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Shaker-type Kv1 channel blockers increase the peristaltic activity of guinea-pig ileum by stimulating acetylcholine and tachykinins release by the enteric nervous system

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1 A constant intraluminal pressure system was used to evaluate the effects of Kv1 channel blockers on the peristaltic activity of guinea-pig ileum.

2 The nortriterpene correolide, a non-selective inhibitor of all Kv1 sub-types, causes progressive and sustained reduction of the pressure threshold for eliciting peristaltic contractions.

3 Margatoxin (MgTX), alpha-dendrotoxin (α-DTX) and dendrotoxin-K (DTX-K), highly selective peptidyl inhibitors of certain Kv1 sub-types, cause immediate reduction of the pressure threshold. This effect subsides with time, irrespective of the peptides' concentration in the bath. In preparations pretreated with saturating concentrations of MgTX, correolide further stimulates the peristaltic activity.

4 Iberiotoxin (IbTX), a selective inhibitor of the high-conductance Ca^{2+}-activated K^+ (BK) channels, and charybdotoxin (ChTX), which inhibits Kv1.2 and Kv1.3 as well as BK channels, fail to stimulate the peristaltic activity.

5 Blockade of muscarinic receptors by atropine reduces, and occasionally suppresses the peristaltic activity of guinea-pig ileum. In atropine-treated preparations, correolide and MgTX retain their abilities to reduce the pressure threshold and are able to restore the peristaltic reflex in the preparations where this reflex was suppressed by atropine.

6 The stimulatory effect of correolide and MgTX in atropine-treated preparations is abolished by subsequent addition of selective antagonists of both NK1 and NK2 receptors.

7 In conclusion, blockade of Kv1, particularly Kv1.1 channels, increases the peristaltic activity of guinea-pig ileum by enhancing the release of neurotransmitters at the enteric nervous system. In contrast, stimulation of the myogenic motility by blockade of BK channels does not affect the threshold for the peristaltic reflex.

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Abbreviations: BK, high-conductance Ca^{2+} activated K^+ channels; ChTX, charybdotoxin; α-DTX, alpha-dendrotoxin; DTX-K, dendrotoxin-K; ENS, enteric nervous system; IbTX, iberiotoxin; MgTX, margatoxin; PSS, physiological saline solution; TTX, tetrodotoxin

Introduction

Peristaltic activity is regulated by the enteric nervous system (ENS), which comprises intrinsic sensory neurons and interneurons, as well as excitatory and inhibitory motor neurons (Furness & Costa, 1987). This complex network enables the gut to perform intrinsic autonomic motor reflexes such as the peristaltic reflex (Barthó & Holzer, 1995). The frequency and propagation characteristics of peristaltic contractions depend on membrane-potential oscillations ('slow-waves'), generated at the interstitial cells of Cajal (Huizinga *et al*., 1997). The slow-waves propagate into electrically coupled smooth muscle cells and, when the membrane potential rises above the threshold for activation of L-type Ca^{2+} channels, an action potential is generated and muscle contraction is initiated.

Several members of the large family of voltage-dependent K^+ channels (Kv channels) have been identified in smooth muscles, where they provide pathways for repolarizing outward currents, which affect the resting membrane potential and membrane excitability. We have previously demonstrated that blockade of Kv1 channels present in preganglionic neurons at the ENS, leads to enhanced release of the excitatory neurotransmitters, acetylcholine and tachykinins, which in turn stimulate contractility of guinea-pig ileum (Suarez-Kurtz *et al*., 1999; Vianna-Jorge *et al*., 2000). These results suggest that Kv1 channels might play an

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important modulatory role in the peristaltic reflex. In the present study we used a constant intraluminal perfusion system to evaluate the effects of selective Kv1 channel blockers on the pressure threshold for eliciting peristaltic contractions of guinea-pig ileum. One of these compounds, the nortriterpene correolide blocks all Kv1 sub-types, while displaying negligible affinity for other families of voltage-dependent K channels (Felix *et al*., 1999). In contrast to correolide, the peptydil blockers, DTX-K, α-DTX and MgTX are selective for one or more of Kv1 channel sub-types. Thus, DTX-K has been reported as specific for Kv1.1, when tested at low nanomolar concentrations (Racape *et al*., 2002), whereas α-DTX inhibits Kv1.1, Kv1.2 and Kv1.6 (Robertson *et al*., 1996), and MgTX is a high-affinity blocker of Kv1.1, Kv1.2 and Kv1.3 (Garcia-Calvo *et al*., 1993a). We have also investigated the effects on the peristaltic activity of two other peptidyl inhibitors of K channels, namely IbTX and ChTX. These peptides have been reported to increase markedly the contractility of guinea-pig ileum, by virtue of their inhibitory effect on the high-amplitude Ca^{2+}-activated K^+ (BK) channels of the smooth muscle fibers (Suarez-Kurtz *et al*., 1991). IbTX is a selective BK channel blocker (Galvez *et al*., 1990), whereas ChTX blocks BK (Vazquez *et al*., 1989) and the intermediate conductance Ca-activated K^+ channels (Jensen *et al*., 1998) as well as Kv1.2 and Kv1.3 channels (Grissmer *et al*., 1994).

Methods

Preparations

Adult guinea-pigs of either sex and 350–500 g of body weight were kept following the precepts of humane care, in rooms with temperature control and light/dark cycle, and were subjected to euthanasia with CO_2. Peristalsis was studied with a constant intraluminal perfusion system adapted from Costall *et al*. (1993). Briefly, ileal segments (approximately 10 cm in length) were excised, flushed of luminal contents, and cannulated with two plastic tubings, which were used to secure the segment vertically in a cylindrical organ bath of 10 ml capacity, the aboral end of the ileum facing the bottom of the bath. The bath was filled with a physiological saline solution (PSS) kept at 37°C and oxygenated with a mixture of 95% O_2 and 5% CO_2. The upper extremity of the outlet tubing inserted into the aboral end of the segment was adjusted to be 2.5–3 cm above the fluid level of the organ bath. Pre-warmed (37°C) PSS was continuously infused (0.8–1.0 ml min^{-1}) into the intestinal lumen from the oral end, causing gradual filling of the segment and a slow rise of the intraluminal pressure (Barthó & Holzer, 1995). The intraluminal pressure was recorded at the aboral end, using a pressure transducer (model PTA 100, Grass Instruments, Quincy, MA, U.S.A.), coupled to a polygraph (model 2200, Gould, Cleveland, OH, U.S.A.).

When the pressure threshold for peristaltic contraction is reached, an aborally moving wave of circular muscle contraction is triggered, propelling the intraluminal fluid to leave the system, causing emptying of the segment and a spike-like increase in intraluminal pressure. The pressure threshold for peristaltic contraction was used to quantify the drug effects on the peristaltic activity (Costall *et al*., 1993; Holzer & Maggi, 1994), decrease and increase of this threshold reflecting facilitation or inhibition of peristalsis, respectively. Drugs were added to the bathing medium, i.e. to the serosal surface of the ileum segments, after a stable pattern of peristaltic activity was recorded for at least 15 min.

Solutions and chemicals

The PSS was a modified Krebs–Henseleit solution, and had the following composition (in mM): NaCl 120, KCl 5.9, $CaCl_2$ 2.5, $MgCl_2$ 1.1, $NaHCO_3$ 15, NaH_2PO_4 1.2, glucose 11, HEPES 10. The pH after equilibration with 95% O_2 and 5% CO_2 was 7.4 at 37°C. Correolide was obtained as described by Goetz *et al*. (1998) and stock solutions (20 mM) were made up in DMSO. The final concentration of DMSO in the bathing medium (<0.05%) has no effect on the peristaltic activity. MgTX was purified as previously described (Garcia-Calvo *et al*., 1993a), ChTX and IbTX were obtained from Peninsula Laboratories (Belmont, CA, U.S.A.), DTX-K was purchased from Alomone Labs (Jerusalem, Israel), while α-DTX was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). These peptidyl K channel blockers were made up in 100 mM NaCl aqueous solution, containing 0.1% bovine serum albumin (BSA). BSA was also added to the bathing medium to a final concentration of 0.1%, to prevent binding of the peptides to the glass. Control experiments indicated that 0.1% BSA does not affect the peristaltic activity. TTX and atropine sulphate, from Sigma Chemical Co., and the tachykinin antagonists GR 82334 (pGlu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Pro(spiro-lactam)Leu-Trp-NH_2; Hall *et al*., 1992) and GR 94800 (Phenyl-CO-Ala-Ala-D-Trp-Phe-D-Pro-Pro-Nle-NH_2, McElroy *et al*., 1992), from Research Biochemicals International (Natick, MA, U.S.A.) were made up in aqueous solutions.

Statistical analysis

Paired *t*-test was used to compare the values of pressure threshold obtained after each treatment with their respective controls. Student's *t*-test was used to compare values of pressure threshold obtained with two different treatments. Statistical significance was set at $P<0.05$.

Results

Correolide increases the peristaltic activity of guinea-pig ileum

The nortriterpene correolide, a non-selective inhibitor of all Kv1 sub-types, causes a concentration-dependent (>100 nM) reduction of the pressure threshold for eliciting peristaltic contractions (Figure 1). This effect is detectable after a latency of 5–15 min, reaches its maximum after 30–60 min of exposure to correolide and shows no subsequent decline in the continuous presence of the drug (observed up to 3 h). The possibility that the effect of correolide on the pressure threshold involves direct stimulation of muscle contractility was excluded by the observation that either interruption of the perfusion of the ileum segment or blockade of the ENS by TTX (1 μM) abolishes the peristaltic contractions in correolide-treated preparations (Figure 2).

Effects of peptidyl inhibitors of Kv1 channels on the peristaltic activity

With the purpose of identifying which sub-type(s) of the Kv1 channel family accounts for stimulation of peristalsis by correolide, we tested peptidyl inhibitors with different patterns of selectivity for Kv1 sub-types. The results showed that MgTX (1–100 nM), α-DTX (30–100 nM) and DTX-K (30–100 nM), all of which block Kv1.1 channels, either selectively or in addition to Kv1.2 and Kv1.3 (see Introduction), reduce the pressure threshold for peristalsis. In marked contrast, ChTX (10–100 nM), which blocks Kv1.2 and Kv1.3, but not Kv1.1, fails to reduce the pressure threshold for peristalsis (1 h observation). Together, these observations point to Kv1.1 as the target for the effect of MgTX, α-DTX and DTX-K on peristalsis. The recording shown in Figure 3A is a typical example of the time course of the effects of Kv1.1 channel blockers on peristalsis. Addition of MgTX (1 nM) to the bath causes immediate reduction of the pressure threshold for eliciting the peristaltic wave, the maximal effect occurring within 2 min. This effect subsides progressively in the continuous presence of the peptide, and is not restored by raising the MgTX concentration in the bathing medium to 100 nM. The effects of α-DTX and DTX-K on the pressure threshold displayed similar time courses (not shown).

Pooled data from several experiments showed that maximum effects of Kv1.1 inhibitors on the pressure threshold are obtained with 10 nM MgTX, 30 nM α-DTX or 100 nM DTX-K. These data are plotted in Figure 3B and, for comparative purposes, the results are expressed as percent of the respective control values. The values obtained for MgTX (44.7±6.9%), α-DTX (51.7±5.6%), or 100 nM DTX-K (50.2±5.2%) are significantly larger ($P<0.05$, Student's *t*-test) than the corresponding values for 1000 nM correolide (4.4±5.2%, see Figure 1). This finding, plus our previous observation that correolide intensifies the twitching of ileum segments exposed to saturating concentrations of MgTX (Vianna-Jorge *et al*., 2000), led us to investigate the pharmacological interaction of these two Kv1 channel blockers in relation to the pressure threshold for peristalsis. The results indicate that pre-exposure to saturating concentrations of MgTX (100 nM: 30 min) did not affect the ability of correolide to reduce the pressure threshold to less than 10% of the control (pre-MgTX) values ($n=4$, not shown).

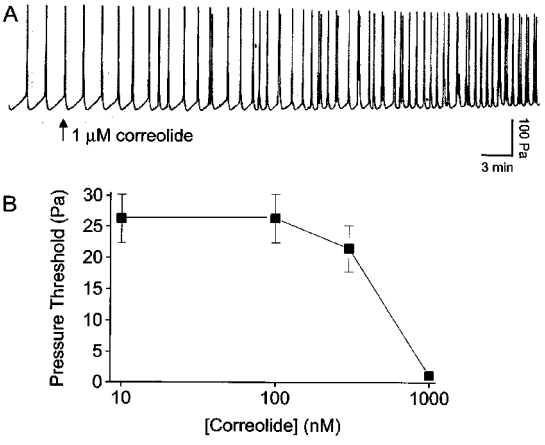

Figure 1 Effects of correolide on the peristaltic activity of a guinea-pig ileum segment. (A) Representative experiment showing the time-course of the correolide-induced reduction of the pressure threshold. (B) Concentration–response curve: Data points (means±s.e.mean, $n=4$ for each correolide concentration) refer to the pressure thresholds measured after 45–60 min of drug exposure.

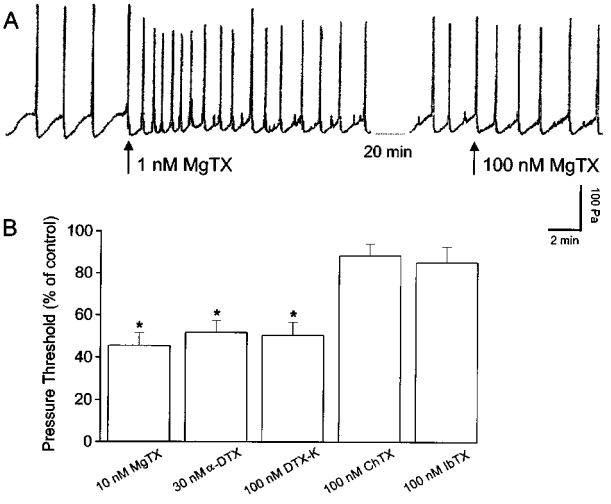

Figure 3 (A) Representative experiment showing the time-course of the effects of MgTX on the peristaltic activity of guinea-pig ileum. (A) Addition of MgTX (1 nM) to the bath caused immediate reduction of the pressure threshold for eliciting a peristaltic contraction, leading to an increase in the frequency of contractions. The effect subsided partially with time in the continuous presence of the toxin, and was not restored by increasing MgTX concentration to 100 nM. (B) Effects of various peptidyl inhibitors of Kv1 and/or BK channels on the peristaltic activity. The mean pressure thresholds recorded within 5 min after administration of MgTX, α-DTX or DTX-K, or 30–45 min after the administration of IbTX or ChTX were used for quantification. The results are expressed as percent of the mean pressure threshold of the respective control, i.e. recorded during 5 min before addition of the peptide. *$P<0.05$ (paired *t*-test for the changes in pressure threshold elicited by each peptide).

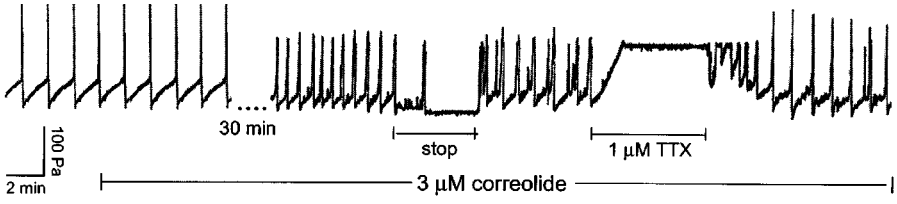

Figure 2 Influence of perfusion flow and TTX on the effects of correolide. The peristaltic activity recorded in the presence of correolide was abolished by either stopping the intraluminal perfusion or by blocking the ENS excitability with TTX (1 μM).

Effects of peptidyl inhibitors of BK channels on the peristaltic activity

Because ChTX blocks BK channels, in addition to Kv1.2 and Kv1.3, its failure in reducing the pressure threshold for peristalsis (Figure 3B) prompted us to examine the effects of IbTX, a selective BK channel blocker (Galvez *et al*., 1990), on peristalsis. The results indicated that IbTX (100 nM, 30–45 min) has no significant effect on the pressure threshold (Figure 3B).

Effects of Kv1 inhibitors in atropine-treated preparations

Atropine (1 μM) consistently increased the pressure threshold for peristalsis and in three out of 15 experiments abolished the peristaltic waves. Correolide (1000 nM, $n=7$) and MgTX (10 nM, $n=8$) retain their abilities to stimulate the peristaltic activity in atropine-treated preparations, whether or not the peristaltic reflex was abolished by atropine (Figure 4A,B). Pooled data from the 12 experiments in which atropine raised the pressure threshold, but did not abolish peristalsis are presented in Figure 4C. In the presence of atropine, the pressure threshold (83.1 ± 13.4 Pa) was significantly greater ($P<0.05$) than in the control (37.1 ± 6.2 Pa). Addition of either MgTX (10 nM, $n=6$) or correolide (1000 nM, $n=6$) significantly reduced the pressure threshold to 59.9 ± 13.4 Pa and 15.9 ± 0.2 Pa, respectively. These values are significantly higher ($P<0.05$) than those obtained for each Kv blocker in the absence of atropine: MgTX, 23 ± 3.2 Pa and correolide, 1.1 ± 0.2 Pa.

The ability of MgTX and correolide to restore the peristaltic reflex or to reduce the threshold for peristalsis in atropine-treated preparations suggests the involvement of other excitatory mediator(s), in addition to acetylcholine. Likely candidates are the tachykinins, which contribute to the contractile effects of correolide on longitudinal segments of guinea-pig ileum (Vianna-Jorge *et al*., 2000). This possibility was investigated by using the tachykinin antagonists GR 82334 (Hall *et al*., 1992) and GR 94800 (McElroy *et al*., 1992), which block NK1 and NK2 receptors, respectively. GR 82334 and GR 94800 (1 μM each) were added, in either sequence ($n=3$, each), to preparations previously treated with atropine and correolide. The results indicate that both tachykinin antagonists are required to abolish the peristaltic activity (Figure 5).

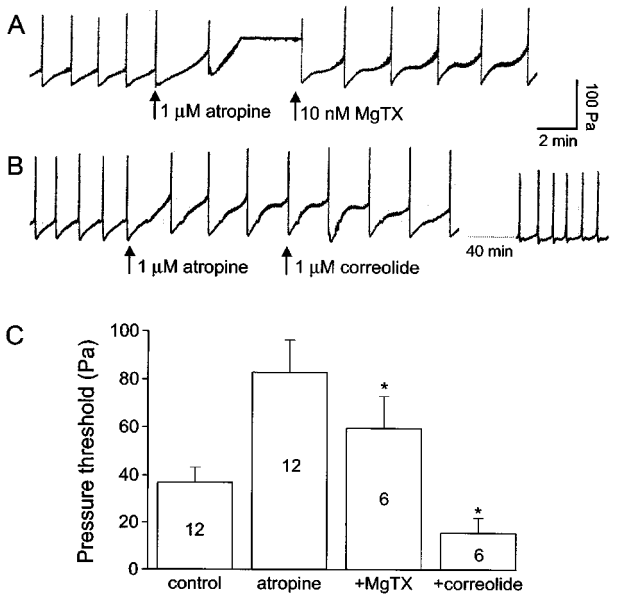

Figure 4 The effects of MgTX and correolide in ileum segments treated with atropine (1 μM). A and B are different preparations. (A) Atropine blocked the peristaltic reflex, which was restored by addition of MgTX (10 nM). (B) Atropine increased the pressure threshold and reduced the frequency of peristaltic contractions. Addition of correolide (3 μM) reversed these effects of atropine. (C) Quantification of the effects of atropine and of MgTX or correolide in atropine-treated preparations: The experimental protocol, similar to that shown in (B), consisted of: After an initial control period (>60 min), atropine (1 μM) was applied in the bathing medium. Ten to fifteen min later, either MgTX (10 nM) or correolide (1000 nM) was added to the atropine-containing bathing medium. The columns show the mean pressure threshold ($\pm$s.e.mean) recorded during the last 10 min of the control period, after 10–15 min of exposure to atropine, and during the first 5 min of exposure to MgTX or after 45–60 min of exposure to correolide. *$P<0.05$ (paired t-test for the reduction in pressure threshold elicited by MgTX or correolide).

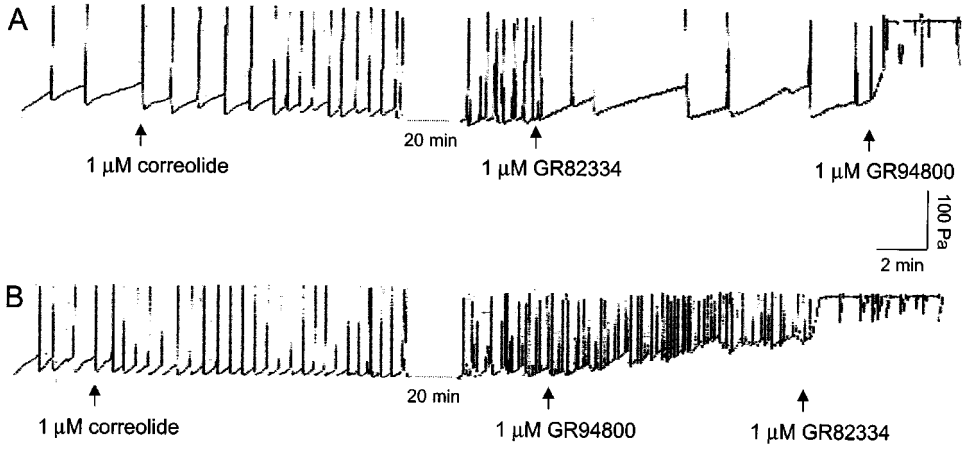

Figure 5 The effects of tachykinin antagonists on the peristaltic activity stimulated by correolide, on two ileum segments (A, B). At the beginning of the recordings, the preparations had been exposed to atropine (1 μM) for 15 min. Correolide (1000 nM), and subsequently GR 82334 and GR 94800 (1 μM each) were applied in the sequences indicated.

Discussion

The present results extend our previous observations (Suarez-Kurtz *et al*., 1999; Vianna-Jorge *et al*., 2000) that Kv1 channels modulate excitation-contraction coupling in guinea-pig ileum. Our previous studies dealt with the ability of peptidyl and non-peptidyl blockers of Kv1 channels to elicit repetitive twitching in guinea-pig ileum strips. This effect was attributed to blockade of Kv1 channels, especially Kv1.1, at preganglionic neurons of the ENS, leading to increased neuronal excitability and enhanced release of acetylcholine and tachykinins, which in turn stimulate the ileum smooth muscle fibres. This proposal, based on the use of selective pharmacological tools, gained support from the subsequent observation that Kv1.1 channels are expressed in the ENS, but not in the smooth muscle fibres of guinea-pig ileum (Hatton *et al*., 2001). We now propose that the same sequence of events, described above for the twitches elicited by Kv1 blockers, accounts for the stimulation of the peristaltic activity induced by correolide, MgTX, α-DTX and DTX-K in guinea-pig ileum. Accordingly, the peristalsis recorded in the presence of these Kv1 blockers is abolished by TTX, which points to involvement of the ENS, and by the association of atropine and blockers of both NK1 (GR 94800; McElroy *et al*., 1992) and NK2 (GR 82334; Hall *et al*., 1992) receptors. The latter observation supports the notion that both acetylcholine, acting on muscarinic receptors, and tachykinins, acting on NK receptors, mediate the stimulation of peristalsis by correolide and by peptidyl inhibitors of Kv1 channels.

In contrast to correolide, which is a selective blocker of the Kv1 channel family, but does not discriminate among Kv1 sub-types (Felix *et al*., 1999) the peptidyl inhibitors tested in the present study differ in their affinity for these sub-types. DTX-K has been reported as a specific inhibitor of Kv1.1 (Robertson *et al*., 1996; Racape *et al*., 2002), and its ability to reduce the pressure threshold for peristalsis would therefore point to this sub-type as a major target for modulating peristalsis in guinea-pig ileum. This notion is supported by the combined observations with three other peptidyl blockers: MgTX and α-DTX, which block Kv1.1, in addition to Kv1.2 and, in the case of MgTX, Kv1.3 channels (Garcia-Calvo *et al*., 1993a), reduced the pressure threshold for peristalsis, whereas ChTX, which blocks Kv1.2 and Kv1.3, but not Kv1.1 (Grissmer *et al*., 1994), failed to do so. A major role for Kv1.1 in modulating the excitability of ENS neurons, and thereby the peristaltic activity, is consistent with our previous results (Suarez-Kurtz *et al*., 1999; Vianna-Jorge *et al*., 2000) and with the demonstrated expression of Kv1.1 channels in the ENS (Hatton *et al*., 2001).

The correolide-induced stimulation of peristalsis is consistent with blockade of Kv1.1 channels. However, in view of the greater magnitude of the effects of correolide on the pressure threshold, as compared to MgTX, α-DTX or DTX-K, it could be suggested that other Kv1 sub-types, which are not targeted by these peptides, are involved. We have previously speculated that Kv1.4 and/or Kv1.5 channels might underlie the greater and more sustained release of excitatory neurotransmitters induced by correolide, as compared to MgTX, α-DTX or DTX-K in guinea-pig ileum (Vianna-Jorge *et al*., 2000).

The effects of the peptidyl blockers of Kv1.1 and of correolide on the pressure threshold for peristalsis differed not only in magnitude but also in time course. Thus, whereas the effect of correolide was detectable after a latency of several minutes, and reached a steady state after 30–60 min, those of peptides were maximal within 5 min, declining subsequently. The slow onset of the correolide effect on peristalsis might be related to the kinetics of this drug interaction with the Kv1 channels in the ENS neurons. Felix *et al*. (1999) demonstrated that correolide binds with high affinity to open and C-type inactivated Kv1.3 channels, but not to channels in the closed state. In firing neurons, the Kv1 channel states which display high-affinity for correolide may be present for only brief periods of time, thus explaining the slow onset of correolide inhibition. Another possible factor for the latency of the correolide effect on peristalsis is that the binding sites for correolide in Kv1 channels appear to be accessible from the intracellular side of the channel. Site-directed mutagenesis studies by Hanner *et al*. (1999) indicated that correolide binds to a transmembrane region which forms the pore. Molecular modeling of this region led to the suggestion that the binding sites for correolide are within a water-filled cavity below the selectivity filter to a hydrophobic pocket (Hanner *et al*., 2001). Thus, blockade of Kv1 channels by correolide requires penetrating across the plasma membrane, which might account for the delayed onset observed in the present study. In contrast, the peptidyl blockers of Kv1 channels bind to the external vestibule of the pore (Hanner *et al*., 1999), which could explain their faster onset of action.

Differently from correolide, which caused a sustained stimulation of peristalsis, the stimulatory effect of MgTX, α-DTX and DTX-K was transient, declining in the continuous presence of the peptides. This decline cannot be ascribed to peptide inactivation, because it is not reversed by increasing the peptides' concentration after the effect has subsided. Taken together, these observations are consistent with our proposal (see above) that the effect of correolide on peristalsis involves other mechanisms, in addition to blockade of Kv1.1 channels.

Finally, the results observed with the high-affinity blockers of BK channels, IbTX and ChTX, suggest that these channels do not modulate peristalsis in guinea-pig ileum. This contrasts markedly with the increase in tonus and myogenic activity of ileum strips, due to blockade of BK channels in the smooth muscle fibres by IbTX and ChTX, or by the indole alkaloids, paxilline and paspalitrem-C (De Farias *et al*., 1996; Suarez-Kurtz *et al*., 1997; 1999). Together, these observations support the notion that the main target of K channel blockers in modulating peristalsis resides in the enteric nervous system, rather than the smooth muscle fibres.

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