Peptidyl Inhibitors of Shaker-Type Kv1 Channels Elicit Twitches in Guinea Pig Ileum by Blocking Kv1.1 at Enteric Nervous System and Enhancing Acetylcholine Release

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ABSTRACT
Potent and selective peptidyl blockers of the Shaker-type (Kv1) voltage-gated potassium channels were used to determine the role of these channels in regulating the spontaneous motility of smooth muscle preparations. Margatoxin (MgTX), kaliiotoxin, and agitoxin-2 at 1 to 10 nM and agitoxin-1 at 50 to 100 nM induce twitches in guinea pig ileum strips. These twitches are abolished by tetrodotoxin (TTX, 0.5 μM), atropine (1 μM), hexamethonium (10 μM), or nifedipine (0.1 μM). It is proposed that blockade of Kv1 channels by MgTX, kaliiotoxin, or the agitoxins increases excitability of intramural nerve plexuses in the ileum, promoting release of acetylcholine from excitatory motor nerve terminals. This, in turn, leads to Ca2+-dependent action potentials and twitching of the muscle fibers. MgTX does not induce twitches in several other guinea pig and/or rat vascular, genitourinary, or gastrointestinal smooth muscles, although small increases in spontaneous myogenic activity may be seen in detrusor muscle exposed to >30 nM MgTX. This effect is not reversed by TTX or atropine. The TTX- and atropine-sensitive twitches of guinea pig ileum are also induced by nanomolar concentrations of α-dendrotoxin, a selective blocker of Shaker K1.1 and 1.2 subtypes, or stichodactylatoxin, a peptide isolated from sea anemone that displays high affinity for Kv1.1 and 1.3, but not by charybdotoxin, which blocks Kv1.2 and 1.3 but not 1.1. The data taken together suggest that high-affinity blockade of Kv1.1 underlies the ability of MgTX, kaliiotoxin, agitoxin-1, agitoxin-2, α-dendrotoxin, and stichodactylatoxin to elicit TTX-sensitive twitches in guinea pig ileum.

The spontaneous motility and tonus of smooth muscle tissues are modulated by K+ channels, which provide pathways for repolarizing outward currents and thereby affect resting membrane potential and membrane excitability. K+ channels comprise a large family of proteins that differ in their biophysical and pharmacological properties. Several members of this family have been identified in smooth muscle, but their precise function in most tissues has not been established. The availability of potent, selective modulators of various K+ channels provides a pharmacological approach for exploring the physiological and possible pathophysiologic function of the respective conductance pathways in different target tissues. Several peptides purified from scorpion venoms interact with specific types of K+ channels. Charybdotoxin (ChTX), a 37-amino acid peptide isolated from the Old World scorpion Leirus quinqueniatritus var hebraeus (Gimenez-Gallego et al., 1988), is one of the best-studied peptides. Originally described as a selective inhibitor of the high-conductance Ca2+-activated K+ (maxi-K) channel (Miller et al., 1985), ChTX was later found to inhibit several small conductance Ca2+-activated K+ channels (Hermann and Erxleben, 1987) and voltage-gated K+ channels (Sands et al., 1989; Christie et al., 1989; Leonard et al., 1992). In each case, channel inhibition occurs with similar potency, in the low nanomolar range. A related peptide, iberiotoxin (IbTX), which shares 68% sequence homology with ChTX, is a selective blocker of the maxi-K channel (Galvez et al., 1990). Suarez-Kurtz et al. (1991) used IbTX and ChTX to examine the role of the maxi-K channel in regulating the contractility of different smooth muscle tissues isolated from the guinea pig. Their results revealed that the maxi-K chan-

ABBREVIATIONS: Kv1 channels, Shaker-type voltage-gated K+ channels; maxi-K channel, high-conductance Ca2+-activated K+ channel; ChTX, charybdotoxin; IbTX, iberiotoxin; MgTX, margatoxin; KTX, kaliiotoxin; AgTX1, agitoxin-1; AgTX2, agitoxin-2; α-DaTX, α-dendrotoxin; ShK, stichodactylatoxin; ShKDAP22, Stichodactyla helianthus mutant toxin.
nel affects excitation-contraction coupling processes in smooth muscle in a tissue-specific fashion. Comparison of results obtained in different species (guinea pig versus rat) led to the suggestion that the role played by the maxi-K channel in smooth muscle myogenic activity may also be species dependent.

This study was initiated to investigate the effects of several other peptidyl K\(^+\)-channel blockers, namely, margatoxin (MgTX, from Centruroides margaritatus; Garcia-Calvo et al., 1993), kaliotoxin (KTX, from Androctonus mauretanicus mauretanicus; Crest et al., 1992), and the agitoxins AgTX\(_1\) and AgTX\(_2\) (from Leiurus quinquestriatus var hebraeus; Garcia et al., 1994) on the spontaneous motility of isolated guinea pig and rat smooth muscle tissues. These peptides are potent inhibitors of Shaker-type voltage-gated K\(^+\) channels (K\(_v1.1\)), especially 1.1, 1.2, and 1.3 subtypes, but display no affinity for the mammalian maxi-K channel (reviewed by Garcia et al., 1997). This study reveals that nanomolar concentrations of MgTX, KTX, AgTX\(_1\), and AgTX\(_2\) induce twitches in guinea pig ileum but not in various other smooth muscle preparations from guinea pig or rat. This selective effect of MgTX, KTX, and both agitoxins in ileum strips differs markedly, both in its time course and sensitivity to blockade by tetrodotoxin (TTX), atropine, or hexamethonium, from the previously reported stimulation of spontaneous myogenic activity of bladder detrusor and other smooth muscle preparations by the maxi-K channel blockers IbTX and ChTX (Suarez-Kurtz et al., 1991). Moreover, other peptidyl blockers of K\(_v1.1\) channels, such as \(\alpha\)-dendrotoxin (\(\alpha\)-DaTX, from the snake Den\(d\)rao\(s\)pis angu\(s\)te\(c\)eps; Duf\(f\)ton and Harvey, 1998) and stichodactylatoxin (ShK, from the sea anemone Stichodactyla heli\(a\)ntus; Kalman et al., 1998), reproduced the effects of MgTX on the guinea pig ileum. Given the specificity of all of these different peptides, it appears that high-affinity blockade of K\(_v1.1\) underlies the ability of MgTX, KTX, AgTX\(_1\), AgTX\(_2\), \(\alpha\)-DaTX, and ShK to stimulate excitatory motor neuron pathways in the enteric nervous system and elicit TTX-sensitive twitches in guinea pig ileum. Our data support the contention (Suarez-Kurtz et al., 1991) that peptidyl blockers, because of their selectivity and high-affinity binding to distinct K\(^+\) channels, provide excellent pharmacological tools for investigating the functional role(s) of the targeted channels in excitation-contraction coupling in smooth muscle.

Materials and Methods

Preparations. Experiments were performed at 37°C on tissues obtained from adult guinea pigs or Wistar rats (Charles River) after death by ether inhalation. Guinea pigs provided portal vein, vas deferens, duodenum, taenia coli, ileum, and urinary bladder strips; rats provided portal vein, duodenum, uterus, and bladder strips. A 1-g load was initially applied to all preparations, because previous studies from our laboratories have shown that this allows stable tension recordings from all preparations for several hours. For recording muscle tension, the preparations were mounted between two metal stirrups, of which the lower was fixed and the upper was attached to a rigid wire connected to a force-displacement transducer (Grass FT-03; Grass Instruments Co., Quincy, MA). The transducer signals were amplified and recorded on a Grass polygraph (model 7). In some experiments, the amplified signals were fed into an integrator (Grass T7P10) for quantitation of the myogenic activity, as described by Suarez-Kurtz et al. (1991). Briefly, the zero level for integration was set at 5 to 10% of the average amplitude of the “basal” spontaneous tension oscillations, recorded between 40 and 60 min after mounting the preparations in the muscle chamber and immediately before exposure to the lowest concentration of the toxin being tested. Integrated activity after exposure to the toxins is expressed relative to the basal (toxin-free) activity. Electrical stimulation of motor neurons of the enteric nervous system in guinea pig ileum was applied via two platinum ring electrodes, placed around the strips 6 mm apart. The contractions evoked by trains (4 s, 1–8 Hz) of square-wave pulses of 0.2-ms duration and supramaximum intensity, applied at 1- to 2-min intervals, could be completely blocked by 1 \(\mu\)M TTX.

Solutions and Chemicals. The physiological saline solution, a modified Krebs-Henseleit solution, had the following composition: 120 mM NaCl, 5.9 mM KCl, 2.5 mM CaCl\(_2\), 1.1 mM MgCl\(_2\), 15 mM NaHCO\(_3\), 1.2 mM NaH\(_2\)PO\(_4\), 11 mM glucose, and 10 mM HEPES. The pH of this solution after equilibration with 95% O\(_2\) and 5% CO\(_2\) was 7.3 at 37°C. MgTX, AgTX\(_1\), AgTX\(_2\), and KTX were expressed in Escherichia coli as part of a fusion protein, cleaved, and purified as described (Koch et al., 1997; Koschak et al., 1998). ChTX, IbTX, and ShK were obtained from Peninsula Laboratories (Belmont, CA); \(\alpha\)-DaTX, TTX, atropine sulfate, and hexamethonium hydrobromide from Sigma Chemical Co. (St. Louis, MO); and suramin from Calbiochem (La Jolla, CA).

Pooled data from identical experiments are presented as means ± S.E.M.

Results

Effects of MgTX on Smooth Muscle Contractility. Previous studies (Winquist et al., 1989; Suarez-Kurtz et al., 1991) have shown that the concentrations of IbTX and ChTX required for stimulation of the spontaneous motility of guinea pig and rat smooth muscle preparations are in the range 10 to 100 nM. Thus, we initially tested these concentrations of MgTX on the force of contraction of different smooth muscle preparations, and results of these experiments are illustrated in Fig. 1. MgTX has no detectable effect on guinea pig portal vein but increases the spontaneous motility of bladder and ileum. However, the response of the latter two tissues to MgTX differs in several aspects: 1) unequivocal stimulation of the bladder detrusor muscle requires 100 nM MgTX but is evident in the ileum on exposure to 10 nM MgTX. Indeed, increasing the peptide concentration

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**Fig. 1.** Effect of MgTX on contractility of guinea pig smooth muscles. Isometric tension recordings were made from portal vein and strips of urinary bladder and ileum. Integrated mechanical activity is shown below tension recordings. Preparations were exposed to increasing concentrations of MgTX at 10-min (C) or 20-min (A, B) intervals. Calibration bars: horizontal, 5 min (A, B) or 2.5 min (C); vertical, 1 g.
to 30 or 100 nM has no additional stimulatory effect in the ileum (see below). 2) In ileum, but not in bladder, MgTX induces fast twitches of relatively large amplitude but little or no change in baseline tension or in amplitude of the slow tension oscillations. In contrast, in detrusor muscle, MgTX increases both the baseline tension and the amplitude of the slow tension oscillations. 3) The time course of development of the stimulatory effects of MgTX in ileum and bladder differs markedly. Whereas the myogenic activity of detrusor muscle increases progressively throughout the 20-min exposure to MgTX, twitching in ileum was maximum within seconds after addition of the peptide to the bathing medium. The pattern of the contractile response of guinea pig ileum to MgTX was not observed in various other preparations such as guinea pig or rat portal vein, detrusor muscle, or duodenum; guinea pig vas deferens; or rat uterus. Some of these tissues, however, respond to 100 nM MgTX in a manner similar to that seen in guinea pig bladder (Fig. 1). Integration of the isometric tension data (see Materials and Methods) reveals that the largest increases in spontaneous myogenic activity occur in the detrusor muscle of guinea pig (2.4 ± 0.5, N = 7) and rat (1.8 ± 0.4, N = 4). These relatively small effects, compared with the response of detrusor muscle to the maxi-K channel blocker IbTX (Suarez-Kurtz et al., 1991), were not investigated further in this study. Thus, the experiments described in the following sections deal with the specific response of guinea pig ileum to MgTX.

**MgTX-Induced Twitching in Guinea Pig Ileum Strips.** As shown in Fig. 1C, increasing the concentration of MgTX above 10 nM causes no additional stimulation of twitching; i.e., the response appears to saturate at 10 nM. This pattern was observed in 14 of 19 guinea pig ileum strips. Of the 5 strips that did not respond to 10 nM MgTX, 3 developed twitches when the toxin concentration was raised to 30 nM, but 2 failed to respond even when challenged with 100 nM MgTX. In 8 other strips, exposed sequentially at 10-min intervals to 1 and 3 nM MgTX, twitching was induced in 3 strips exposed to 1 nM (Fig. 2A) and in 2 others when the toxin concentration was raised to 3 nM. Occasionally, the ileum strips exhibited spontaneous twitching in the control recording conditions; in these strips, MgTX (1–10 nM) increases the frequency of the twitches, with no effect on either their amplitude or time course (Fig. 2, B and C). The time course of these twitches was comparable to that elicited by short (5-s; 4-Hz) trains of pulses of 0.2-ms duration and supramaximal amplitude, which stimulate the enteric excitatory motor neurons (Fig. 2C). The twitches recorded in the absence and presence of MgTX were abolished by nifedipine (0.1 μM; not shown), which is consistent with their dependence on the activity of L-type Ca2+ channels to promote Ca2+ influx across the sarcolemma.

MgTX (1–100 nM, 10- to 30-min exposure) has no effect on the time course or amplitude of the contractions elicited by electrical stimulation of the enteric motor nerve terminals, with trains (2–8 Hz) of supramaximal pulses (see Materials and Methods). The electrically evoked contractions were abolished by nifedipine (0.1 μM) or atropine (0.1–0.5 μM) but were only partially (<20%) reduced by hexamethonium (50 μM).

**Pharmacological Interaction between MgTX and TTX in Guinea Pig Ileum.** The high-affinity interaction of MgTX with certain Kv1 channels in mammalian nervous tissue (Knaus et al., 1995; Helms et al., 1997; Koschak et al., 1998) led us to investigate whether peptide-induced twitches in guinea pig ileum could result from increased excitability of excitatory motor pathways in the enteric nervous system, secondary to Kv1-channel blockade in the plasmalemma. The results shown in Fig. 3A support this idea, because TTX (0.1–0.5 μM) causes a concentration-dependent inhibition of MgTX-induced twitches but does not affect the spontaneous myogenic activity of the strip. TTX, however, does not prevent (Fig. 3A) or reverse the IbTX-induced increase in spontaneous contractility of ileum strips (not shown). Thus, different mechanisms must underlie the stimulatory effects of MgTX and IbTX on ileum contractility, consistent with their selective effects on Kv1 and maxi-K channels, respectively. In fact, as shown in Fig. 3B, blockade of these two types of K+ channels with MgTX and ChTX results in additive effects on muscle contractility, although the contribution of each toxin can be clearly recognized by differences in the time course of tension responses and by their distinct sensitivity to TTX blockade. Figure 3C provides confirmatory evidence that TTX does not affect the ChTX-induced stimulation of myogenic activity; in contrast, nifedipine abolishes the stimulatory effect of ChTX. The finding that ChTX does not reproduce the MgTX-induced twitching is significant because ChTX blocks with similar potency the maxi-K channel and the Kv1.2 and 1.3 channel subtypes. This suggests that ChTX-induced tensions are probably caused by interaction with maxi-K channels and that Kv1.2 and Kv1.3 are not the channels involved in MgTX-induced twitching in ileum strips (see Discussion).
Effects of Suramin, Atropine, and Hexamethonium on MgTX-Induced Twitches in Guinea Pig Ileum. MgTX-induced twitches are unaffected by suramin (100 μM), a nonselective purinergic antagonist (not shown), but can be abolished by the muscarinic antagonist atropine (1.0 μM, Fig. 4A) or by the ganglionic blocking agent hexamethonium (10 μM, Fig. 4B). These observations suggest that the MgTX-induced twitches require the functional integrity of nicotinic receptors in ganglion cells of the enteric nervous system and release of acetylcholine by excitatory motor neurons. The possibility that increased sensitivity of muscarinic receptors to the neurotransmitter acetylcholine might contribute to the MgTX-induced twitching was ruled out by the experimental observation that MgTX (10–30 nM) does not affect the tonic tension induced by acetylcholine (0.5 μM) in TTX-treated ileum strips (not shown).

In contrast to their effects on the MgTX-induced twitching, atropine (1.0 μM) and hexamethonium (50 mM) do not reverse the stimulation of contractility induced by peptidyl (ChTX and IbTX) or nonpeptidyl blockers of maxi-K channels in guinea pig ileum strips (DeFarias et al., 1996).

Effects of KTX and Agitoxins in Guinea Pig Ileum and Bladder Strips. The availability of other high-affinity peptidyl inhibitors of K_v channels, i.e., KTX, AgTX_1, and AgTX_2, led us to investigate their effects on the contractility of guinea pig ileum and bladder detrusor muscle. These preparations were chosen because of their sensitivity to MgTX or to peptidyl blockers of the maxi-K channel, respectively. Figure 5 shows that either KTX or AgTX_2 at 10 nM or AgTX_1 at 50 nM stimulates the contractility of guinea pig ileum, and this effect shares many of the characteristics described above for MgTX; e.g., 1) twitches of large amplitude and fast time course are elicited within seconds after addition of each peptide to the bath; 2) no increase in baseline tension or amplitude of the slow tension oscillations is observed during 10- to 20-min exposure to the peptides; and 3) the peptide-induced twitches are abolished by TTX.

In contrast to their stimulatory effect in guinea pig ileum, KTX and AgTX_1 (1–100 nM) do not affect the spontaneous motility of detrusor muscle (N = 4 for each toxin), whereas AgTX_2 (100 nM) causes a 2.2 (±0.3, N = 4) increase in this muscle’s integrated myogenic activity. This effect was not reversed by either TTX or atropine (not shown).

Effects of α-DaTX and ShK in Guinea Pig Ileum. Our observation (Fig. 3A) that ChTX, a potent blocker of K_v1.2 and K_v1.3 channels, does not elicit twitching in ileum strips suggested that other K_v subtypes must be the target for the TTX-sensitive twitches elicited by MgTX, KTX, or the agitoxins.
ins. Because these peptides display high affinity for Kv1.1, we investigated whether their effects on the contractility of ileum strips are reproduced by other peptidyl blockers of Kv1.1, such as α-DaTX and ShK. The results indicated that 10 to 30 nM α-DaTX or 3 to 10 nM ShK induce TTX-sensitive twitches in guinea pig ileum (Fig. 6). The mutant ShKDAP22, which displays lower affinity than ShK for Kv1.1 (Kalman et al., 1998), was less effective at eliciting twitches than wild-type ShK, with concentrations of 50 to 100 nM ShKDAP22 being required for this effect (Fig. 6). The twitches elicited by α-DaTX or the wild-type ShK or ShKDAP22 are abolished by 1.0 μM atropine or 10 μM hexamethonium (not shown).

Discussion

This study reveals a selective pharmacological effect of high-affinity peptidyl blockers of the Shaker-type Kv channels, that is, their ability to elicit twitches in guinea pig ileum. This effect was consistently observed with 1 to 10 nM MgTX, KTX, AgTX2, α-DaTX, or ShK or with 50 to 100 nM AgTX1 or ShKDAP22 but was not reproduced by MgTX (100 nM) in other gastrointestinal, vascular, or genitourinary smooth muscle preparations isolated from guinea pig or rat. The twitches induced in guinea pig ileum by MgTX, KTX, AgTX1, α-DaTX, ShK, and ShKDAP22 are blocked by TTX, suggesting that Kv channels in the enteric nervous system rather than the smooth muscle fibers are the peptide’s targets. Consistent with this idea, twitches elicited in the absence of TTX are abolished by hexamethonium, a ganglionic blocking agent, or by atropine, a muscarinic antagonist. All the data taken together strongly suggest that the muscle twitches require functional integrity of nicotinic ganglionic transmission and release of acetylcholine from excitatory motor neurons and that this release is the result of blockade of Kv1.1 channels in the enteric nervous system.

The idea that Kv1.1 channels are responsible for enhanced acetylcholine release can be easily understood in view of the specificity of the peptides used in this study. Thus, contributions of channels other than Kv1.1, such as maxi-K or Kv2, Kv3, or Kv4 channels, can be initially eliminated. Within the Kv1 family of channels, only Kv1.1, Kv1.2, and Kv1.3 are sensitive to MgTX. However, the fact that ChTX, a high-affinity blocker of Kv1.2 and Kv1.3, does not reproduce the effect of MgTX suggests that Kv1.1 is the relevant target. Consistent with this idea are the results obtained with all other peptides investigated herein (AgTX, AgTX2, ShK, ShKDAP22, KTX, and α-DaTX). An alternative approach to identifying the channels responsible for MgTX-induced twitches involves the labeling of these channels with 125I-MgTX and their solubilization followed by immunoprecipitation with specific site-directed antibodies. Only in this way will it be possible to directly determine whether the target channel exists in a homo- or heteromeric structure. Notably, the peptides used in this study would usually block Kv1.1 regardless of whether this subunit exists with other Kv1.X subunits in the tetrameric K+ channel structure.

The selective stimulatory effect of MgTX on the contractility of isolated ileum strips may provide an explanation for the diarrhea observed in Yucatan and Hanford miniature pigs treated with MgTX i.v. as part of a study of this toxin’s immunosuppressive effects (Koo et al., 1997). Significantly, atropine, which inhibited MgTX-induced stimulation of isolated ileum motility, opposed the diarrhea observed in vivo (G. Koo, unpublished observations).

MgTX, KTX, and the agitoxins induced no or small (<2.5-fold) increases in integrated myogenic activity of the various preparations tested, the detrusor muscle of rat or guinea pig being the most sensitive to these peptides. This contrasts with the much greater (>10-fold) stimulation of spontaneous contractility of detrusor muscle induced by maxi-K channel blockers (Suarez-Kurtz et al. 1991; DeFarias et al., 1996). Thus, Kv1.1 channels seem to play a relatively minor functional role in excitation-contraction coupling in the smooth muscles examined here, with the notable exception of guinea pig ileum. Because the stimulatory effects of MgTX or AgTX2 in detrusor muscle were insensitive to TTX or atropine, it appears that the targeted Kv channels are in the smooth muscle fibers rather than in nervous tissue. However, we did not investigate the mechanism(s) underlying the stimulatory effects of Kv1 peptidyl blockers on detrusor muscle contractility.

In conclusion, we have demonstrated a selective effect of peptidyl blockers of Kv1 channels on the motility of guinea pig ileum, which we ascribe to blockade of voltage-dependent Kv1.1 channels in the plasmalemma of nerve fibers in intramural plexuses. These data support our contention (Suarez-Kurtz et al., 1991) that peptidyl inhibitors, because of their selectivity and high-affinity binding to distinct K+ channels, provide excellent pharmacological tools for investigating the functional role(s) of the targeted channels in excitation-contraction coupling in smooth muscle.

References


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