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Pharmacological analysis of the effects of Bay K 8644 and organic calcium antagonists on the mouse isolated distal colon

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1 Bay K 8644 (10^{-8} to 10^{-6} M) induced concentration-related contractions of the longitudinal muscle of the mouse distal colon. The maximal responses were enhanced and the EC_{50} was lowered in the presence of tetrodotoxin (TTX; 1.5×10^{-7} M). The responses were not affected by atropine (10^{-7} M), mepyramine (2.5×10^{-7} M), methysergide (5×10^{-7} M), propranolol (10^{-6} M), phentolamine (10^{-6} M) or naloxone (4×10^{-7} M). By contrast, the contractile responses were inhibited by Ca^{2+} entry blockers (verapamil, nifedipine) and abolished in Ca^{2+}-free EGTA solution. These observations indicate that the contractile effects of Bay K 8644 are dependent on its ability to promote Ca^{2+} influx.

2 At 10^{-4} M, Bay K 8644 provoked a slow relaxation of the preparation. Moreover, from 10^{-5} M, Bay K 8644 markedly reduced the contractile responses to ACh and K^+ depolarization. These inhibitory effects were comparable with those produced by nifedipine. Such data suggest that, at high concentrations, Bay K 8644 could act in part as a dihydropyridine Ca^{2+} channel antagonist.

3 Bay K 8644 (10^{-9} M) preferentially enhanced, while nifedipine (10^{-10} to 10^{-8} M) as well as verapamil (3×10^{-9} to 10^{-6} M) preferentially inhibited, the tonic component of the contractile response evoked by K^+ depolarizing solution. This may indicate that different populations of voltage-sensitive Ca^{2+} channels are involved in the biphasic response to K^+ depolarization.

4 The biphasic contractile activity induced by ACh was barely enhanced by Bay K 8644 (10^{-9} M) and was less sensitive to Ca^{2+} entry blockers than the responses to KCl. These findings are discussed in terms of receptor-operated channels and mobilization of bound calcium.

Introduction

It is generally believed that a rise in the concentration of internal ionized Ca^{2+} generates tension in the contractile proteins of smooth muscle (Bolton, 1979; Loutzenhiser *et al.*, 1985). An increase in the myoplasmic concentration of free Ca^{2+} may result either from intracellular or extracellular Ca^{2+} mobilization. The extracellular Ca^{2+} mobilization probably occurs through several distinct pathways, including potential or voltage-sensitive calcium channels and receptor-operated calcium channels (Bolton, 1979; Loutzenhiser *et al.*, 1985).

Organic and inorganic Ca^{2+} channel blockers have been extensively used to study Ca^{2+} channel properties in many cell types including smooth muscle (Hurwitz *et al.*, 1980; van Breemen *et al.*, 1980; Hagiwara & Byerly, 1981; Meisheri *et al.*, 1981; Glossman *et al.*, 1982; Spedding, 1985; Godfraind *et al.*, 1986). Recently, it has been shown that structural modifications of dihydropyridine molecules generate a novel class of compounds (Bay K 8644, CGP 28392, YC 170), which have been proposed to act as Ca^{2+} channel activators instead of inactivators (Schramm *et al.*, 1983; Kokubun & Reuter, 1984; Schramm & Towart, 1985; Spedding, 1985).

In the present study, the effects of Bay K 8644 have been investigated on the mouse distal colon which represents a sensitive tool to study the mechanisms modulating intestinal tone (Fontaine *et al.*, 1984; Fontaine & Lebrun, 1985). Furthermore, and in ordef to gain further insight into the properties of the Ca^{2+} channels of the colonic smooth muscle

cells, the effects of Bay K 8644 were compared with those evoked by acetylcholine and K^+ depolarization.

Methods

Swiss Webster mice (30–40 g) were stunned and killed by exsanguination. The terminal colon was dissected out and suspended under an initial load of 1 g in an organ bath containing 10 ml of a Krebs-Henseleit solution of the following composition (mM): NaCl 118.1, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, glucose 5. Bathing media containing no calcium salt were also used (Ca^{2+}-free solution) with or without 0.5 mM ethylene glycol *bis*-(β-aminoethyl-ether)N,N'-tetraacetic acid (EGTA). Tetrodotoxin (TTX) (1.5×10^{-7} M) was added to some bathing media in order to block the neuronal activity of the muscle preparation (Gershon, 1967). The physiological solutions were maintained at 36 ± 1°C and equilibrated with a mixture of O_2 (95%) and CO_2 (5%).

The isometric contractions of the longitudinal muscle were measured with a Grass force-displacement transducer. Preparations were allowed to equilibrate for 60 min before adding drugs. Acetylcholine (ACh) 10^{-6} M was added at the beginning of each experiment to test the reactivity of the preparation. ACh and KCl were added to the bath at 15 min intervals whilst Bay K 8644 was added at 45 min intervals. Antagonists were added 15 min before agonists. In some experiments, the preparation was preincubated 3 min in the presence of Bay K 8644 or nifedipine before testing ACh or KCl.

Both Bay K 8644 and nifedipine were first dissolved in dimethylsulphoxide (DMSO) and further diluted in the experimental medium so that the final concentration of the organic solvent never exceeded 0.1% (v/v). DMSO up to 0.1% did not affect the mechanical activity of the longitudinal muscle. Experiments were conducted with minimal light to prevent photodegradation of the dihydropyridines.

The statistical significance of differences between mean experimental and control data was assessed by use of Student's *t* test.

Drugs

The bathing medium contained, as required: acetylcholine hydrochloride (ACh; Astra), atropine sulphate (Fluka), Bay K 8644 (methyl-1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate, Bayer), mepyramine maleate (Rhône-Poulenc), methysergide hydrogen maleate (Sandoz), naloxone hydrochloride (Endo), nifedipine (Bayer), phentolamine methane sulphonate (Ciba), (±)-propranolol hydrochloride (ICI), tetrodotoxin (TTX; Calbiochem), (±)-verapamil hydrochloride (Knoll).

Results

Contractile effects of Bay K 8644, ACh and KCl on the mouse distal colon

At concentrations ranging from 10^{-8} to 10^{-6} M. Bay K 8644 induced concentration-related contractions of the terminal colon (Figure 1a). The responses were rapid in onset, an initial peak (phasic) being followed by a more sustained contraction (tonic). The maximal responses (tonic) induced by 10^{-6} M Bay K 8644 averaged $37 \pm 4\%$ (1.25 ± 0.17 g) ($n = 8$) of the tension elicited in the same preparations by 10^{-6} M ACh.

The addition of 10^{-5} M Bay K 8644 also induced a biphasic contractile response but the tonic tension was of smaller amplitude than that evoked by 10^{-6} M Bay K 8644. At a higher concentration (10^{-4} M), Bay K 8644 evoked a short lasting contraction which was followed by a slow relaxation of the preparation (Figure 1a).

The contractile effects of Bay K 8644 (10^{-6} M) were reproducible at 45 min intervals (the preparation being challenged twice with 10^{-7} M ACh during each interval), but the resting tone of the colon remained somewhat higher after washing out the drug. The contractions were not modified by atropine (10^{-7} M), mepyramine (2.5×10^{-7} M), methysergide (5×10^{-7} M), propranolol (10^{-6} M), phentolamine (10^{-6} M) or naloxone (4×10^{-7} M).

The contractile responses to 10^{-7} M Bay K 8644 were markedly reduced in Ca^{2+}-free solution and totally abolished in Ca^{2+}-free EGTA solution (data not shown).

When concentration-effect curves to Bay K 8644 were performed in the presence of tetrodotoxin (TTX, 1.5×10^{-7} M) in the bathing medium, several differences were observed (Figures 1b and 2, and Table 1).

Firstly, and as previously observed, the spontaneous tone of the longitudinal muscle was increased (Fontaine *et al.*, 1984). Secondly, the threshold concentration for Bay K 8644 effects was lower in the presence than in the absence of TTX. Thirdly, the biphasic responses to Bay K 8644 were enhanced in the presence of TTX.

Table 1 clearly indicates that the maximal phasic and tonic tension recorded respectively at 10^{-5} and 10^{-6} M were enhanced in the presence of TTX. Furthermore, the EC_{50} values for the phasic and tonic response to Bay K 8644 were significantly lower in the presence of the toxin (Table 1).

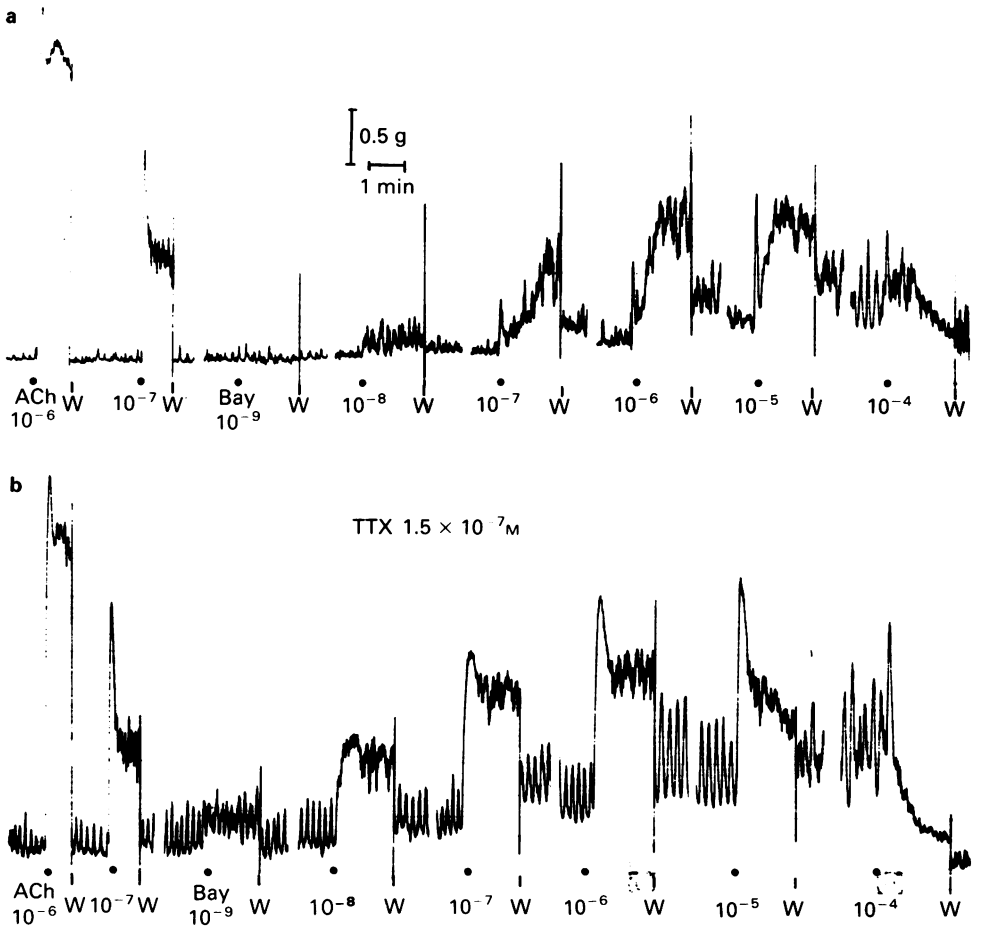

Figure 1 Effects of acetylcholine (ACh) (10^{-6}, 10^{-7} M) and increasing concentrations of Bay K 8644 (10^{-9} to 10^{-4} M) on the mouse distal colon incubated in the absence (a) and presence (b) of tetrodotoxin (TTX, 1.5×10^{-7} M). The preparation was challenged twice with ACh 10^{-7} M between 2 successive additions of Bay K 8644. W = washout.

The spasmogenic effects of increasing concentrations of Bay K 8644 have been compared with those induced by ACh and KCl. In the absence and presence of TTX (1.5×10^{-7} M), ACh (10^{-9} to 10^{-5} M) and KCl (10^{-3} to 4×10^{-2} M) provoked concentration-dependent responses characterized by an initial phasic contraction followed by a lower and sustained plateau (tonic component).

The presence of TTX in the medium did not modify the maximal phasic or tonic component of the contractile response evoked by ACh and their EC_{50}s (Figure 2, Table 1). By contrast, in the presence of TTX, the maximal phasic and tonic tensions developed by KCl were significantly increased whilst their EC_{50} values were lower (Figure 2, Table 1).

Effects of organic Ca^{2+} channel blockers on agonist-induced contractions

The effects of increasing concentrations of verapamil and/or nifedipine on Bay K 8644 (10^{-7} M)-, ACh (10^{-7} M)- and KCl (2×10^{-2} M)-induced contractile activity, are illustrated in Figure 3. The experiments were performed in the presence of TTX (1.5×10^{-7} M). The effects of verapamil and nifedipine have been quantified on both the phasic and tonic component of the mechanical response to ACh and K^+ depolarization. As in most experiments 10^{-7} M Bay K 8644 only evoked a monophasic increase in tension, the response has been analysed in terms of tonic component.

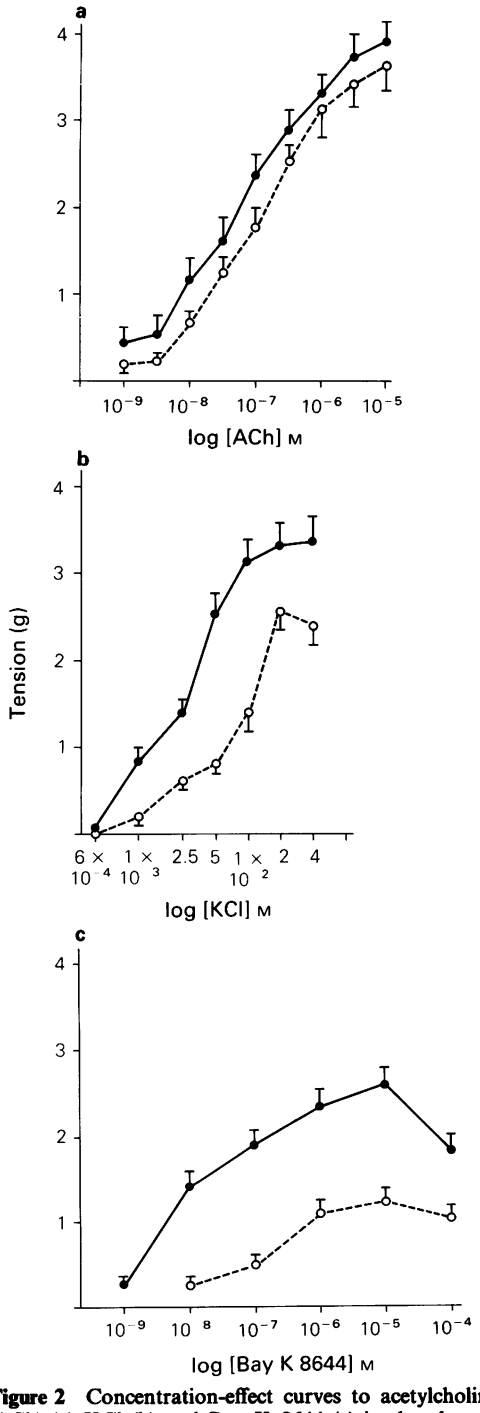

Figure 2 Concentration-effect curves to acetylcholine (ACh) (a) KCl (b) and Bay K 8644 (c) in the absence (○----○) and presence (●——●) of tetrodotoxin (1.5×10^{-7} M). Each point represents the mean of at least 4 individual determinations of the phasic response to each agonist; vertical lines indicate s.e.mean.

Table 1 Maximal phasic and tonic tension and EC_{50} developed by acetylcholine (ACh), KCl or Bay K 8644

Line	*Agonist*	*Contractile response*	*TTX* (M)	*Maximal tension* (g)	P	EC_{50} (M)	P
1	ACh	phasic	—	3.7 ± 0.5 (4)		$1.0 \times 10^{-7} \pm 0.2$	
2	ACh	phasic	1.5×10^{-7}	4.0 ± 0.5 (5)	1 vs 2	$7.5 \times 10^{-8} \pm 1.0$	1 vs 2 NS
3	ACh	tonic	—	3.4 ± 0.4 (4)		$2.2 \times 10^{-7} \pm 0.5$	
4	ACh	tonic	1.5×10^{-7}	3.0 ± 0.3 (5)	3 vs 4 NS	$1.0 \times 10^{-7} \pm 0.3$	3 vs 4 NS
5	KCl	phasic	—	2.6 ± 0.2 (8)		$8.7 \times 10^{-3} \pm 0.7$	
6	KCl	phasic	1.5×10^{-7}	3.4 ± 0.3 (8)	5 vs 6 $P < 0.05$	$3.0 \times 10^{-3} \pm 0.6$	5 vs 6 $P < 0.001$
7	KCl	tonic	—	1.9 ± 0.1 (8)		$1.3 \times 10^{-2} \pm 0.2$	
8	KCl	tonic	1.5×10^{-7}	3.0 ± 0.3 (8)	7 vs 8 $P < 0.005$	$5.8 \times 10^{-3} \pm 0.8$	7 vs 8 $P < 0.005$
9	Bay K 8644	phasic	—	1.2 ± 0.2 (8)		$2.0 \times 10^{-7} \pm 0.6$	
10	Bay K 8644	phasic	1.5×10^{-7}	2.6 ± 0.3 (7)	9 vs 10 $P < 0.005$	$1.0 \times 10^{-8} \pm 0.3$	9 vs 10 $P < 0.025$
11	Bay K 8644	tonic	—	1.2 ± 0.1 (8)		$2.0 \times 10^{-7} \pm 0.7$	
12	Bay K 8644	tonic	1.5×10^{-7}	1.9 ± 0.3 (7)	11 vs 12 $P < 0.05$	$4.0 \times 10^{-9} \pm 0.5$	11 vs 12 $P < 0.025$

Concentration-effect curves have been constructed in the absence and presence of tetrodotoxin (TTX, 1.5×10^{-7} M). Values are expressed as means ± s.e.mean with the number of individual experiments in parentheses. NS = non significant.

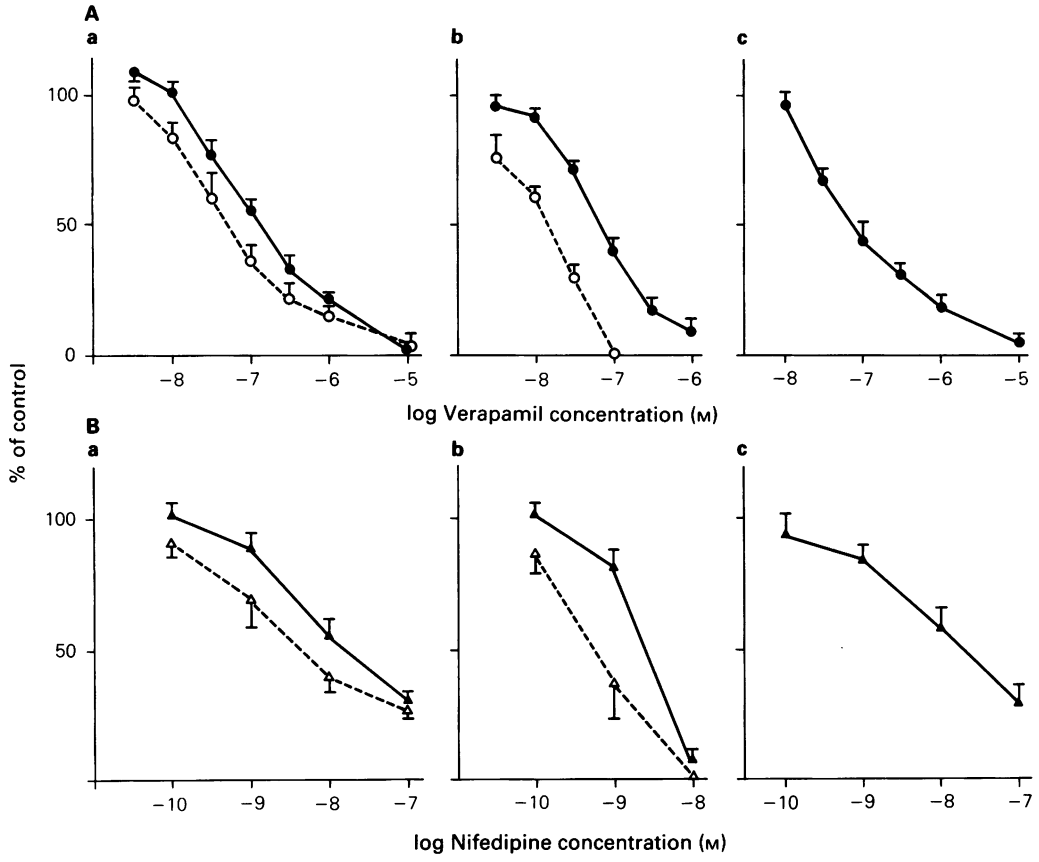

Figure 3 Concentration-response curves for the inhibitory effects of verapamil (A) and nifedipine (B) on contractile activity induced by (a) acetylcholine (ACh, 10^{-7} M), (b) KCl (2×10^{-2} M) and Bay K 8644 (10^{-7} M). (●——●) or (▲——▲) Refer to the phasic and (○----○) or (△----△) to the tonic component of the responses evoked by ACh or KCl. The contractile responses to Bay K 8644 (10^{-7} M) were monophasic. Results are expressed as % (mean with vertical lines indicating s.e.mean) of the contractile responses induced by each agonist in the absence of Ca^{2+} channel blockers (n = 4–12). Tetrodotoxin (1.5×10^{-7} M) was present in the physiological solution.

The Ca^{2+} channel blockers caused a dose-related inhibition of Bay K 8644, ACh and KCl-induced contractions (Figure 3).

Both verapamil and nifedipine inhibited more markedly the contractile effects of KCl than that induced by Bay K 8644 or ACh. Indeed, in the presence of 10^{-8} M nifedipine, the tonic component of the KCl response was completely suppressed (n = 4), whilst the contraction induced by Bay K 8644 and the tonic component of the ACh response averaged, respectively, 59 ± 6% (n = 10) and 40 ± 6% (n = 8) of the control values.

In the presence of 10^{-7} M verapamil in the bathing medium, the tonic response to KCl was totally abolished. The contraction induced by Bay K 8644 and the tonic component of the ACh response averaged, respectively, 45 ± 6% (n = 9) and 36 ± 6% (n = 5) of those evoked by the agonists in the absence of verapamil in the physiological solution.

Both verapamil and nifedipine also inhibited more markedly the KCl-induced phasic response than the ACh-evoked phasic contraction (Figure 3).

On the other hand, the present results also indicate that the tonic component of the contractile response evoked by K^+ depolarization displays a much greater sensitivity towards Ca^{2+} antagonists than the phasic component (Figure 3).

Effects of nifedipine and different Bay K 8644 concentrations on ACh and KCl-induced contractile activity

In this series of experiments performed in the presence of TTX (1.5×10^{-7} M), the preparation was

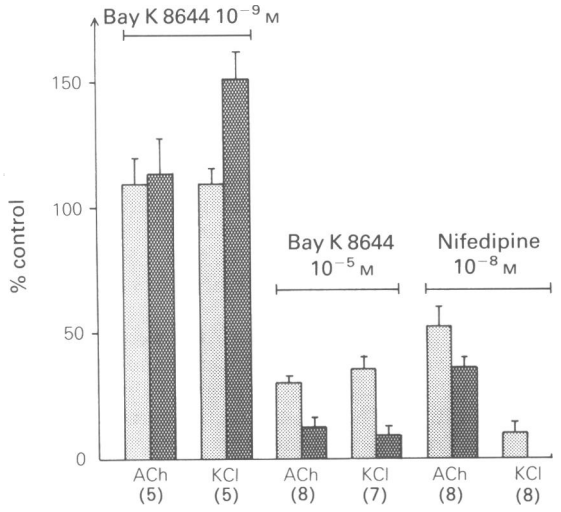

Figure 4 Effects of Bay K 8644 (10^{-9} and 10^{-5} M) and nifedipine (10^{-8} M) on the contractile activity evoked by acetylcholine (ACh, 10^{-7} M) or KCl (2×10^{-2} M). The stippled columns represent the phasic component whilst the cross hatched columns represent the tonic component of the contractile responses. Results are expressed as % (mean with vertical lines indicating s.e.mean) of control responses measured in the absence of Bay K 8644 or nifedipine ($n = 5$–8). Tetrodotoxin (1.5×10^{-7} M) was present in the physiological solution.

preincubated 3 min in the presence of nifedipine (10^{-8} M) or Bay K 8644 (10^{-9} and 10^{-5} M) before adding ACh (10^{-7} M) or KCl (2×10^{-2} M).

Bay K 8644 (10^{-9} M) increased slightly but not significantly both the phasic and tonic component of the contractile response to ACh ($P > 0.5$ and $P > 0.4$) (Figure 4). The dihydropyridine (10^{-9} M) enhanced the tonic but not the phasic response evoked by K^+ depolarization ($P < 0.05$ and $P > 0.2$) (Figure 4).

At a higher concentration (10^{-5} M), Bay K 8644 exerted inhibitory effects on the ACh and KCl induced contractions (Figure 4). For both agonists, the tonic responses displayed a greater sensitivity towards the inhibitory effect of 10^{-5} M Bay K 8644 than the phasic responses.

An identical situation was recorded in the presence of 10^{-8} M nifedipine in the bathing solution (Figure 4). The tonic component of the contractile responses evoked by ACh and KCl was more inhibited than the phasic tension. Furthermore, and as previously described, the ACh-induced responses were less sensitive to the inhibitory effect of nifedipine than the KCl-induced responses. Thus, in the presence of 10^{-8} M nifedipine, the phasic and tonic component of the contractile response to ACh averaged, respectively, 52 ± 8 and $36 \pm 4\%$ ($n = 8$) of the control values. The phasic tension evoked by K^+ depolarizing solution averaged $10 \pm 4\%$, whilst the tonic response was totally abolished ($n = 8$).

Discussion

The present study reveals that Bay K 8644 exerts potent spasmogenic effects on the mouse isolated distal colon.

The contractile responses to Bay K 8644 were antagonized by two organic calcium channel blockers, were reduced in Ca^{2+}-free solution and totally abolished in Ca^{2+}-free EGTA solution. These observations indicate that the myogenic effects of Bay K 8644 are highly dependent on extracellular calcium and suggest that, in colonic smooth muscle like in other tissues, the dihydropyridine derivative enhances calcium influx (Schramm & Towart, 1985).

It is interesting to note that Bay K 8644 contracted the distal colon in the absence of any contractile agent. Since previous observations clearly showed that the dihydropyridine failed to affect quiescent voltage-sensitive Ca^{2+} channels and did not induce spontaneous Ca^{2+} channel openings without depolarizing pulses, our data may indicate that, in mouse colonic smooth muscle cells, the fraction of active Ca^{2+} channels is relatively high in resting conditions (Freedman & Miller, 1984; Kokubun & Reuter, 1984). This suggestion is reinforced by the knowledge that the mouse distal colon exhibits spontaneous activity (Fontaine *et al.*, 1984; Fontaine & Lebrun, 1985).

The effects of Bay K 8644 deserve two other comments. First, the presence of TTX in the physiological solution lowered the threshold concentration for Bay K 8644 actions and enhanced the Bay K 8644 responses. TTX also increased the spontaneous activity of the longitudinal muscle and increased the contractile effects of K^+ depolarizing solution without modifying the responses evoked by ACh. These findings may support the view that the toxin interferes with inhibitory nerves causing constant hyperpolarization of the colonic smooth muscle cells. TTX, by reducing the hyperpolarization mediated by the neural influence, would therefore enhance the ability of Bay K 8644 to affect the voltage-sensitive Ca^{2+} channels.

Alternatively, it could be postulated that TTX, a Na^+ channel blocker, may slightly reduce the Na-K pump activity as a consequence of a decreased intracellular sodium activity. This inhibition of the electrogenic sodium pump could also lead to a depolarization of the membrane and indirectly increase the fraction of active Ca^{2+} channels which represent the target sites for Bay K 8644 action

(Thomas, 1972; Freedman & Miller, 1984; Kokubun & Reuter, 1984).

Second, Bay K 8644 in high concentrations (10^{-5}; 10^{-4} M) relaxed the preparation and reduced the contractile activity evoked by ACh or K^+ depolarization. These inhibitory effects of Bay K 8644 on the induced contractions were comparable with those produced by 10^{-8} M nifedipine. This capacity of Bay K 8644 to exhibit calcium antagonistic properties at high concentrations, has been described in other tissues (Thomas *et al.*, 1984; Coruzzi & Poli, 1985; Lebrun & Atwater, 1985; Towart *et al.*, 1985; Bechem & Schramm, 1987). Such a feature does not appear to result from the use of racemic Bay K 8644 containing the (−)(activator) and the (+)(inactivator) enantiomer. Indeed, it has been shown that the effects of (−)-Bay K 8644 were indistinguishable from the racemic compound (Bechem & Schramm, 1987). The effects of high Bay K 8644 concentrations could rather be explained by assuming that voltage-sensitive calcium channels have more than one binding site or state for dihydropyridines (Thomas *et al.*, 1984; Vaghy *et al.*, 1984; Lee *et al.*, 1987). However, an alternative explanation could be that high concentrations of Bay K 8644 interfere with intracellular events (Movsesian & Adelstein, 1984; Towart *et al.*, 1985).

The present investigation was also undertaken to characterize the sensitivity of ACh- or KCl-induced contractions towards organic calcium antagonists and Bay K 8644.

The tonic component of the contractile activity induced by K^+ depolarization displayed a much greater sensitivity towards the Ca^{2+} channel blockers and towards Bay K 8644 than the phasic component. Such findings suggest that two different K-activated Ca^{2+} channels are involved in the mechanical responses (Rosenberger *et al.*, 1979; Hurwitz *et al.*, 1980; Bolger *et al.*, 1983; Morel *et al.*, 1987).

Recent electrophysiological studies identified three types (L, T and N) of voltage-sensitive Ca^{2+} channels which can be distinguished by their pharmacological and kinetic properties (Nilius *et al.*, 1985; Nowycky *et al.*, 1985). The pharmacological analysis of these ionic channels indicated that only the L channels were affected by dihydropyridine agonists and antagonists. Thus, it is tempting to speculate that the tonic component of the contractile response to K^+ could be mediated by the modulation of Ca^{2+} channels, which share similar characteristics with the L channels previously described in neuronal and cardiac cells (Nilius *et al.*, 1985, Nowycky *et al.*, 1985).

The contractile responses to ACh were less sensitive to the Ca^{2+} channel blockers and to Bay K 8644 than the mechanical responses induced by K^+ depolarization. These data are in agreement with previous results showing that receptor-operated channels initially activated by ACh have a smaller sensitivity towards verapamil and dihydropyridines than voltage-operated channels (Bolton, 1979; Meisheri *et al.*, 1981; Godfraind, 1983).

On the other hand, the initial phasic component of the contractile activity evoked by ACh was slightly more resistant towards the Ca^{2+} entry blockers than the tonic component. This phasic response may be rather resistant to calcium antagonists because phasic tension could be dependent in part on the release of bound calcium by muscarinic receptor activation (Bolton, 1979; Loutzenhiser, 1985).

In summary, our results indicate that low concentrations of Bay K 8644 contract the mouse distal colon in the absence of any contractile agent. The analysis of the biphasic mechanical response evoked by ACh or K^+ depolarization reveals that the tonic and phasic tension induced by these agonists are differently sensitive to Bay K 8644, nifedipine and verapamil.

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