, $n = 5$). Bay K 8644 (0.1 nM to 3 μM) also promoted concentration-dependent and reversible twitch potentiation. Comparison of the mean results illustrated in Figure 2 indicates that Bay K 8644 was 1000 fold more potent than verapamil; EC_{50} values were 17.3 nM (9.0–33.3) and 17.5 μM (13.7–22.5), respectively. Figure 2 also shows that maximal twitch potentiation afforded by Bay K 8644 (952.3 ± 208.8%) was 3 fold greater than that caused by verapamil (314.7 ± 35.3%). In contrast, nifedipine (0.1 nM to 1 μM)-induced inhibition of field stimulation evoked twitches, yielding an IC_{50} of 7.7 nM (5.0–11.8) and complete inhibition at 1 μM (Figure 2). This effect of nifedipine was concentration-dependent and reversed by washout.

Interactions between nifedipine and Bay K 8644 or verapamil

Figure 3 shows the inhibitory concentration-response curves obtained for nifedipine in the absence or presence of a maximally potentiating con-

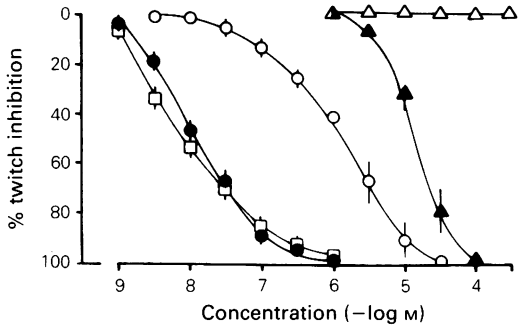

Figure 3 Mean concentration-response curves to nifedipine: control curve (□); in presence of verapamil 30 μM (●); or Bay K 8644, 1000 nM (○); to yohimbine: alone (△); in presence of Bay K 8644, 1000 nM (△) or in presence of verapamil 30 μM (▲) in inhibiting twitch tension in the field-stimulated mouse vas deferens. Note that yohimbine did not modify basal twitch tension up to 300 μM in the absence or presence of Bay K 8644. Each point represents the mean of 5 to 8 experiments and the vertical lines indicate s.e.mean.

centration of Bay K 8644 (1 μM) or verapamil (30 μM). The presence of Bay K 8644 caused a marked parallel rightward shift of the concentration-response curve to nifedipine. The IC_{50} determined for nifedipine in the presence of Bay K 8644 (0.9 μM, 0.3–2.2) was 120 fold greater than that determined in the absence of dihydropyridine agonist (7.7 nM, 5.0–11.8). In sharp contrast, simultaneous exposure to verapamil failed to affect significantly the inhibitory concentration-response curve to nifedipine (IC_{50} 12.6 nM, 8.0–19.7).

When twitches were abolished by nifedipine (1 μM), cumulative additions of Bay K 8644 initially at 0.1 or 0.3 nM restored the twitch responses to their original magnitude and at higher concentrations (1 to 1000 nM) caused concentration-dependent potentiations of twitch tension (EC_{50} 5.2 nM, 1.8–15.1; maximal response 1137.3 ± 381.8%; $n = 6$), similar to those observed in the absence of nifedipine (EC_{50} 17.3 nM, 9.0–33.3; maximal response 952.3 ± 208.8%; $n = 8$; $P > 0.05$). Interestingly, the presence of nifedipine (1 μM) rendered the preparation completely insensitive to the potentiating effects of verapamil (1 to 100 μM, $n = 4$). In addition, concentrations of verapamil in excess of 100 μM caused concentration-dependent sustained increases in tone of preparations bathed in nifedipine 1 μM (results not shown, $n = 4$).

Inhibition of verapamil-induced potentiation by yohimbine

The selective α_2-adrenoceptor antagonist yohimbine (up to 300 μM) did not affect basal twitch tension ($n = 3$, results not shown). However, Figure 3 shows that yohimbine (1 to 100 μM) caused a concentration-dependent inhibition of twitches potentiated by a maximally effective concentration of verapamil (30 μM) (IC_{50} 13.6 μM, 7.0–24.9; $n = 5$). In contrast, yohimbine failed to inhibit twitches potentiated by a maximally effective concentration of Bay K 8644 (1 μM, $n = 4$) (Figure 3).

Interactions between Bay K 8644 and verapamil

Previous incubation with verapamil (30 μM) enhanced the responsiveness of the preparation to Bay K 8644 (0.1 to 10 nM). As shown in Figure 4, the twitch potentiations observed in the presence of both compounds were significantly greater than those expected from the mere summation of their respective effects when given alone. Therefore, in the presence of verapamil, the potency of Bay K 8644 was enhanced nearly 60 fold at the EC_{50} level ($P < 0.05$) from 17.3 nM (9.0–33.3) to 0.3 nM (0.2–0.6). The maximal response to Bay K 8644 in the presence of verapamil (1442.0 ± 348.4%) was not significantly different ($P > 0.05$) from that detected in the absence of this drug (952.3 ± 208.8%).

When experiments were performed in the reverse conditions, i.e., the preparations were exposed to Bay K 8644 before cumulative additions of verapamil (1 to 30 μM), the results depended on the dose of Bay K 8644 employed. At 1 nM, a concentration that

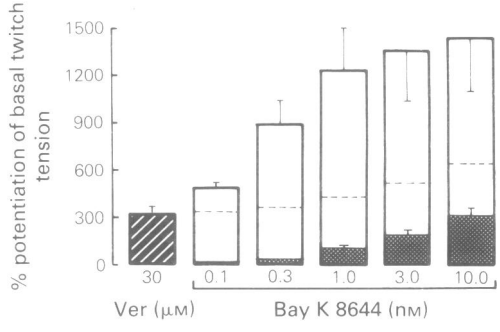

Figure 4 Influence of preincubation with verapamil (30 μM, hatched column) on the potentiation by Bay K 8644 (0.1 to 10 nM) of field stimulation-evoked twitches of the mouse isolated vas deferens. Cross-hatched columns represent the effect of Bay K 8644 alone and open columns the effect of Bay K 8644 in the presence of verapamil 30 μM. The broken lines indicate the values to be expected from simple addition of the individual effects of each agent alone. Each column represents the mean of 4 to 8 experiments and vertical bars the s.e.mean. Note that verapamil markedly potentiated the action of Bay K 8644 when compared to the control responses obtained in the absence of the calcium antagonist.

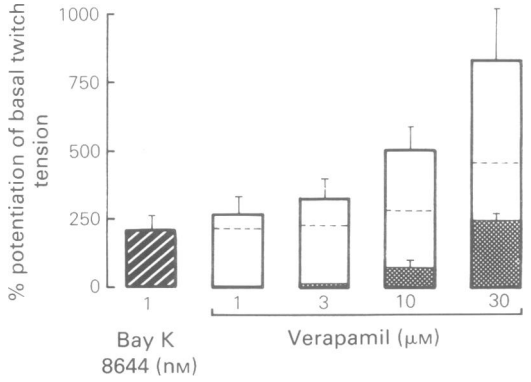

Figure 5 Influence of preincubation with Bay K 8644 (1 nM, hatched column) on the potentiation by verapamil (1 to 30 μM) of field stimulation-evoked twitches of the isolated mouse vas deferens. Cross-hatched columns represents the effect of verapamil alone and open columns the effect of verapamil in the presence of Bay K 8644 1 nM. The broken lines indicate the values to be expected from simple addition of the individual effects of each agent alone. Each column represents the mean of 6 to 12 experiments and vertical bars the s.e.mean. Note that in presence of Bay K 8644, the effect caused by verapamil (3, 10 and 30 μM) was potentiated when compared to the control responses obtained in the absence of Bay K 8644.

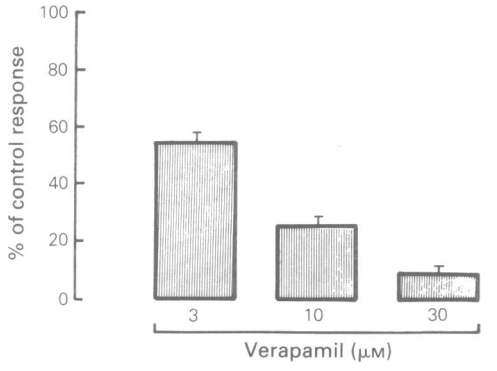

Figure 6 Inhibition by verapamil (3 to 30 μM) of noradrenaline-induced contractions of the mouse isolated vas deferens. Each column represents the mean of 11 experiments and the vertical bars indicate s.e.mean.

potentiated twitches to a lesser extent than maximally effective concentrations of verapamil, Bay K 8644 produced a supra-additive potentiation of the effect of verapamil (Figure 5), displacing its concentration-response curve to the left about 3 fold ($P < 0.05$), reducing the EC_{50} from 17.5 μM (13.7–22.5) to 5.3 μM (3.8–7.3) and increasing the maximal response from 314.7 $\pm$ 35.3% to 778.8 $\pm$ 186.2% ($P < 0.05$). However, at 10 nM, a concentration producing about half maximal twitch potentiation, Bay K 8644 potentiated the response to verapamil in a merely additive fashion ($n = 6$, results not shown). Thus, true potentiation by Bay K 8644 of the responses to verapamil was observed only when the former compound was incubated at a low concentration.

Effects of verapamil on noradrenaline- and acetylcholine-induced contractions

In the absence of field stimulation contractions of the mouse vas deferens to maximally effective concentrations of noradrenaline (100 μM) were depressed in a concentration-dependent manner by exposure to verapamil (3 to 30 μM) (Figure 6), yielding a mean pD'_2 value of 5.50 $\pm$ 0.13. Similar results were obtained with verapamil (1 to 10 μM) against responses to maximally effective concentrations of acetylcholine (1 mM), giving a mean pD'_2 value of 5.68 $\pm$ 0.09 ($n = 6$, results not shown).

Discussion

It is clear from the present results that verapamil induces a concentration-dependent enhancement of twitches evoked by electrical stimulation of the mouse isolated vas deferens at 0.1 Hz. This effect was essentially similar to that produced by Bay K 8644, but the potency of and maximal response to verapamil were 1000 and 3 fold smaller, respectively, than those seen with the dihydropyridine agonist. Almost identical findings have been obtained with verapamil in the rat vas deferens (French & Scott, 1983; Hay & Wadsworth, 1983; Moritoki *et al.*, 1987). In contrast, Zetler & Kaschube (1985) reported that nerve-mediated contractions of the mouse vas deferens elicited by high frequency field stimulation (trains of 0.03–0.8 ms pulses at 8–15 Hz for 1 s every 30 s) are depressed by verapamil. Indeed, when we applied trains of 0.5 ms pulses at 10 Hz for 0.5 s every 10 s, addition of verapamil only caused twitch inhibition (results not shown). Therefore, it appears that the twitch enhancing effect of verapamil only occurs at low frequencies of field stimulation.

Moritoki *et al.* (1987) suggested that, in the rat vas deferens, twitch potentiation by verapamil is mediated through α_2-adrenoceptors and binding of verapamil to α_2-adrenoceptors has been demonstrated (Van Meel *et al.*, 1981; Baranathan *et al.*, 1982). However it is difficult to envisage that the calcium antagonist elicits twitch potentiation by interaction with these receptors. In the mouse vas deferens, which appears to contain only prejunctional α_2-adrenoceptors (Baker & Marshall, 1983), field stimulation-evoked contractions are inhibited by the

α_2-adrenoceptor agonist clonidine and this effect is antagonized by yohimbine (Rae & De Moraes, 1984). In the present study, yohimbine failed to modify basal twitch tension but antagonized the potentiating effect of verapamil. Since clonidine and verapamil promote opposite yohimbine-sensitive effects on twitch tension, it seems likely that yohimbine antagonized verapamil-induced twitch potentiation through mechanisms independent of α_2-adrenoceptors.

Motor innervation of the mouse vas deferens appears to consist mainly, if not exclusively (Jones & Spriggs, 1975), of sympathetic neurones which release noradrenaline, ATP and neuropeptide Y (Stjärne & Lundberg, 1986; Forsyth & Pollock, 1988). In this preparation, exogenous administration of these co-transmitters each produces a distinct pattern of contraction (Stjärne *et al.*, 1986). Verapamil enhances the release of [^{3}H]-noradrenaline from sympathetic neurones in rat and guinea-pig vasa deferentia and in the aorta and tail artery of the rat (Karaki *et al.*, 1984; Zsóter *et al.*, 1984; Beattie *et al.*, 1986; Moritoki *et al.*, 1987). In the present study, however, the selective α_1-adrenoceptor antagonist prazosin (1 μM) failed to modify basal twitch tension or the twitch enhancing effect of verapamil. Moreover, verapamil markedly inhibited contractions induced by exogenous maximally effective concentrations of noradrenaline and acetylcholine. Taken together, these findings would indicate that twitches evoked by field stimulation at 0.1 Hz are due mainly to the action of a non-adrenergic transmitter, as suggested by Jenkins *et al.* (1975) and Marshall *et al.* (1978), and that verapamil acts to enhance the release of this transmitter. It is also tempting to speculate that ATP is the neurotransmitter preferentially released by field stimulation at low frequency, since this substance induces twitch-like phasic contractions of the mouse vas deferens, contrasting with the slowly developing sustained contractions caused by neuropeptide Y (Stjärne *et al.*, 1986).

Although verapamil and Bay K 8644 both increased twitch tension, there is evidence to suggest that different mechanisms underlie the effects of these drugs. Yohimbine, which antagonized twitch potentiation induced by verapamil, did not affect the response to Bay K 8644. Pre-incubation with a maximal inhibitory concentration of nifedipine (1 μM) abolished the twitch-enhancing effect of verapamil but did not modify the potency of or maximal potentiation induced by Bay K 8644. Conversely, nifedipine was equally effective in inhibiting basal or verapamil (30 μM)-potentiated twitches, but was 120 fold less potent against twitches maximally potentiated by Bay K 8644 (1 μM).

The observation that pre-incubation with Bay K 8644 antagonized nifedipine in a competitive manner whereas no antagonism occurred in the reverse condition deserved some consideration. In depolarized smooth muscle preparations contracted with calcium, competitive interaction between these dihydropyridines has been reported to occur both ways (Spedding & Berg, 1984; Spedding, 1985). Perhaps such differences are related to the lack of a persistent depolarized state in the present experiments (Sanguinetti & Kass, 1984; Middlemiss & Spedding, 1985).

One of the more interesting findings of the present study was that preincubation with verapamil (30 μM) markedly potentiated the twitch-enhancing effect of Bay K 8644, i.e. the concentration-response curve to Bay K 8644 was shifted to the left and its EC_{50} reduced 60 fold. Therefore, the effects produced by the combination of both compounds was clearly greater than that expected by summation of the effects produced by each drug alone. This result suggests that verapamil and Bay K 8644 enhance twitches through different mechanisms. When the reverse experiments were performed i.e. the preparation was pre-incubated with a low concentration of Bay K 8644 (1 nM, but not 10 nM) and then exposed to verapamil, a significant but less marked synergism was observed (verapamil EC_{50} reduced 3 fold). However, the maximal response observed when Bay K 8644 (1 nM) and verapamil (30 μM) were combined was the same, irrespective of the sequence of drug administration, and did not differ significantly from that caused by Bay K 8644 alone at a concentration of 1 μM.

Together, these results indicate that verapamil potentiates twitches evoked by low frequency field stimulation of the mouse vas deferens through a mechanism(s) distinct from that of Bay K 8644. Moreover, it is suggested that, in this preparation, twitch potentiation by verapamil may reflect a prejunctional action leading mainly to enhanced release of a non-adrenergic co-transmitter.

We are grateful to Bayer A.G. for the gift of Bay K 8644, to Mrs Elza A. Bernardini Ramos for technical assistance, to Mrs Mariza M. Ciarallo and to Elizabete Ramos Canzer for secretarial assistance and to Paulo C.A. Neves for graphical work. This study was supported by grants from CNPq and FINEP.

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(Received January 15, 1988
Revised September 19, 1988
Accepted September 29, 1988)