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Similarity of relaxations evoked by BRL 34915, pinacidil and field-stimulation in rat oesophageal tunica muscularis mucosae

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1 In the rat oesophageal tunica muscularis mucosae (TMM) the potassium channel openers, BRL 34915 and pinacidil, raised the threshold for concentration-dependent K^+ contractions, suppressed contractions evoked by field stimulation of the TMM in the presence of tetrodotoxin (TTX) and tetraethylammonium (TEA), and relaxed tonic contractions resulting from muscarinic cholinoceptor stimulation.

2 BRL 34915 and both (+)- and (−)-pinacidil increased ^{86}Rb efflux from tracer-loaded tissues; nifedipine abolished this effect.

3 Relaxations produced by potassium channel openers were inhibited by a temperature drop from 37°C to 26.5°C, an increase in extracellular K^+ concentration to 64 mM, and treatment with the calcium channel antagonist, nifedipine. The same treatments also blocked field stimulation-evoked TTX-insensitive relaxations.

4 It is concluded that field stimulation of rat oesophageal smooth muscle in the presence of cholinoceptor-induced tone results in an increase in K^+ permeability that is directly or indirectly coupled to Ca^{2+} influx through potential-operated channels.

Introduction

In the preceding paper (Akbarali *et al.*, 1988), we presented evidence suggesting that the tetrodotoxin (TTX)-insensitive relaxation of rat oesophageal smooth muscle elicited by field-stimulation depends on Ca^{2+} influx through channels which are similar to those mediating contractile responses to K^+ depolarization. One of the possible mechanisms for such a Ca^{2+}-entry-dependent relaxation is the stimulation of Ca^{2+}-activated potassium channels, which have been shown to exist in smooth muscle (see Meech, 1978). Previous attempts to test this hypothesis by the use of apamin, a specific inhibitor of certain Ca^{2+}-activated potassium channels (Banks *et al.*, 1979), and tetraethylammonium (TEA), a non-selective potassium channel blocker (Hille, 1984), were unsuccessful (Akbarali *et al.*, 1986). However, the potassium channels implicated in relaxations evoked by field stimulation may be atypical, and resemble those described in the lower oesophageal sphincter of the opossum oesophagus (Jury *et al.*, 1985) and guinea-pig circular muscle of the intestine (Yamanaka *et al.*, 1985), in so far as they are not involved in maintaining resting membrane potential, or generation of non-adrenergic, non-cholinergic (NANC)-nerve mediated inhibitory junctional potentials in the guinea-pig taenia coli (Den Hertog & Jager, 1975).

BRL 34915 ((+)-6-cyano-3,4-dihydro-2,2-dimethyl-trans-4-(2-oxo-1-pyrrolidyl)-2H-benzo(b) pyran-3-ol) and pinacidil (N″-cyano-N-4 pyridyl-N′-1,2,2-trimethylpropylguanidine), have recently been shown to open potassium channels in a number of smooth muscle preparations including guinea-pig taenia caeci (Weir & Weston, 1986a), rabbit aorta (Kreye & Weston, 1986; Cook *et al.*, 1987; Southerton *et al.*, 1987), rat uterus (Edwards *et al.*, 1987), rat portal vein (Weir & Weston, 1986b); and rabbit mesenteric artery (Coldwell & Howlett, 1987). Furthermore BRL 34915-induced relaxations were not inhibited by apamin in the guinea-pig taenia caeci (Weir & Weston, 1986a) or the trachealis muscle (Allen *et al.*, 1986), and were dependent on extracellular Ca^{2+} in the rabbit aorta (Kreye &

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Weston, 1986). We therefore examined the effects of these putative potassium channel openers in the rat oesophageal muscularis mucosae with a view to elucidating potassium channel involvement in TTX-insensitive relaxation. Our findings suggest that stimulation of Ca^{2+}-activated potassium channels may constitute a common mechanism for relaxant responses to both field-stimulation, BRL 34915, and pinacidil.

Methods

Preparations

The rat tunicae musculares mucosae were dissected and set up for field-stimulation and recording of longitudinal isometric tension as described by Bieger & Triggle (1985). Taenia caeci were obtained from male Hartley guinea-pigs and set up in similar fashion. For eliciting oesophageal smooth muscle contractions, field-stimulation parameters selected were 40 V, 0.5 ms pulse-width and 8 Hz pulse rate; relaxations were examined at 40 V, 2 ms pulse-widths and 4 Hz pulse rate after contractile responses were blocked with TTX (0.1–0.3 μM) and tonus induced with a muscarinic agonist, *cis*-2 methyl-4-dimethylamino-methyl-1,3 dioxolane methiodide (*cis*-dioxolane) at 0.1 μM. Concentration-response curves to K^+ were constructed by cumulative addition to KCl to the organ bath with bath K^+ concentrations raised at the peak of the preceding phasic response to K^+.

^{86}Rb efflux

Segments of the oesophageal smooth muscle (2 cm long) were mounted on stainless steel rods (diameter 1.0 mm), inserted into the lumen, and incubated in Tyrode solution and gassed with 95% O_2/5% CO_2 at 37°C for 30 min. After equilibration, tissues were loaded with ^{86}Rb (1 μCi ml^{-1}) for 90 min. Each tissue was briefly rinsed to remove excess radioactivity and then sequentially passed at 2 min intervals through a series of test tubes containing 2 ml Tyrode solution at 37°C. In some experiments, tissues were exposed to *cis*-dioxolane and BRL 34915 or pinacidil for three collection intervals after 28 min of efflux. When BRL 34915 and pinacidil were used, appropriate ethanol control experiments were performed. At the end of the efflux period, 500 μl aliquots of each sample of wash-out medium were counted for radioactivity. Each tissue was blotted dry and solubilised with Protosol (0.5 M) in order to estimate the residual tissue-bound radioactivity. The efflux data were expressed in terms of a rate coefficient (fractional loss of Rb from the tissue standardized for a 1 min period and expressed as a percentage).

Both BRL 34915 and pinacidil were made up as 10 mM stock solutions in 70% ethanol and subsequent dilutions were made in Tyrode solution.

The bath temperature was controlled by means of a Lauda K2/R thermostat-regulated circulator pump equipped with a refrigerator which permitted rapid cooling.

Drugs

BRL 34915 was generously donated by Beecham Pharmaceuticals, U.K., *cis*-dioxolane by Dr D.J. Triggle, State University of New York, Buffalo, and racemic and (+)- and (−)-pinacidil by Leo Pharmaceutical Products, Ballerup, Denmark, Bay K 8644 by Dr A. Scriabine, Miles Labs., New Haven, Ct, U.S.A. and nifedipine from Bayer A.G., F.R.G. Indomethacin was purchased from Sigma, St Louis; tetrodotoxin and (−)-hyoscine methylbromide from Calbiochem, U.S.A.

Results

Smooth muscle relaxant effects of BRL 34915

BRL 34915 diminished the contractility of rat oesophageal smooth muscle as evidenced by its ability: (1) to raise the threshold for K^+ contractions, (2) to suppress contractions evoked by field stimulation, and (3) to relax tonic contractions resulting from muscarinic cholinoceptor stimulation.

Maximal contractions to K^+ (84 mM) attained 50% of the maximal response to *cis*-dioxolane (1 μM). There were no regional differences in the EC_{50} values; however, as previously shown for *cis*-dioxolane- and field stimulation-evoked contractions, the maximal tension generated was greater in the distal than in the proximal segment (Akbarali *et al.*, 1986; 1987). Thus the EC_{50} values for distal and proximal segments were 43 mM and 46 mM while the maximal amplitudes of K^+ contractions were 1.3 ± 0.09 g and 0.74 ± 0.08 g, respectively. In the presence of BRL 34915 (10 μM) the foot of the K^+ log concentration-response curve was displaced to the right while the maximum remained unaltered (Figure 1). Similar results with BRL 34915 were obtained when K^+ concentration-response curves were determined in the presence of 0.01 μM (−)-hyoscine methylbromide.

In the presence of TTX, oesophageal smooth muscle failed to show a mechanical response to field-stimulation; however, upon addition of 5 mM TEA they became mechanically responsive such that single contractions occurred to field-stimulation followed by after-contractions (Figure 2). These TTX-

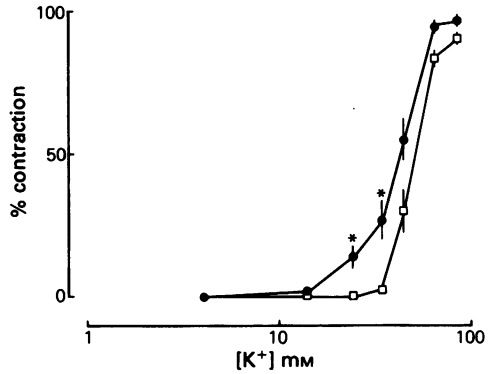

Figure 1 Effect of BRL 34915 on concentration-response curve to KCl in rat oesophageal smooth muscle. Control responses (●); responses in the presence of BRL 34915, 10 μM (□), applied 10 min before the addition of KCl. Each point is a mean derived from 8 experiments; vertical bars show s.e.mean. $*P < 0.05$ (Student's t test).

insensitive contractions were not affected by 0.01 μM (−)-hyoscine methylbromide but were reduced by 1 μM nifedipine. BRL 34915, 1 μM, completely suppressed such responses or upon pretreatment prevented them. In the absence of TTX, 1 mM TEA diminished field stimulation-evoked contractions; however, at >5 mM TEA enhancement was noted. The relaxant effect of TEA showed tachyphylaxis.

cis-Dioxolane-precontracted oesophageal smooth muscle, in the presence of TTX, relaxed to BRL 34915 in a concentration-dependent manner (Figure 3). Relaxations were generally slow in onset with a maximal relaxation of 82 ± 6% being observed at 100 μM. By comparison, the guinea-pig taenia caeci was more sensitive and responded more rapidly to the relaxant action of BRL 34915. Responses to pinacidil were essentially similar, the (−)-isomer [IC_{50} 1.4 μM] being more potent than the (+)-isomer [IC_{50} 7 μM].

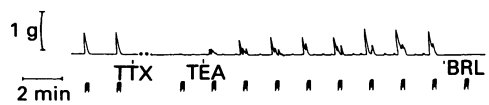

Figure 2 BRL 34915 inhibition of tetraethylammonium (TEA)-dependent oesophageal smooth muscle contraction. The top trace is a continuous record with a 10 min gap indicated by two dots. Initial contractions evoked by field-stimulation (indicated in bottom trace) were blocked by tetrodotoxin (TTX) (0.1 μM). Contractile responses reappeared after additional application of TEA (5 mM) which were blocked by BRL 34915 (1 μM). Field-stimulation parameters: train length 10 s, pulse frequency 8 Hz, pulse width 0.5 ms.

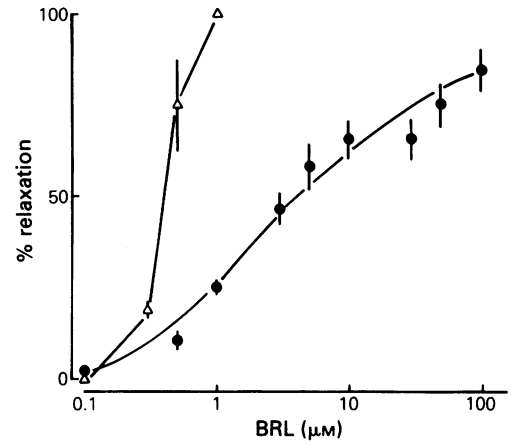

Figure 3 Concentration-response relationships of relaxant effects of BRL 34915 in the rat oesophageal tunica muscularis mucosae (●) and guinea-pig taenia caeci (△). Relaxations are expressed as % of maximal relaxation in the presence of tone induced by *cis*-dioxolane (0.1 μM) for both tissues. Vertical bars show s.e.mean; rat oesophageal smooth muscle $n = 8$, guinea-pig taenia caeci $n = 4$.

^{86}Rb efflux

BRL 34915 accelerated ^{86}Rb efflux (Figures 4, 5) from oesophageal smooth muscle, producing a maximum stimulation at a concentration of 30 μM (Figure 4). However, the muscarinic receptor agonist, *cis*-dioxolane, in concentrations up to 3 μM, was without effect on ^{86}Rb efflux. In the presence of 3 μM *cis*-dioxolane and 30 μM BRL 34915, stimulation of ^{86}Rb efflux was unaltered (1.39 ± 0.10 vs 1.43 ± 0.03 % min^{-1}).

Nifedipine, 0.1 μM, inhibited the BRL 34915-induced ^{86}Rb efflux (Figure 5). Pinacidil, 10 and

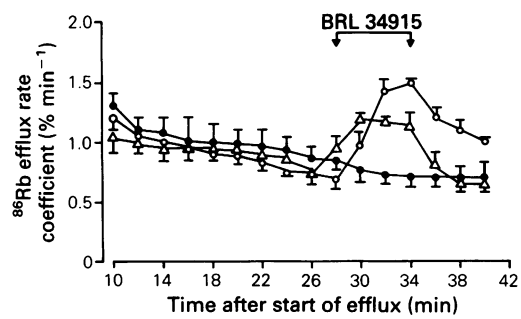

Figure 4 Effect of BRL 34915 on ^{86}Rb efflux in rat oesophageal smooth muscle. Curves were obtained for controls (●, $n = 6$) and in the presence of 10 μM (△, $n = 6$) and 30 μM (○, $n = 8$) BRL 34915. Vertical bars represent s.e.mean.

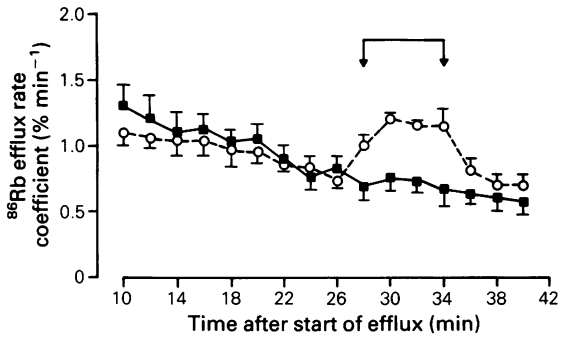

Figure 5 Antagonism of BRL 34915-induced ^{86}Rb efflux from rat oesophageal smooth muscle by nifedipine; (○) BRL 10 μM; (■) BRL 10 μM plus nifedipine 0.1 μM.

30 μM, had essentially the same effect on ^{86}Rb efflux as that described for BRL 34915 (Figure 6). The calcium channel activator Bay K 8644 at a concentration of 10 μM had no effect on ^{86}Rb efflux.

Suppression of BRL 34915- and pinacidil-induced relaxations

Three conditions which have been shown to abolish field-stimulation evoked TTX-insensitive relaxations were examined with respect to BRL 34915-induced relaxation. These were: high K^+, cooling, and treatment with the calcium channel antagonist, nifedipine.

Neither BRL 34915 nor pinacidil relaxed muscularis mucosae segments contracted by 64 mM K^+. This was also reflected in the inability of BRL 34915 to alter the maxima of the K^+ concentration-response curve; pinacidil was not tested.

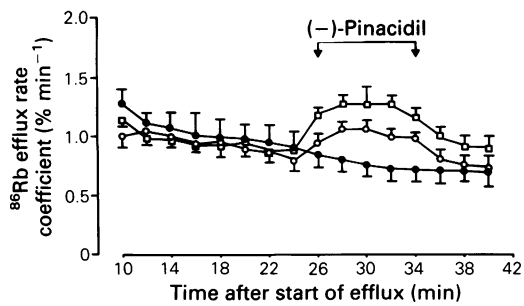

Figure 6 Effect of (−)-pinacidil on ^{86}Rb efflux in rat oesophageal smooth muscle. Curves were obtained for controls (●, $n = 6$) and in the presence of 10 μM (○, $n = 8$) and 30 μM (□, $n = 8$) (−)-pinacidil. Vertical bars represent s.e.mean.

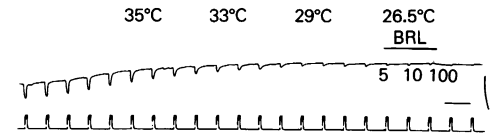

Figure 7 The effects of cooling on tetrodotoxin (TTX)-insensitive relaxations and BRL 34915-induced relaxation. TTX-insensitive relaxations were observed as transient drop in isometric tension (top trace) during application of field-stimulation (bottom trace). Muscle tension was induced by *cis*-dioxolane (0.1 μM) in presence of TTX (0.1 μM) applied 10 min before the start of record. Note decrease in TTX-insensitive relaxation responses as bath temperature is lowered and absence of relaxant response to BRL 34915 (5–100 μM). Stimulation parameters: 10 s train length, 2 ms pulse width; 4 Hz pulse rate). Vertical calibration 500 mg and horizontal bar 1 min.

Both BRL 34915 and pinacidil also failed to relax precontracted oesophageal smooth muscle at temperatures of 26.5°C–28°C ($n = 8$) as shown for BRL 34915 in Figure 7. In some tissues, indomethacin pretreatment was required to maintain tone of the cooled preparation (Akbarali *et al.*, 1986); however, this did not alter BRL 34915-induced relaxations at 37°C.

As reported in the preceding paper, TTX-insensitive relaxations were abolished in the presence of nifedipine. Similarly, pretreatment ($n = 8$) with 0.05 μM nifedipine blocked BRL 34915-induced relaxations (Figure 8). Pinacidil-induced relaxations were also blocked by nifedipine (not shown).

Effect of BRL 34915 on field stimulation evoked TTX-insensitive relaxations

As shown in Figure 9, BRL 34915 at concentrations greater than 10 μM blocked TTX-insensitive relax-

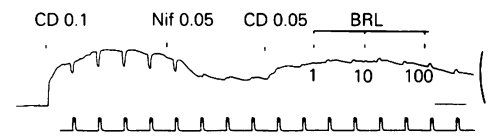

Figure 8 Block of tetrodotoxin (TTX)-insensitive relaxations and antagonism of BRL 34915 by nifedipine. Isometric tension recording from rat oesophageal smooth muscle. Following pretreatment with TTX (0.1 μM) and induction of active tonus with *cis*-dioxolane (0.1 μM) the tissue displayed TTX-insensitive relaxations during application of field-stimulation (bottom trace). The decrease in active tone, but not the relaxation amplitude, after nifedipine (Nif) (0.05 μM) was overcome by addition of *cis*-dioxolane (CD, 0.05 μM). Note failure of BRL 34915 (1–10 μM) to induce a relaxation. Vertical bar indicates 1 g and horizontal bar 2 min. Stimulation parameters are same as in Figure 5.

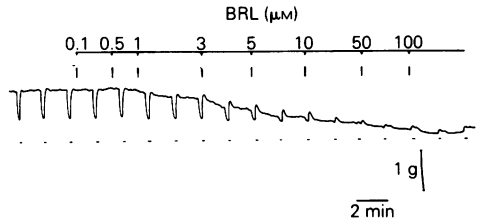

Figure 9 Relaxation induced by BRL 34915 (BRL) in rat oesophageal smooth muscle. Tissue was pretreated with tetrodotoxin (TTX, 0.1 μM) and tonus induced by *cis*-dioxolane (0.1 μM). Note conversion of relaxations to contractions. Dashes indicate field-stimulation as in Figures 5 and 6. Calibration: Vertical bar 1 g.

ations. Pretreatment with BRL also produced similar results; however, 4–5 stimulations were required to achieve blockade suggesting a 'use-dependent' block and continued stimulation of the tissue then resulted in contraction. The effects of pinacidil were not determined.

Discussion

Relaxation responses to BRL 34915 of rat oesophageal smooth muscle resembled TTX-insensitive relaxations in that both were blocked in high K^+, by cooling and by nifedipine. Furthermore, like the TTX-insensitive relaxations, BRL 34915-induced relaxations were TTX-insensitive. These results therefore support the hypothesis that (1) TTX-insensitive relaxations may be a consequence of potassium channel opening and (2) both forms of relaxation are dependent upon Ca^{2+} influx through calcium channels which have properties similar to those of potential-operated calcium channels. In other words, the common denominator of both relaxation responses is the activation of a dihydropyridine-sensitive, Ca^{2+}-activated potassium conductance.

BRL 34915 effects on the K^+ concentration-response curve observed in the present study are consistent with the postulated opening of potassium channels by this compound (Hamilton *et al.*, 1986; Weir & Weston, 1986a; Edwards *et al.*, 1986; Allen *et al.*, 1986; Cook *et al.*, 1987). The block of TTX-insensitive relaxations by high concentrations of BRL 34915 would at first glance be incompatible with the foregoing hypothesis. However, maximal activation of potassium channels by BRL 34915 may preclude further relaxations by field stimulation.

Our hypothesis receives further support by the finding that BRL 34915 and another putative potassium channel opener, pinacidil, increased $^{86}Rb^+$ efflux in oesophageal smooth muscle, an effect previously demonstrated in the guinea-pig taenia caeci (Weir & Weston, 1986a), rat vascular smooth muscle (Hamilton *et al.*, 1986; Weir & Weston, 1986b) and rabbit mesenteric arteries (Coldwell & Howlett, 1987). An increase in $^{86}Rb^+$ efflux was not observed when the actions of BRL 34915 were studied in the rat uterus (Hollingsworth *et al.*, 1987). In contrast to the longitudinal muscle of the guinea-pig intestine (Bolton & Clark, 1981), muscarinic receptor activation also did not stimulate $^{86}Rb^+$ efflux in oesophageal smooth muscle, suggesting the absence of an overt depolarizing action of the muscarinic agonist. Furthermore, muscarinic activation of guinea-pig ileal longitudinal muscle is highly sensitive to inhibition by calcium channel antagonists (Triggle *et al.*, 1979), whereas in rat oesophageal smooth muscle only a minor component of the response to *cis*-dioxolane is sensitive to nifedipine, and such inhibition is readily surmountable by increasing agonist concentration (Akbarali *et al.*, 1988; preceding paper). In this context, it is noteworthy that the nifedipine antagonism towards *cis*-dioxolane-induced contractions in the lower oesophageal sphincter region of the rat oesophagus is not surmountable (unpublished data). The comparative insensitivity to nifedipine and lack of effect on ^{86}Rb efflux in rat oesophageal smooth muscle strongly suggest that depolarization plays but a small part in the response of this preparation to muscarinic receptor activation.

The results obtained with TEA lead to the conclusion that the muscularis mucosae is mechanically unresponsive to direct electrical stimuli unless potassium channels are blocked. As expected, such contractions were sensitive to inhibition by BRL 34915 and pinacidil. In contrast, under the conditions of a maintained tone induced by *cis*-dioxolane, it was possible to initiate relaxations with BRL 34915, pinacidil, and also field stimulation in the presence of TTX. These data suggest that (i) relaxations are brought about by the opening of potassium channels, and (ii) under the conditions of maintained *cis*-dioxolane tone, K^+ conductance is not fully activated. The implied increment in K^+ conductance activated by the potassium channel openers or field-stimulation is highly sensitive to the 1,4-dihydropyridine calcium channel antagonists, suggesting that Ca^{2+} influx is required for its functional expression.

The question which then must be answered is whether there is a maintained Ca^{2+} entry during *cis*-dioxolane maintained tone which should activate such a K^+ conductance. Our available data do not allow us to reach a firm conclusion in this regard; however, others (Morgan & Morgan, 1984; Himpens & Castels, 1987) have shown that Ca^{2+} entry into smooth muscle following receptor stimulation is a transient, but that during K^+-mediated depolar-

ization is a maintained signal. Furthermore, digital imaging of fura-2 fluorescence in single heart cells (Wier *et al.*, 1987) suggests that changes in intracellular Ca^{2+} levels during activation may not be homogeneous throughout the cell. It is thus perhaps not unreasonable to consider that local changes in intracellular Ca^{2+} levels at the intracellular membrane surface could trigger altered potassium channel function.

As regards the apparent antagonism between potassium channel openers and the 1,4-dihydropyridines, we suggest that the sites of action of these two groups of drugs are distinct. In support of our hypothesis is the recent evidence indicating that BRL 34915 does not displace specific [^{3}H]-nitrendipine binding from rat heart membranes (Coldwell & Howlett, 1987). Kreye & Weston (1986) have also reported a calcium channel antagonist-sensitive effect of BRL 34915-induced increases in ^{86}Rb efflux from the rabbit aorta, although in their study a concentration of 1–10 μM nifedipine was required in order to observe inhibition. In contrast, Quast (1987) reported that BRL 34915-induced increases in ^{86}Rb efflux from the guinea-pig portal vein were insensitive to inhibition by 1,4-dihydropyridine calcium channel antagonists. Ca^{2+}-activated potassium channels in the guinea-pig taenia caeci, mediating relaxation, are apamin-sensitive, whereas BRL 34915-induced relaxations in the same preparation were found to be apamin-insensitive (Weir & Weston, 1986a). Nonetheless, apamin-insensitive Ca^{2+}-activated potassium channels may occur in the lower oesophageal sphincter of the opossum (Jury *et al.*, 1985) and may also be present in rat oesophageal smooth muscle. Thus tissue and species differences with respect to the association between Ca^{2+} entry through dihydropyridine-sensitive calcium channels and the actions of the potassium channel openers can be anticipated.

It might be anticipated that if TTX-insensitive relaxations do result from the opening of calcium antagonist-sensitive Ca^{2+}-operated potassium channels, then the calcium channel agonist Bay K 8644 might be expected to mimic, in the presence of active tone, the effect of field stimulation. However, we have not been able to demonstrate such an effect of Bay K 8644. Furthermore, Bay K 8644 was without effect on ^{86}Rb efflux, and this finding is, again, at variance with our hypothesis concerning the opening of Ca^{2+}-activated potassium channels in rat oesophageal smooth muscle.

In conclusion, we postulate that field stimulation in the presence of muscarinic cholinoceptor activation exerts a direct effect on the rat oesophageal tunica muscularis mucarae, leading to a Ca^{2+} influx and a secondary opening of a class of potassium channels. The coupling of this event to mechanical relaxation could be due to phosphorylation of intracellular proteins that bind intracellular Ca^{2+} (Ignarro & Kadowitz, 1985) or by other as yet undefined mechanisms. Further work is needed to define the cellular processes involved.

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