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Selectivity of Ca^{2+} channel blockers in inhibiting muscular and nerve activities in isolated colon

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1 Potency and efficacy of nifedipine, verapamil and diltiazem and of Bay K 8644 in modifying propulsion and nerve or smooth muscle activities have been compared in the guinea-pig isolated distal colon. Both the neuronal and muscular effects of Ca^{2+} channel blockers seem to develop at concentrations that are devoid of any significant effect apart from that on Ca^{2+} channels.

2 Nifedipine, verapamil and diltiazem were all able to impair propulsion, resting and stimulated acetylcholine (ACh) release and smooth muscle contractility in a concentration-dependent way. However, some degree of selectivity for neuronal and muscular effects could be observed. Nifedipine was more than 500 fold more potent than verapamil in relaxing musculature but less than twice as potent in reducing ACh release. On the other hand, verapamil was the most efficacious Ca^{2+} channel blocker tested in inhibiting ACh release, its effects being inversely correlated to the external Ca^{2+} concentration, and completely abolished by Bay K 8644.

3 By comparing the potencies exhibited by each drug against peristaltic reflex, smooth muscle contractility and ACh release, verapamil proved to be almost as potent in slowing the peristaltic reflex as in reducing ACh release, while nifedipine was about 100 fold more potent against the peristaltic reflex than against ACh release, but nearly equal against the peristaltic reflex and smooth muscle tone. Therefore, interference with cholinergic neurotransmission is likely to play a major role in the antipropulsive effect of verapamil, while peristaltic reflex impairment by nifedipine is likely to be dependent on inhibition of smooth muscle.

4 A facilitatory effect of Bay K 8644 on both the efficiency of the peristaltic reflex and the non-adrenergic, non-cholinergic (NANC) nerve-mediated relaxation could be observed at concentrations at least 10 fold lower than those required to affect ACh release or smooth muscle.

5 It is concluded that the effects of Ca^{2+} channel blockers on neurotransmitter release may be relevant to their effects on the gastrointestinal motor function.

Introduction

There is growing evidence that the high affinity binding sites for organic Ca^{2+} channel blockers, which are known to be present in high concentrations in gastrointestinal neuromuscular tissues, are associated with functional calcium channels. Indeed, a good correlation between functional effects and competition for nitrendipine binding sites has been shown for a series of dihydropyridine analogues (Bolger *et al.*, 1983; Godfraind & Wibo, 1985). However, although Ca^{2+} channel blockers are reported to be at least as potent in inhibiting intestinal smooth muscle contractility as they are in relaxing small blood vessels (Rosenberg *et al.*, 1979; Barone *et al.*, 1986; Godfraind *et al.*, 1986; Yousif & Triggle, 1986), at present there seems to be little scope for the currently available drugs of this class to be employed in motor disorders of the gastrointestinal tract (Traube & McCallum, 1984; Castell, 1985; Prior *et al.*, 1987). Nevertheless, constipation is not infrequently associated with long term treatment with verapamil (Hedner, 1986).

It is well known that in the gastrointestinal tract most myogenic and nerve-sustained motor activity show a high degree of Ca^{2+} dependence (Wood, 1987). Synaptic events not dependent on external Ca^{2+} seem not to play a significant role in sustaining neurotransmission in the enteric plexuses, as suggested by the strict dependence of both neurotransmitter release and neuronal excitation on Ca^{2+} entry (Alberts & Stjarne, 1982; Yokoyama *et al.*, 1986; Wood, 1987). However, only recently has the functional role of drug-sensitive Ca^{2+} channels at the level of enteric neuronal synapses and neuromuscular junctions received attention (Bornstein *et al.*, 1985; Miller *et al.*, 1988; Katsuragi *et al.*, 1990).

In the present study the effects of drugs, representative of the major classes of L-type Ca^{2+} channel blockers, were investigated for their ability to modify some neuromuscular functions in the distal portion of the guinea-pig large intestine.

Methods

Experiments were carried out in the guinea-pig isolated distal colon. The capability of Ca^{2+} channel blockers and of the Ca^{2+} channel activator Bay K 8644 to interfere with responses related to enteric neurone activation was studied by measuring peristaltic reflex, non-adrenergic, non-cholinergic (NANC) nerve-mediated relaxation of the longitudinal muscle in response to transmural stimulation (TS) and resting and stimulated acetylcholine (ACh) release. Moreover, the ability of drugs to modify circular muscle resting tone and contractility was also assessed.

Isolated specimens of terminal colon 5–7 cm long were taken from male guinea-pigs weighing 300–400 g, mounted in organ baths perfused with Tyrode solution under a tension of 0.5–1.5 g through an isotonic force transducer.

The peristaltic reflex was elicited by a radial distension of the lumen applied at the proximal end of the specimens by means of an intraluminal balloon. Longitudinal muscle movements and displacement of the balloon towards the distal end of the colon were recorded. Since the velocity of propulsion is dependent on the degree of distension (Frigo & Lecchini, 1970), supramaximal stimulation was employed and the maximal velocity of propulsion taken as a measure of the efficiency of the peristaltic reflex. Changes in peristaltic action are indicated as the percentage variation of the propulsion velocity with respect to the control value and were estimated as described by Frigo *et al.* (1984).

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Resting and stimulated ACh release was measured by incubating the specimens in a 3 ml organ bath with physostigmine sulphate (1.5×10^{-5} M) during 20 min collection periods. Samples were obtained during resting and stimulation periods in a sequence similar to that described by Lecchini *et al.* (1969). Stimulation-induced release was obtained by applying rectangular pulses of supramaximal strength, 1 ms duration and 1 Hz frequency delivered by coaxial silver wire electrodes for the first 10 min in a way similar to that described by Frigo *et al.* (1984). The 1 Hz frequency of stimulation was chosen because of its relative selectivity in stimulating the enteric cholinergic neurones (Cowie *et al.*, 1978). ACh was assayed on the guinea-pig isolated ileum incubated with morphine sulphate (6.6×10^{-6} M) and physostigmine sulphate (7.7×10^{-9} M) by the method of Paton & Vizi (1969). To allow for any effects that modifying drugs might have on the ACh assay, the concentrations of such drugs in the test samples were duplicated in the standard ACh solutions during the assay. The effects of compounds on ACh release were expressed as percentage variations with respect to the control level.

Transmural stimulation was used to excite NANC intrinsic nerves by delivering trains of rectangular pulses of supramaximal strength, 0.5 ms duration and frequencies ranging from 0.2 to 2 Hz for 20 s through coaxial silver wire electrodes to specimens pretreated with hyoscine sulphate (3×10^{-6} M) and guanethidine sulphate (3×10^{-6} M). Suppression by tetrodotoxin (TTX, 5×10^{-7} M) was used to check that NANC relaxation was entirely neurogenic. Electrical stimulations were applied at 10 min intervals and inhibitory responses of the longitudinal muscle indicated as percentage of the maximal responses. Maximal response was the greatest response obtained independent of the frequency employed. Frequency-response relationships for TS-induced NANC nerve-mediated relaxations were obtained over the range of 0.2–1 Hz. The effect of Ca^{2+} channel blockers used at the highest concentrations that were ineffective on longitudinal muscle resting tone was tested on frequency-response relationships.

Circular muscle activity was measured in isolated strips of circular muscle deprived of the mucosal layer and connected to an isotonic force transducer under a tension of 0.5–1 g. The basal tension chosen during the experiments was that which allowed rhythmic activity to develop after setting up the preparation. The tone of the circular muscle was fairly constant during the experiment. In any case tension variations such as those produced mechanically by changing the load applied to the preparation did not significantly change the amplitude of carbachol- or $BaCl_2$-induced contractions. In order to exclude any neurogenic component in the drug-induced variation of resting tone, the experiments were carried out in the presence of TTX (5×10^{-7} M). Maximal contraction to both $BaCl_2$ and carbachol were used to test the effect of both Bay K 8644 and Ca^{2+} channel blockers on muscular contractility. For this purpose, the concentrations of $BaCl_2$ and carbachol were in the range of $5–20 \times 10^{-7}$ M and $1–5 \times 10^{-4}$ M, respectively. The Ca^{2+} channel blocker-induced reduction and Bay K 8644-induced increase of circular muscle resting tone were measured as a fraction of the maximal relaxation induced by papaverine and as a fraction of carbachol-induced maximal contraction, respectively. The effects of compounds on carbachol- or $BaCl_2$-induced contractions were expressed as percentage variation with respect to the control value.

The effects of both Ca^{2+} channel blockers and of the Ca^{2+} channel activator Bay K 8644 were evaluated after a preincubation period of at least 20 min for each concentration of the compounds.

The Tyrode solution was of the following composition (mM): NaCl 136.9, KCl 2.7, $MgCl_2$ 1.04, $NaHCO_3$ 11.9, NaH_2PO_4 0.4 and glucose 5.5. Unless otherwise indicated, the experiments were carried out in solutions containing Ca^{2+} 1.8 mM.

The dependence of the efficiency of the peristaltic reflex, resting ACh release and NANC nerve-mediated relaxation on external Ca^{2+} was studied by changing the Ca^{2+} concentration in the Tyrode solution from 0 to 8 mM. The effect of each Ca^{2+} concentration was evaluated after 20 min incubation. Ca^{2+}-free solution was made by omitting $CaCl_2$. No osmolar compensation was made for Ca^{2+} modified solutions.

The drugs used were: acetylcholine chloride (ACh, Sigma); carbamylcholine chloride (carbachol, Sigma); diltiazem hydrochloride (Sigma); hyoscine sulphate (Sigma); guanethidine sulphate (Ciba); morphine hydrochloride (SIFAC); papaverine hydrochloride (Sigma); physostigmine sulphate (Sigma); tetrodotoxin (TTX, Sankyo); yohimbine hydrochloride (Sigma). Nifedipine and Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) were kindly supplied by Dr Thomas (Bayer, Milano) and (±)-verapamil hydrochloride by Knoll (Italia). Dihydropyridines were dissolved in Tween 80 prior to dilution in distilled water. All solutions were protected from light and experiments performed under yellow light.

Concentration-effect relationships were obtained in order to calculate the concentrations able to produce half maximum effects (EC_{50}) and the 30% or 50% inhibitory concentrations (IC_{30} or IC_{50}). Linear regression analysis was performed and potency ratios calculated according to Finney (1978).

For each Ca^{2+} channel blocker, the ratio of the potencies exhibited in inhibiting circular muscle tone versus resting ACh release, as derived from the analysis of log concentration-response relationships, was calculated and indicated as a muscular versus neuronal selectivity index.

Antagonism by Bay K 8644 of nifedipine, verapamil and diltiazem effects was studied according to the method of Arunlakshana & Schild (1959).

Statistical significance of differences between groups was analyzed by applying Student's *t* test for unpaired data.

Results

Ca^{2+}-dependence of acetylcholine release and nerve-mediated motor activities

The peristaltic reflex, resting ACh release and NANC nerve-mediated relaxation of longitudinal muscle were dependent on the external Ca^{2+} concentration. In effect, although a small fraction of the ACh release and NANC relaxation still persisted in Ca^{2+}-free solution, a Ca^{2+} concentration-related increase of the velocity of propulsion, the resting ACh release and the amplitude of NANC relaxation was observed in the concentration range of 0–1.8 mM (Figure 1). All the parameters considered reached a maximum in the concentration range of 1.5–2 mM Ca^{2+}, while the peristaltic reflex and ACh release rapidly declined above 4 mM Ca^{2+}.

Effects of the Ca^{2+} channel activator Bay K 8644

Opposite effects of Bay K 8644 on both the peristaltic reflex and the amplitude of TS-induced NANC relaxation could be observed depending on the concentration employed. A slight but significant increase in the peristaltic reflex and in the amplitude of NANC relaxation, reaching a maximum of 20 and 33% of the controls, respectively, could be observed in the concentration-range of $0.8–8 \times 10^{-9}$ M (Figures 2 and 3). Propulsion was reduced by a concentration of 3×10^{-8} M and was prevented by concentrations of 3×10^{-6} M. Concentrations of Bay K 8644 able to facilitate both peristaltic reflex and NANC relaxation were at least 10 fold lower than the concentrations required to affect both circular and longitudinal smooth muscle.

In fact, Bay K 8644 ($3 \times 10^{-8}–3 \times 10^{-6}$ M) increased in a concentration-dependent manner both the circular muscle resting tone and the contractions in response to $BaCl_2$ and carbachol. The average maximum increase of the maximal $BaCl_2$- and carbachol-induced contractions were 61.2 ± 8.6 and 18.4 ± 1.9 (mean ± s.e.mean, $n = 5$), respectively.

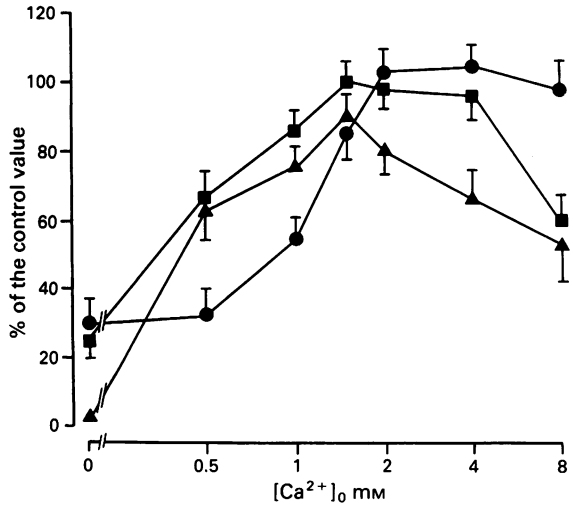

Figure 1 Ca^{2+}-dependence of some nerve-mediated functions in guinea-pig isolated colon. Effect of varying external Ca^{2+} concentration on propulsion velocity (▲); resting acetylcholine release (■); amplitude of TS-induced NANC relaxation of longitudinal muscle (0.5 Hz, supramaximal strength) (●). Values are expressed as percentages of the controls and are plotted against log Ca^{2+} concentration. Controls are considered the values obtained in standard Tyrode solution containing 1.8 mM Ca^{2+}. Each value represents the mean of 5 experiments. Vertical bars indicate s.e.mean.

Bay K 8644 produced a concentration-dependent increase of resting ACh release in the concentration range of 8×10^{-8}–3×10^{-6} M. The calculated EC_{50} value (with 95% confidence limits) was 2.01 (1.20–3.35) $\times 10^{-7}$ M. The Bay K 8644-induced increase of ACh release could be antagonized by verapamil and nifedipine. The reduction of the Bay K 8644-induced increase of ACh release was linearly related to concentration only for nifedipine (Figure 4).

Effects of Ca^{2+} channel blockers

Peristaltic reflex Ca^{2+} channel blockers were able to impair propulsive activity in a concentration-dependent manner. Log concentration-response relationships of nifedipine, verapamil and diltiazem in reducing the peristaltic reflex and the effect of

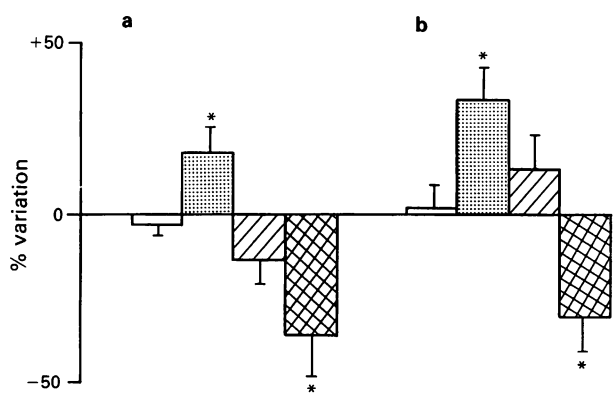

Figure 2 Bay K 8644-induced changes of the NANC nerve-mediated inhibition of longitudinal muscle and of the peristaltic reflex in guinea-pig isolated colon. Columns represent the percentage variation of the velocity of propulsion (a) and of the TS-induced NANC relaxation (2 Hz, supramaximal strength) (b) with respect to the control values in the presence of Bay K 8644: 3×10^{-10} M (open columns); 3×10^{-9} M (stippled columns); 3×10^{-8} M (hatched columns); 3×10^{-7} M (cross-hatched columns). Each column represents the mean of 5 experiments. Vertical bars indicate s.e.mean. * Significantly different from the control value ($P < 0.05$).

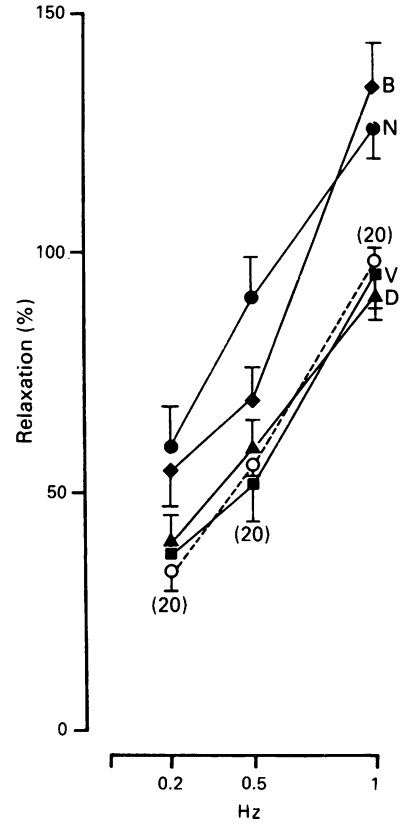

Figure 3 Effect of Ca^{2+} channel blockers and Bay K 8644 on NANC nerve-mediated relaxation of longitudinal muscle in guinea-pig isolated colon. Log frequency-response relationships for TS-induced NANC relaxation in the absence (○) and in the presence of nifedipine (N) 3×10^{-8} M (●); verapamil (V) 7×10^{-7} M (■); diltiazem (D) 7×10^{-7} M (▲) or Bay K 8644 (B) 3×10^{-9} M (◆). Values on the ordinate scale are expressed as percentage of the maximal response. Unless otherwise indicated, each point represents the mean of 5 experiments. Vertical bars indicate s.e.mean. For each Ca^{2+} channel blocker, the concentration chosen was devoid of any effect on muscle tone.

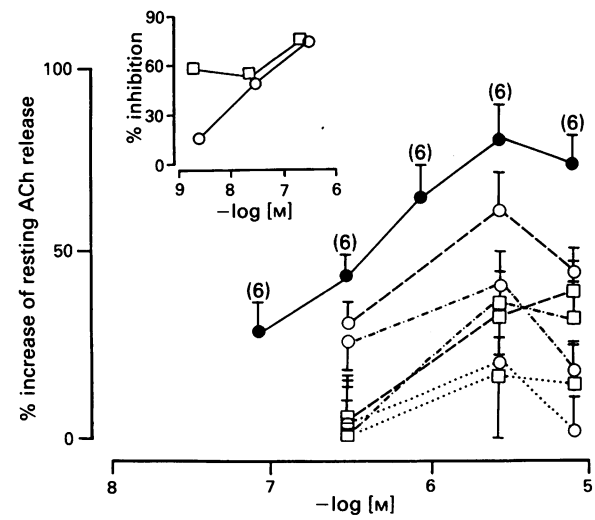

Figure 4 Bay K 8644-induced increase of resting acetylcholine (ACh) release in guinea-pig isolated colon and its modification by organic Ca^{2+} channel blockers. Log concentration-response relationships for Bay K 8644 (●——●) and for Bay K 8644 plus nifedipine (○) or verapamil (□) at the concentrations of 3×10^{-9} (---), 3×10^{-8} (-·-·) and 3×10^{-7} (·····) M in increasing resting acetylcholine release. Log concentrations are plotted against percentage increase of acetylcholine release. Unless otherwise indicated, each point represents the mean of 4 experiments. Vertical bars indicate s.e.mean. The concentration dependence of the nifedipine but not of the verapamil effect is shown by plotting concentration against percentage reduction of Bay K 8644 maximally stimulated acetylcholine release (inset); (○) $r = 0.99$; (□) r = p.75 (NS).

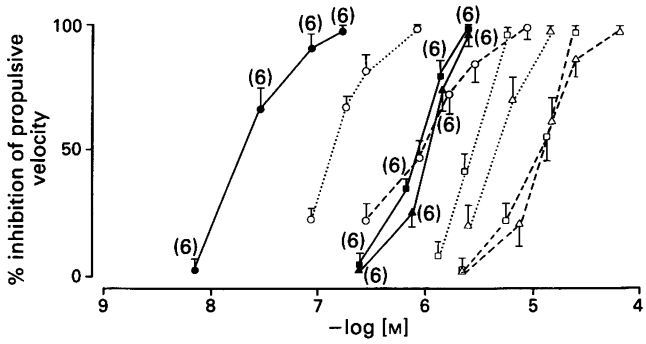

Figure 5 Bay K 8644 antagonism towards Ca^{2+} channel blocker-induced inhibition of peristaltic reflex in guinea-pig isolated colon. Log concentrations of Ca^{2+} channel blockers are plotted against percentage inhibition of velocity of propulsion. Log concentration-response relationships were obtained for nifedipine (●), verapamil (■) and diltiazem (▲) and for nifedipine (○), verapamil (□) and diltiazem (△) plus Bay K 8644 at the concentrations of 3×10^{-8} (·····) and 3×10^{-7} (– – –) M. Unless otherwise indicated, each point represents the mean of 4 experiments. Vertical bars indicate s.e.mean.

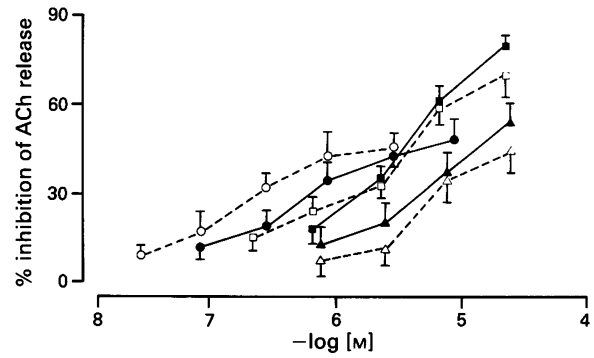

Figure 6 Log concentration response relationships of Ca^{2+} channel blockers in reducing acetylcholine (ACh) release from guinea-pig isolated colon. Log concentrations of nifedipine (●, ○), verapamil (■, □) and diltiazem (▲, △) are plotted against percentage inhibition of acetylcholine release during rest (——) and during TS stimulation (1 Hz, supramaximal strength) (– – –). Each point represents the mean of 6 experiments. Vertical bars indicate s.e.mean.

Bay K 8644 are shown in Figure 5. The IC_{50} values with 95% confidence limits were 2.70 (1.10–6.64) $\times 10^{-8}$ M for nifedipine, 7.04 (4.76–10.42) $\times 10^{-7}$ M for verapamil and 8.50 (4.95–14.95) $\times 10^{-7}$ M for diltiazem, the corresponding potency ratios being: nifedipine (1) > verapamil (0.038) ⩾ diltiazem (0.031). Higher concentrations were required to prevent the onset of the peristaltic reflex. The average blocking concentrations (± s.e.mean) were 8.48 (± 0.24) $\times 10^{-8}$ M for nifedipine, 1.53 (± 0.26) $\times 10^{-6}$ M for verapamil and 2.08 (± 0.21) $\times 10^{-6}$ M for diltiazem. In the presence of blocking concentrations of Ca^{2+} channel blockers the peristaltic reflex could be completely restored by increasing the Ca^{2+} concentration to 6 mM. The effect of nifedipine was antagonized in an apparently competitive way by Bay K 8644, the apparent pA_2 value being 8.22. Bay K 8644 did not fit the requirements for competitive antagonism against verapamil and diltiazem, the slopes of Schild plots being 0.44 and 0.63, respectively.

Acetylcholine release Ca^{2+} channel blockers were found to reduce both resting and TS-induced ACh release in a concentration-dependent manner. The corresponding log concentration-response relationships are depicted in Figure 6. The calculated IC_{30} values (with 95% confidence limits) were 6.51 (3.50–12.13) $\times 10^{-7}$ M for nifedipine, 1.40 (0.97–2.02) $\times 10^{-6}$ M for verapamil and 3.82 (2.05–7.24) $\times 10^{-6}$ M for diltiazem in inhibiting resting ACh release and 2.53 (1.42–4.51) $\times 10^{-7}$ M for nifedipine, 1.53 (0.82–2.84) $\times 10^{-6}$ M for verapamil and 5.13 (2.29–11.50) $\times 10^{-6}$ M for diltiazem in inhibiting TS-induced ACh release. The corresponding potency ratios were: nifedipine (1) ⩾ verapamil (0.47) > diltiazem (0.17); and nifedipine (1) > verapamil (0.16) ⩾ diltiazem (0.049), respectively. Nifedipine was significantly more potent against TS-induced than resting ACh release, the corresponding IC_{30} potency ratio of resting against stimulated ACh release being 2.57. Nifedipine although more potent, was much less efficacious than verapamil. The potency of verapamil was strongly dependent on changes in the external Ca^{2+} concentration. Ca^{2+}-related changes of log concentration-response relationships of verapamil in reducing resting ACh release are shown in Figure 7. Verapamil (6×10^{-6} M)-induced inhibition of resting ACh release was abolished by pretreatment with Bay K 8644 (3×10^{-6} M), but unaffected by pretreatment with yohimbine (1×10^{-6} M).

NANC nerve-mediated relaxation The frequency-response relationships for NANC inhibitory responses in the presence of different Ca^{2+} channel blockers at concentrations insufficient to modify smooth muscle tone but effective in inhibiting

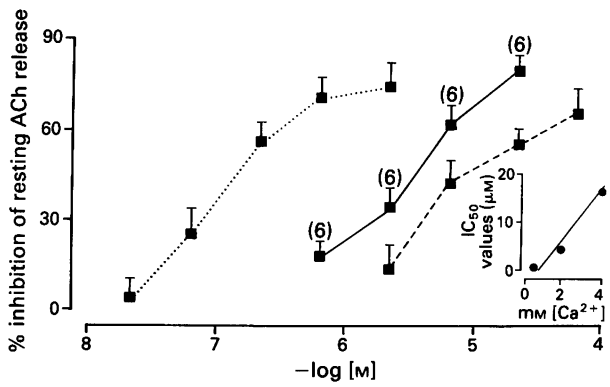

Figure 7 Ca^{2+}-dependence of verapamil-induced inhibition of acetylcholine (ACh) release from guinea-pig isolated colon. Log concentration-response relationships were obtained in organs perfused with Tyrode solution containing 0.5 mM Ca^{2+} (·····); 1.8 mM Ca^{2+} (——); 4 mM Ca^{2+} (– – –). Log concentrations of verapamil are plotted against percentage inhibition of resting acetylcholine release. Unless otherwise indicated, each point represents the mean of 4 experiments. Vertical bars indicate s.e.mean. A significant correlation can be observed between external Ca^{2+} concentration and IC_{50} values of verapamil (inset).

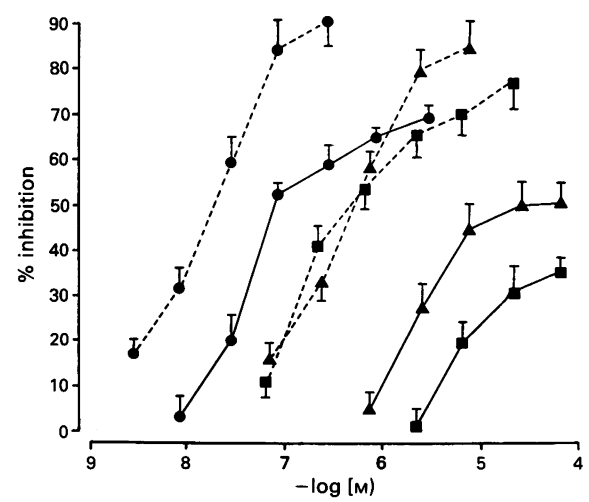

Figure 8 Log concentration-response relationships of Ca^{2+} channel blockers in inhibiting circular muscle contractility in guinea-pig isolated colon. Log concentrations of nifedipine (●); verapamil (■); diltiazem (▲) are plotted against percentage inhibition of contraction elicited by carbachol at supramaximal concentration (– – –) and the fractional tone inhibition (——) in respect to the maximal relaxation produced by papaverine. Each point represents the mean of 5 experiments. Vertical bars indicate s.e.mean.

Table 1 Ratios of the potencies calculated for each Ca^{2+} channel blocker in inhibiting propulsion, acetylcholine (ACh) release and smooth muscle contractility in guinea-pig isolated colon, and muscular versus neuronal selectivity indices, calculated from the analysis of log concentration-response relationships

	Propulsion versus resting ACh release	*Propulsion versus circular muscle tone*	*Carbachol-induced contraction versus resting ACh release*	*Selectivity index**
Nifedipine	107.82 (82.24–141.35)†	3.59 (2.55–5.05)	229.24 (167.22–314.26)	22.69 (14.99–34.35)
Verapamil	5.96 (4.97–7.16)	71.41 (57.51–88.67)	4.51 (3.23–6.30)	0.039 (0.025–0.061)
Diltiazem	9.94 (7.77–12.72)	9.45 (7.30–12.23)	28.83 (20.93–39.71)	1.09 (0.69–1.71)

* Selectivity index for each Ca^{2+} channel blocker was calculated from the log concentration-response relationships in inhibiting circular muscle tone versus resting ACh release.
† (95% confidence limits)

peristaltic reflex are represented in Figure 3. The dihydropyridines nifedipine and Bay K 8644 were both able to increase NANC relaxation, the effect being independent of the frequency employed, while verapamil and diltiazem had no effect.

Circular muscle resting tone and contractility Log concentration-response relationships of nifedipine, verapamil and diltiazem in inhibiting the resting tone and carbachol-induced contraction of circular muscle are shown in Figure 8. All drugs exhibited higher potency and efficacy against contracted than against resting musculature. This feature was more evident for verapamil than for nifedipine. Potency ratios for the inhibition of resting tone and carbachol-induced contraction as derived from IC_{50} or IC_{30} values were: nifedipine (1) > diltiazem (0.012) > verapamil (0.001); and nifedipine (1) > diltiazem (0.034) ≥ verapamil (0.022), respectively.

Discussion

It is well documented that several types of Ca^{2+} channels with some tissue specificity are present in excitable cells, which are believed to contribute to the differences in sensitivity to Ca^{2+} channel blockers exhibited by nervous and muscular tissues (Lee & Tsien, 1983; Hurwitz, 1986; Callewaert *et al.*, 1989). The physiological relevance of dihydropyridine binding sites in neuronal cells has long been questioned (Miller & Freedman, 1984). However, data have recently accumulated suggesting a functional role for drug-sensitive Ca^{2+} channels both in the central and in the peripheral nervous systems (Middlemiss & Spedding, 1985; Turner & Goldin, 1985; Thayer *et al.*, 1986; Miller, 1987; Hirning *et al.*, 1988; Tsien *et al.*, 1988; Miller *et al.*, 1988). Under certain conditions sensitivity of cholinergic as well as adrenergic and glutamatergic neurones to Ca^{2+} channel blockers of different classes has been reported (Starke *et al.*, 1984; Nordstrom *et al.*, 1986; Bartfai & Vizi, 1986). As far as the enteric nervous system is concerned, most neurophysiological studies indicate a low sensitivity of intramural nerves to Ca^{2+} channel blockers (Wood, 1987), although cholinergic and NANC inhibitory neurones have recently been reported to be modulated by dihydropyridines (Bornstein *et al.*, 1985; Katsuragi *et al.*, 1990). On the other hand, although spontaneous myoelectrical and mechanical activities can hardly be affected even by high concentrations of organic Ca^{2+} channel blockers, inhibition of intestinal smooth muscle has been reported to occur at subnanomolar concentrations during K^+ or receptor-mediated excitation (Rosenberg *et al.*, 1979; Golenhofen, 1981; Barone *et al.*, 1986; Terada *et al.*, 1987). However, to what extent smooth muscle inhibition or impairment of neurotransmission in the enteric plexuses could contribute to the end effect of Ca^{2+} channel blockers on intestinal complex motor function (e.g. propulsive activity) remains to be determined. Peristaltic reflex in the isolated intestine is a sensitive and reliable method to assess the viability of enteric neuronal synapses during activation by a physiological stimulus, and ACh release and NANC responses reflect the activity of functionally relevant enteric neurone subpopulations.

In order to interpret the mechanism of Ca^{2+} channel blocker motor inhibitory effects, it is worth noting that in our experiments substances supposed to act by opening or blocking Ca^{2+} channels were able to affect in opposite directions release of ACh from enteric neurones in a concentration-dependent manner. However, a unique profile for each Ca^{2+} channel blocker emerged when the potencies exhibited by each substance against peristaltic reflex, muscle contractility and ACh release were compared (Table 1). Indeed, the muscular versus neuronal selectivity index was about 23 for nifedipine and 0.04 for verapamil. While verapamil proved to be nearly as potent in slowing the peristaltic reflex as it was in reducing ACh release, the potency of nifedipine was about 100 fold higher against the peristaltic reflex than against ACh release, but nearly equal against peristaltic reflex and smooth muscle tone. This is highly suggestive of a substantial contribution of cholinergic neuronal inhibition to the impairment of the peristaltic reflex in the case of verapamil, while it contributes little to the end effect of nifedipine. Whatever the neural or muscular mechanisms responsible for the Ca^{2+} channel blocker-induced inhibition of peristaltic reflex, their effects, which can be easily suppressed both by increasing Ca^{2+} concentration or by Bay K 8644, are likely to be entirely dependent on interference with Ca^{2+} channels. However, the lack of competitive antagonism by Bay K 8644 of the inhibitory effects of verapamil and diltiazem on the peristaltic reflex seems to exclude competition for the same binding site and is compatible with a widely accepted model assuming an interaction with different binding sites on the same channel (Glossmann *et al.*, 1983).

Although Ca^{2+} channel blockers showed similar orders of potency to inhibitors of muscular and of nerve-mediated effects, the highest potency being exhibited in both cases by nifedipine, the ratios of the potencies were markedly different. In fact nifedipine, which was more than 500 fold more potent than verapamil in relaxing musculature, was less than twice as potent as verapamil in reducing ACh release.

Circular muscle sensitivity to verapamil was strongly dependent on the degree of excitation, a feature which occurred only to a minor extent with nifedipine. Indeed, the ratio of potencies in inhibiting carbachol-induced contraction and in relaxing resting circular muscle was about 260 for verapamil and only 7.19 for nifedipine.

The question can be raised as to whether the effect of verapamil on enteric cholinergic neurones is entirely dependent on Ca^{2+} channel blockade. In fact, verapamil is well known to bind to several neurotransmitter receptors, such as α_1- and α_2-adrenoceptors, muscarinic, and opioid receptors, and to block Na^+ channels besides Ca^{2+} channels (see Janis & Scriabine, 1983). All these properties could be responsible for the effects of verapamil on cholinergic neurotransmission. However, the low concentrations able to affect ACh release (see the concentration-response curve at 0.5 mM external Ca^{2+}) and the antagonism both by external Ca^{2+} and Bay K 8644 are all in favour of a selective action of verapamil on neuronal Ca^{2+} channels in our preparation.

The muscular and neuronal effects of Bay K 8644 could also help in interpreting the role and location of Ca^{2+} channels on the intestinal structures. Our experiments are suggestive of a correlation between the improvement of peristaltic reflex efficiency and the facilitatory effect on NANC neurotransmission, which both appear at concentrations far below those required to enhance either contractility or ACh release. A similar facilitatory effect of nifedipine on NANC inhibition, possibly due to a partial agonist activity (Glossmann *et al.*, 1985), has been reported previously in the ileum (Bornstein *et al.*, 1985).

In conclusion, by assuming all the effects of Ca^{2+} channel blockers observed in our preparations result from an interference with Ca^{2+} channels, the following hypotheses can be advanced: (a) Drug-sensitive Ca^{2+} channels are likely to be present on neurones of the enteric plexuses and to be involved in the modulation of both ACh release and NANC inhibition; (b) the Ca^{2+} channels modulating resting ACh release seem to be more efficiently blocked by verapamil-like drugs. However, dihydropyridine sensitive Ca^{2+} channels appear to be involved for a significant fraction (about 50%) of ACh released at rest unless this release is secondary to stretch caused by spontaneous muscle contractions. This fraction increased during Bay K 8644 induced excitation (up to 80%). (c) Ca^{2+} channels located on NANC neurones seem to exhibit the highest sensitivity for dihydropyridine moieties.

Impairment of peristaltic activity could result from blockade of either neuronal or muscular Ca^{2+} channels. While all Ca^{2+} channel blockers were more potent against propulsion than in inhibiting ACh release or the smooth muscle directly, they showed different nervous-muscular activity ratios. The spectrum of activities ranges from the high muscular-low neuronal profile of nifedipine to the low muscular-high neuronal profile of verapamil, diltiazem standing in an intermediate position.

Due to the high sensitivity of enteric neurones to Ca^{2+} channel blockers, in our opinion the neurotropic intestinal effects deserve to be investigated in the search for moieties with higher selectivity for the gastrointestinal tract.

References

ALBERTS, P. & STJARNE, L. (1982). Role of calcium in muscarinic autoinhibition of ^{3}H-acetylcholine secretion in guinea-pig ileum myenteric plexus. *Acta Physiol. Scand.*, **115**, 487–491.

ARUNLAKSHANA, O. & SCHILD, H.P. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BARONE, F.C., WHITE, R.F., ORMSBEE, H.S. & WASSERMAN, M.A. (1986). Effects of calcium channel entry blockers, nifedipine and nivaldipine, on colonic motor activity. *J. Pharmacol. Exp. Ther.*, **237**, 99–106.

BARTFAI, T. & VIZI, E.S. (1986). Prevention by nimodipine, a calcium entry blocker, of the effect of α-adrenoceptor blocking agents on noradrenaline release: differential effects of nimodipine on (^{3}H)noradrenaline and (^{14}C)acetylcholine release measured concomitantly from the guinea-pig ileum. *Arch. Int. Pharmacodyn.*, **284**, 212–224.

BOLGER, G.T., GENDO, P., KLOCKOWSKI, R., SIEGEL, H., JANIS, R.A., TRIGGLE, A.M. & TRIGGLE, D.J. (1983). Characterization of binding of the Ca^{++} channel antagonist, ^{3}H nitrendipine, to guinea-pig ileal smooth muscle. *J. Pharmacol. Exp. Ther.*, **225**, 291–309.

BORNSTEIN, J.C., LEES, G.M. & McBAIN, C.J. (1985). Comparative effects of calcium antagonists on inhibitory junction potentials of circular muscle of guinea-pig ileum. *Br. J. Pharmacol.*, **86**, 724P.

CALLEWAERT, G., HANBAUER, I. & MORAD, M. (1989). Modulation of calcium channels in cardiac and neuronal cells by an endogenous peptide. *Science*, **243**, 663–666.

CASTELL, D.O. (1985). Calcium-channel blocking agents for gastrointestinal disorders. *Am. J. Cardiol.*, **55**, 210B–313B.

COWIE, A.L., KOSTERLITZ, H.W. & WATERFIELD, A.A. (1978). Factors influencing the release of acetylcholine from the myenteric plexus of the ileum of the guinea-pig and rabbit. *Br. J. Pharmacol.*, **64**, 565–580.

FINNEY, D.J. (1978). *Statistical Method in Biological Assay*. 3rd edn. London: Charles Griffin Ltd.

FRIGO, G.M. & LECCHINI, S. (1970). An improved method for studying the peristaltic reflex in the isolated colon. *Br. J. Pharmacol.*, **39**, 346–356.

FRIGO, G.M., LECCHINI, S., MARCOLI, M., TONINI, M., D'ANGELO, L. & CREMA, A. (1984). Changes in sensitivity to the inhibitory effects of adrenergic agonists on intestinal motor activity after chronic sympathetic denervation. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **325**, 145–152.

GLOSSMANN, H., FERRY, D.R., GOLL, A., STRIESSNIG, J. & ZERNIG, G. (1985). Calcium channels: introduction into their molecular pharmacology. In *Cardiovascular Effects of Dihydropyridine-type Calcium Antagonists and Agonists* ed. Fleckenstein, A., Van Breemer, C., Grob, R. & Hoffmeister, F., pp. 113–139. Berlin, Heidelberg: Springer Verlag.

GLOSSMANN, H., FERRY, D.R., LUBBECKE, F., MEWES, R. & HOFMANN, F. (1983). Identification of voltage operated calcium channels by binding studies: differentiation of subclasses of calcium antagonist drugs with 3H-nimodipine radioligand binding. *J. Recept. Res.*, **3**, 177–190.

GODFRAIND, T., MILLER, R. & WIBO, M. (1986). Calcium antagonisms and calcium entry blockade. *Pharmacol. Rev.*, **38**, 321–416.

GODFRAIND, T. & WIBO, M. (1985). Subcellular localization of [^{3}H]-nitrendipine binding sites in guinea-pig ileal smooth muscle. *Br. J. Pharmacol.*, **85**, 335–340.

GOLENHOFEN, K. (1981). Differentiation of calcium activation processes in smooth muscle using selective agonists. In *Smooth muscle: an Assessment of Current Knowledge*. pp. 157–170. ed. Bülbring, E., Brading, A.F., Jones, A.W. & Tomita, T. London: Arnold.

HEDNER, T. (1986). Calcium channel blockers: spectrum of side effects and drug interaction. *Acta Pharmacol. Toxicol.*, **58** (suppl 2), 119–136.

HIRNING, L.D., FOX, A.P., McCLESKEY, E.W., PLIVERA, B.M., THAYER, S.A., MILLER, R.J. & TSIEN, R.W. (1988). Dominant role of N-type Ca^{2+} channels in evoked release of norepinephrine from sympathetic neurons. *Science*, **239**, 57–61.

HURWITZ, L. (1986). Pharmacology of calcium channels and smooth muscle. *Annu. Rev. Pharmacol. Toxicol.*, **26**, 225–258.

JANIS, R.A. & SCRIABINE, A. (1983). Sites of action of Ca^{2+} channel inhibitors. *Biochem. Pharmacol.*, **32**, 3499–3507.

KATSURAGI, T., SHIRAKABE, K., OGAWA, S., SOEJIMA, O. & FURUKAWA, T. (1990). Involvement of dihydropyridine-sensitive Ca^{2+} channels in adenosine-evoked inhibition of acetylcholine release from guinea pig ileal preparation. *J. Neurochem.*, **55**, 363–369.

LECCHINI, S., DEL TACCA, M., SOLDANI, G., FRIGO, G.M. & CREMA, A. (1969). The actions of atropine, tropenziline and N-butyl hyosacine bromide on the isolated distal colon of guinea-pig: a comparison of their activities and mechanisms of action. *J. Pharm. Pharmacol.*, **21**, 662–667.

LEE, K.S. & TSIEN, R.W. (1983). Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendimine in single dialysed heart cells. *Nature*, **302**, 790–794.

MIDDLEMISS, D.N. & SPEDDING, M. (1985). A functional correlate for dihydropyridine binding site in rat brain. *Nature*, **314**, 94–96.

MILLER, R.J. (1987). Multiple calcium channels and neuronal function. *Science*, **235**, 46–52.

MILLER, R.J., EWALD, D.A., FOX, A.P., HIRNING, L.D., McCLESKEY, E.W., PERNEY, T.M., STUREK, M., THAYER, S.A., TSIEN, R.W. & WALKER, M.W. (1988). The effect of calcium channel antagonists on peripheral neurones. *Ann. N.Y. Acad. Sci.*, **521**, 269–277.

MILLER, R.J. & FREEDMAN, S.B. (1984). Are dihydropyridine binding sites voltage sensitive calcium channels? *Life Sci.*, **34**, 1205–1221.

NORDSTROM, O., BRAESCH-ANDERSEN, S. & BARTFAI, T. (1986). Dopamine release is enhanced while acetylcholine release is inhibited by nimodipine (Bay e 9736). *Acta Physiol. Scand.*, **126**, 115–119.

PATON, W.D.M. & VIZI, E.S. (1969). The inhibitory action of noradrenaline and adrenaline on acetylcholine output by guinea-pig longitudinal muscle strips. *Br. J. Pharmacol.*, **35**, 10–28.

PRIOR, A., HARRIS, S.H. & WHORWELL, P.J. (1987). Reduction of colonic motility by intravenous nicardipine in irritable bowel. *Gut*, **28**, 1609–1612.

ROSENBERG, L.B., TICKU, M.K. & TRIGGLE, D.J. (1979). The effects of Ca^{2+} movements in guinea-pig ileal longitudinal muscle. *Can. J. Physiol. Pharmacol.*, **57**, 333–347.

STARKE, K., SPATH, L. & WICHMANN, T. (1984). Effects of verapamil, diltiazem and ryosidine on the release of dopamine and acetylcholine in rabbit caudate nucleus slices. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **325**, 124–130.

TERADA, K., KITAMURA, K. & KURIYAMA, H. (1987). Blocking actions of Ca^{2+} antagonists on the Ca^{2+} channels in the smooth muscle cells membrane of rabbit small intestine. *Pflügers Arch.*, **408**, 552–557.

THAYER, S.A., MURPHY, S.N. & MILLER, R.J. (1986). Widespread distribution of dihydropyridine-sensitive calcium channels in the central nervous system. *Mol. Pharmacol.*, **30**, 505–509.

TRAUBE, M. & McCALLUM, R.W. (1984). Calcium-channel blockers and the gastrointestinal tract. *Am. J. Gastroenterol.*, **79**, 892–896.

TSIEN, R.W., LIPSCOMBE, D., MADISON, D.V., BLEY, K.R. & FOX, A.P. (1988). Multiple types of neuronal calcium channel and their selective modulation. *Trends Neurosci.*, **11**, 431–437.

TURNER, T.J. & GOLDIN, S.M. (1985). Calcium channels in rat brain synaptosomes: identification and pharmacological characterization. *J. Neurosci.*, **5**, 841–849.

WOOD, J.D. (1987). Physiology of the enteric nervous system. In *Physiology of the Gastrointestinal Tract*, ed. Johnson, L.R. pp. 67–109. New York: Raven Press.

YOKOYAMA, K., SHIMIZU, M. & YAGASAKI, O. (1986). Effect of external Ca^{2+} on the spontaneous and the various stimuli-induced acetylcholine release from guinea-pig ileum myenteric plexus. *Jpn. J. Pharmacol.*, **40**, 194–198.

YOUSIF, F.B. & TRIGGLE, D.J. (1986). Inhibitory actions of a series of Ca^{2+} channel antagonists and K^+ depolarization induced responses in smooth muscle: an assessment of selectivity of action. *Can. J. Physiol. Pharmacol.*, **64**, 273–283.

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