MIT_1, a black mamba toxin with a new and highly potent activity on intestinal contraction

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Abstract Mamba intestinal toxin (MIT_1) isolated from *Dendroaspis polylepis* venom is a 81 amino acid polypeptide cross-linked by five disulphide bridges. MIT_1 has a very potent action on guinea-pig intestinal contractility. MIT_1 (1 nM) potently contracts longitudinal ileal muscle and distal colon, and this contraction is equivalent to that of 40 mM K^+. Conversely MIT_1 relaxes proximal colon again as potently as 40 mM K^+. The MIT_1-induced effects are antagonised by tetrodotoxin (1 μM) in proximal and distal colon but not in longitudinal ileum. The MIT_1-induced relaxation of the proximal colon is reversibly inhibited by the NO synthase inhibitor L-NAME (200 μM). ^{125}I-labelled MIT_1 binds with a very high affinity to both ileum and brain membranes (K_d = 1.3 pM and 0.9 pM, and B_{max} = 30 fmol/mg and 26 fmol/mg, respectively). MIT_1 is a very highly selective toxin for a receptor present both in the CNS and in the smooth muscle and which might be an as yet unidentified K^+ channel.

Key words: Venom toxin; Intestinal muscle; Ion channel; Contraction

1. Introduction

Mamba venoms contain an unusually high diversity of pharmacologically active peptides [1]. Two particularly important structural groups have been identified so far. The first group comprises a series of toxins homologous to the basic pancreatic trypsin inhibitor. They are the dendrotoxins which are powerful blockers of some of the Kv1,*n* class of K^+ channels and display a strong central neurotoxicity [2–8]. Besides dendrotoxins and other protease inhibitors with a weaker activity on K^+ channels, this same structural class comprises calcicludins which are blockers of high voltage activated Ca^{2+} channels with a particular potency for L-type Ca^{2+} channels in cerebellar granule neurones [9]. The second structural group contains peptides which are structural homologues to curaremimetic α-neurotoxins. This structural group comprises short and long α-neurotoxins which block the nicotinic receptor, muscarinic toxins which act selectively as agonists or antagonists on one or several of the muscarinic receptor types [10–12], anticholinesterase toxins [13,14], '*angusticeps*-type' peptides with low toxicity and unknown molecular targets, and calciseptins which are specific blockers of L-type Ca^{2+} channels mainly in the cardiovascular system [15]. The remaining group of peptides contained in mamba venoms is heterogeneous both in peptide size and in structural arrangement if known at all. The function of most peptides in this latter class has not yet been elucidated.

This paper deals with a peptide belonging to this latter relatively unexplored class of peptides. The toxin named mamba intestinal toxin 1 (MIT_1) is a polypeptide with 81 amino acids and five disulphide bridges. The solution structure of MIT_1 has been recently determined at a resolution of 0.5 Å and has revealed a new type of folding for venom toxins similar to that of colipase, a protein involved in fatty acid digestion [16]. We show that MIT_1 has the unique property of contracting guinea-pig ileum and distal colon while relaxing the proximal colon. MIT_1 has very high affinity binding sites in membranes from both guinea-pig ileum and rat brain, which appear to be unique to this toxin.

2. Materials and methods

2.1. Purification of MIT_1 and sequence determination

MIT_1 was purified from the venom of the black mamba *Dendroaspis polylepis* as previously described (fraction G4 from Fig. 1 in [17]). The primary structure of MIT_1 was determined by Edman degradation of the complete polypeptide using an Applied Biosystems model 477 A microsequencer. The polypeptide was reduced with 2-mercaptoethanol and pyridylethylated with 4-vinylpyridine before sequencing. The C-terminal part of the polypeptide was obtained by splitting at Met-55 with BrCN (MIT_1 was incubated for 24 h at room temperature with 0.1 mg/ml BrCN in 70% formic acid) and separation on a microbore C_{18} reverse phase column. The C-terminal amino acid was confirmed by digestion with carboxypeptidase. A spontaneous hydrolysis of the C-terminal serine was observed. Lasermat mass spectroscopy shows the progressive disappearance of the peak at 8607 Da and concomitant appearance of a peak at 8506 Da. The molecular weight determined by electrospray ionisation mass spectrometry yielded a value of 8506.4, in accordance with the value deduced from the sequence determination (8506.9) for the polypeptide MIT_1 without the C-terminal serine. MIT_1 is very similar to the polypeptide 'protein A' purified from the same venom [26], the main difference being the inversion of cysteine 18 with serine 22 which may be due to a previous error in the sequence determination. Lysine 55 is deleted in MIT_1 and the labile C-terminal serine is absent in protein A.

2.2. Toxicity measurements

They were carried out as previously described [18].

2.3. Contraction measurements on isolated organs

Adult guinea-pigs were killed by decapitation. Samples of longitudinal muscle of the ileum were taken by pinching and stretching the ileum in the vicinity of the caecum. Pieces of proximal colon were cut off in the vicinity of the caecum and pieces of distal colon in the vicinity of the rectum. All pieces were cut 1.5 cm long and mounted vertically in 3 ml organ baths filled with a Tyrode medium containing

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Abbreviations: MIT_1, mamba intestinal toxin; BSA, bovine serum albumin; TTX, tetrodotoxin; L-NAME, L-N^G-nitroarginine methyl ester; NO, nitric oxide

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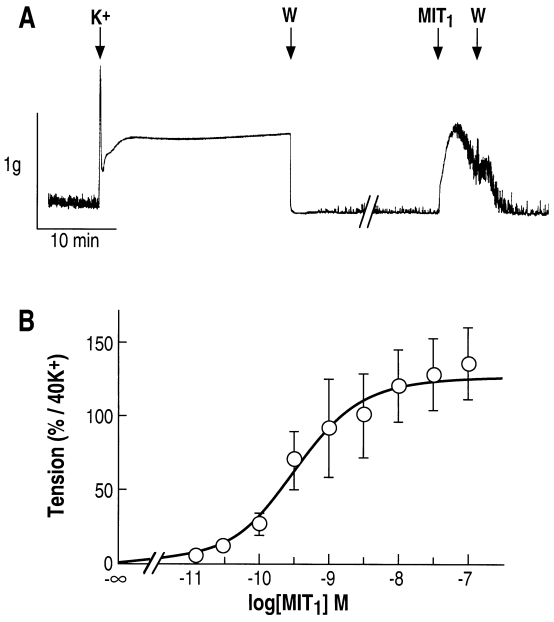

Fig. 1. Effect of MIT_1 on the contractility of guinea-pig ileum longitudinal muscle. A: Contractions of ileum longitudinal muscle by increasing the external K^+ concentration to 40 mM and by 1 nM MIT_1. B: Concentration-response curve of guinea-pig ileum longitudinal muscle contraction obtained with MIT_1. Results are given as % of the maintained contraction obtained with 40 mM K^+. W: wash. The data are mean values from two to eight experiments.

137 mM NaCl, 2.7 mM KCl, 1 mM $MgSO_4$, 0.5 mM NaH_2PO_4, 12 mM $NaHCO_3$, 1.8 mM $CaCl_2$ and 5 mM glucose. This medium was oxygenated by carbogen. Adult rats were killed by decapitation. Their hearts and thoracic aortas were immediately excised and dissected in the above medium for aorta and for heart in Tyrode medium containing 127 mM NaCl, 4 mM KCl, 1 mM $MgSO_4$, 0.5 mM NaH_2PO_4, 12 mM $NaHCO_3$, 1.8 mM $CaCl_2$ and 5 mM glucose which was also oxygenated by carbogen. Right and left atria were mounted vertically in the 3 ml baths as well as rings of de-endothelialised aorta. The media were thermostatted at 37°C. Strips and organs were fixed to a UL5 microbalance screwed to a UC2 isometric force transducer which was connected to a model -50 amplifier. Tensions were recorded on a Gould 2600 polygraph. Initial preloads were 0.5 g for intestine strips, 1 g for atria and 2 g for aorta rings. Left atria were stimulated at 1 Hz through field electrodes using square wave pulses of 2 ms and a voltage which was 50% above threshold.

2.4. Iodination

MIT_1 was iodinated by the iodogen method. MIT_1 (0.5 nmol) was incubated for 15 min at room temperature with 0.5 nmol (1 mCi) of ^{125}INa (Dupont-NEN) in 40 μl of 100 mM Tris-HCl pH 7.4 in an Eppendorf tube coated with 3 nmol iodogen. The reaction was stopped by addition of 400 μl of 1 mg/ml bovine serum albumin (BSA) in 0.1% trifluoroacetic acid (solvent A). BSA and free iodine were removed by a chromatographic step on a Symmetry C_{18} (4.6×250 mm) Waters column. With a gradient from 10% to 40% of solvent B (0.1% trifluoroacetic acid in acetonitrile) in 30 min, ^{125}I-MIT_1 eluted as a thin single peak at 22.5 min, the maximum of radioactivity being superimposed on the maximum of absorbance. Assuming that all the radioactivity found in this peak has been incorporated in 0.5 nmol of MIT_1, we evaluated the specific radioactivity as 1639 cpm/fmol.

2.5. Binding to membranes

Membranes used were P3 pellets obtained by homogenisation and differential centrifugations of whole rat brain and guinea-pig ileum smooth muscle obtained by removing the epithelium by scratching. Incubations and filtrations were done as described earlier [9] but in 20 mM Tris-HCl pH 7.4 with 1 mg/ml BSA as incubation medium.

2.6. Electrophysiological measurements of cloned K^+ channels expressed in Xenopus oocytes

Cloning of cDNAs, synthesis of complementary RNAs, preparation of oocytes, cRNA injections and electrophysiological measurements have been previously described [19].

2.7. Patch-clamp recording of cloned K^+ channels expression in transfected COS cells

COS cells were grown and transfected as described earlier [19]. Cloning of cDNA and subcloning in plasmid have been detailed in [20,21]. Electrophysiological measurements on COS cells were performed 48 h after the transfection using the whole cell configuration of the patch-clamp technique. For K^+ channel recordings, the pipette solution contained KCl 150 mM, $MgCl_2$ 3 mM, EGTA 5 mM, HEPES-KOH 10 mM, pH 7.4. The external solution contained KCl 5 mM, NaCl 150 mM, $CaCl_2$ 1 mM, $MgCl_2$ 3 mM, HEPES-NaOH 10 mM, pH 7.4. For proton-gated Na^+ channels, the pipette solution contained KCl 140 mM, $MgCl_2$ 2mM, $CaCl_2$ 1.8 mM, HEPES-NaOH 10 mM, pH 7.4.

3. Results

3.1. Primary structure and toxicity of MIT_1

MIT_1 is a 81 amino acid polypeptide with 10 cystine residues which form five disulphide bridges. The primary structure of MIT_1 is: A V I T G A C E R D L Q C G K G T C C A V S L W I K S V R V C T P V G T S G E D C H P A S H K I P F S G Q R M H H T C P C A P N L A C V Q T S P K K F K C L S K S. The solution structure has recently been determined and shows that MIT_1 does not belong to any class of proteins isolated from snake venoms so far [16].

Intraperitoneal injection of MIT_1 in mice (5–10 μg/g body weight) produced only transient effects. Injected mice had their hind legs stretched out and lacked muscle tone for about 1 h before they recovered a normal motion. Intracisternal injection of MIT_1 at 0.8 μg/g body weight induced small bounds for less than 2 h. At higher doses (1.6–5 μg/g), animals again had their hind legs stretched out and lacked muscle tone, but these effects lasted more than 17 h and were associated with a reduced motion.

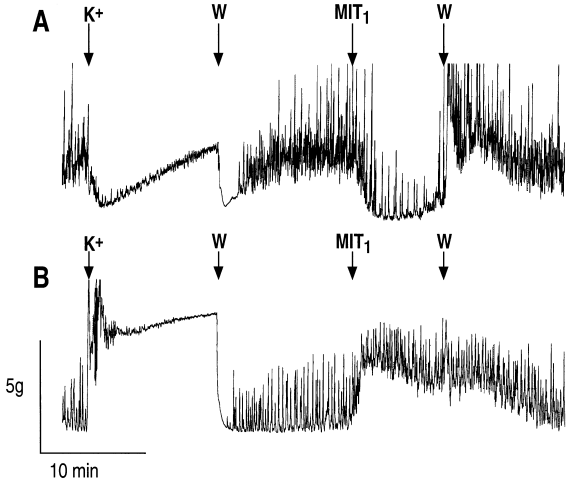

Fig. 2. Effect of MIT_1 on the contractility of guinea-pig colon. A: Relaxation of proximal colon induced by 40 mM K^+ or 1 nM MIT_1. B: Contraction of distal colon induced by 40 mM K^+ or 1 nM MIT_1. The data presented are representative of six to eight experiments.

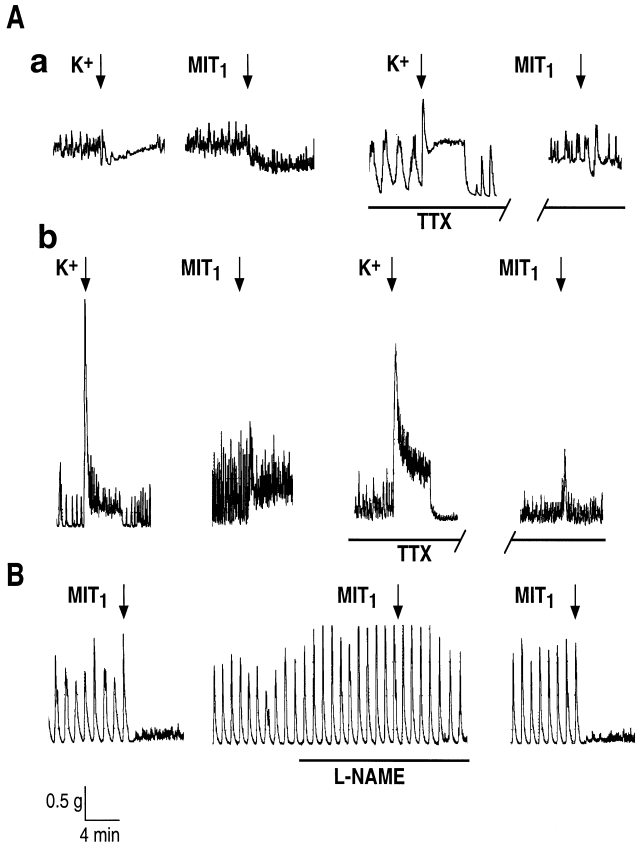

Fig. 3. Inhibition of MIT_1 effects. A: Effect of TTX (1 μM, right panels) on the relaxation of proximal colon induced by 40 mM K^+ or 1 nM MIT_1 (a) and on the contraction of distal colon induced by 40 mM K^+ or 1 nM MIT_1 (b). Control experiments obtained before TTX application are also shown (left panels). B: Effect of L-NAME (200 μM) on relaxation of proximal colon induced by 1 nM MIT_1. Control experiments obtained before application and after removal of L-NAME are also shown. The data presented are representative of at least four to eight experiments.

3.2. Physiological experiments

MIT_1 has a very potent contractile effect on guinea-pig ileum longitudinal muscle. Fig. 1A shows that 1 nM MIT_1 contracts this muscle as strongly as 40 mM K^+. However, the effect is transient while contraction evoked by K^+ is sustained. A concentration-response curve of this effect is shown in Fig. 1B. The ED_{50} is 0.3 nM. At the same low concentration of 1 nM, MIT_1 has a differential effect on whole strips of colon. It relaxes proximal colon (Fig. 2A) but contracts distal colon (Fig. 2B). Fig. 2 also shows the difference in the effect of 40 mM K^+ in the proximal and distal colon. In the proximal colon, K^+ induced a relaxation followed by a slow increase in the tone (Fig. 2A) whereas it produced a maintained contraction in the distal colon (Fig. 2B).

In order to identify the target of MIT_1 in the intestine and, in particular, to separate presynaptic from postsynaptic effects, the effects of tetrodotoxin (TTX) were analysed. TTX (1 μM), which is known to block action potential propagation [22], had no effect on the MIT_1-induced contraction in the longitudinal muscle from guinea-pig ileum (not shown). In contrast, both the relaxing effect of MIT_1 in the proximal colon (Fig. 3Aa) and the contracting effect of MIT_1 in the distal colon (Fig. 3Ab) were inhibited in the presence of TTX (1 μM), indicating that a major part of the toxin action takes place presynaptically. The relaxing effect of MIT_1 was further investigated by using the Ca^{2+}-dependent K^+ channel blocker apamin and the nitric oxide (NO) synthase inhibitor L-N^G-nitroarginine methyl ester (L-NAME). Apamin (1 nM) had no effect (not shown) whereas L-NAME (200 μM) reversibly antagonised the MIT_1-induced relaxation (Fig. 3B). The MIT_1-induced contraction of longitudinal ileum or distal colon was abolished by methoxyverapamil (D600, 10 μM), suggesting the involvement of Ca^{2+} entry through voltage-dependent Ca^{2+} channels as a component of the MIT_1 effect (Fig. 4). The contracting effect of MIT_1 was inhibited when the tone was previously raised by 60 mM external K^+ and was abolished in the presence of K^+ channel openers (cromakalim or pinacidil, 10 μM; Fig. 4).

Although it has a potent action on the intestine, MIT_1 appears to be inactive on the cardiovascular system. At a concentration of 0.2 μM, it neither contracts de-endothelialised rat thoracic aorta nor relaxes aorta precontracted with 40 mM K^+. At the concentration of 0.1 μM it does not affect the contractions of left and right rat atria (not shown).

3.3. Binding experiments

^{125}I-MIT binds with a very high affinity to specific receptors in ileum and brain membranes. Fig. 5 shows the saturation curves and the Scatchard plots corresponding to these binding experiments. Binding parameters are: $K_d = 1.3$ pM and $B_{max} = 30$ fmol/mg for guinea-pig ileum longitudinal muscle membranes (Fig. 5A) and $K_d = 0.9$ pM and $B_{max} = 26$ fmol/mg for rat brain membranes (Fig. 5B).

Competition experiments with the unlabelled toxin confirm

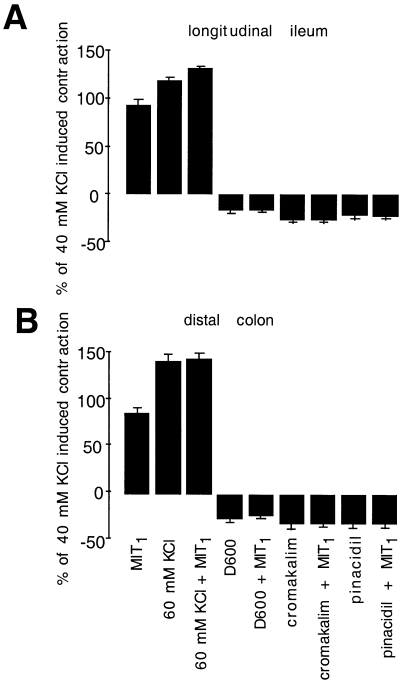

Fig. 4. Effect of the L-type voltage-dependent Ca^{2+} channel antagonist D600 (10 μM), the K^+ channel openers cromakalim (10 μM) and pinacidil (10 μM) and increased external K^+ concentration (60 mM) on the contraction induced by (1 nM) MIT_1 on guinea-pig longitudinal ileum muscle (A) and guinea-pig distal colon (B). Each condition is expressed as a percentage of the maintained contraction induced by 40 mM K^+. Results are expressed as mean values of four to eight experiments.

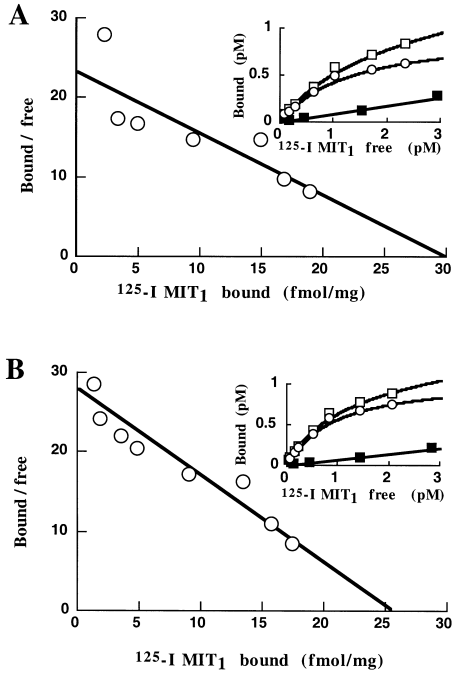

Fig. 5. Binding of ^{125}I-MIT$_1$ to ileum and brain membranes. Membranes were incubated in 5 ml of the buffer with ^{125}I-MIT$_1$ (0.1–3 pM) for 2 h at 25°C. Insets: Saturation curves for ^{125}I-MIT$_1$ binding (○) obtained after subtraction of non-specific ^{125}I-MIT$_1$ binding (■) (determined by including 2 nM MIT in the incubation medium) from the total ^{125}I-MIT$_1$ binding (□). Main panels: Corresponding Scatchard plots (○). Results are means of two determinations. Bound/free is expressed as pmol/mg of protein×nM. A: Guinea-pig ileum membranes (32 μg/ml). B: Rat brain membranes (42 μg/ml).

the existence in both ileum and brain membranes of high affinity specific binding sites in the picomolar range (Fig. 6). At a low concentration (3.3 pM), ^{125}I-MIT$_1$ binds to guinea-pig ileum with a $K_{0.5}$ value of 3.7 pM (Fig. 6A) corresponding to a K_d of 1.2 pM, almost identical to the value obtained from the Scatchard plot (1.3 pM). In rat brain, the $K_{0.5}$ value is 4 pM (Fig. 6B), corresponding to a K_d value of 1 pM, also very close to the K_d of 0.9 pM obtained from the Scatchard plot.

3.4. Electrophysiological measurements

MIT$_1$ was tested for possible activity against outward voltage-sensitive K^+ channels (Kv channels) expressed in *Xenopus* oocytes or in COS cells. None of the cloned channels Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, Kv2.1, Kv3.4 expressed in *Xenopus* oocyte was affected by 100 nM MIT$_1$ ($n=2$ or 3 for each condition). Similarly, neither the cloned cardiac channels Kv4.2, HERG nor the human KCNQ1 channel coexpressed with IsK [21,23] nor KCNQ2 and KCNQ3 nor the two P domains K^+ channel TREK-1, TASK-1 and TRAAK [20,24] recorded in COS transfected cells were affected by 100 nM MIT$_1$. Inward rectifier K^+ channels such as IRK1, IRK2, ROMK1, and the heteropolymers of G-protein-gated inward rectifiers GIRK1,2 and GIRK1,4 were also tested. These cloned channels expressed in *Xenopus* oocytes were also insensitive to 100 nM MIT$_1$ ($n=2$ or 3 for each channel type).

MIT$_1$ was also tested against the proton-gated Na^+ channels ASIC 1a, ASIC 2a and ASIC 3 that are involved in sensing tissue acidosis [25]. None of these channels, transfected in COS cells, was sensitive to 100 nM MIT$_1$.

4. Discussion

The results of this study show that MIT$_1$ is a polypeptide toxin that has a potent effect on intestinal contractility. MIT$_1$ at an extremely low concentration (0.1 nM) relaxes proximal colon whereas it contracts distal colon and longitudinal ileal muscle. A particularly interesting aspect of MIT$_1$ is that it is a toxin with a new type of folding. Its overall three-dimensional structure resembles that of colipase [16] although there is only 20% sequence homology between the two structures. Unlike colipase, MIT$_1$ does not activate lipase and unlike MIT$_1$, colipase, even at the high concentration of 10 μM, does not have any effect on intestinal contraction (not shown).

The MIT$_1$-induced relaxation was inhibited by TTX, suggesting that the toxin stimulates inhibitory enteric neurones. Recent studies have suggested that neural inhibition of contraction in the gastrointestinal tract involved at least two transmitter substances. NO was clearly identified as one of the inhibitory neurotransmitters [27–29]. NO is synthesised and released during stimulation of inhibitory nerves. Postjunctional mechanisms involving smooth muscle guanylate cyclase transduce the inhibitory transmitter signal to produce cyclic GMP-dependent membrane hyperpolarisation through opening of the K^+ channel and inhibition of the contraction. Other transmitter substances mediate nerve-evoked inhibition of contraction through a NO-independent pathway, by activa-

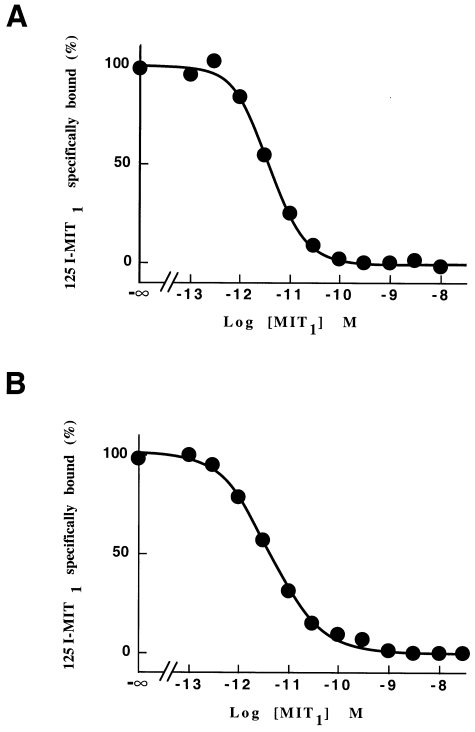

Fig. 6. Concentration-response curves for the inhibition by MIT$_1$ of ^{125}I-MIT$_1$ binding to guinea-pig ileum and rat brain membranes. The unlabelled peptide was first incubated at different concentrations with the membranes (65 μg/ml) in 2 ml buffer and then ^{125}I-MIT$_1$ was added and the membranes were incubated for 3.5–5 h at 22–23°C. A: Guinea-pig ileum membranes with 3.3 pM of ^{125}I-MIT$_1$. B: Rat brain membranes with 3.4 pM of ^{125}I-MIT$_1$. ●: MIT$_1$.

tion of apamin-sensitive K^+ channels [30]. Both NO-dependent and apamin-sensitive components of hyperpolarisation may then contribute to the closing of voltage-dependent Ca^{2+} channels, leading to a decrease in intracellular Ca^{2+} levels and relaxation of smooth muscle. It is also likely that membrane potential-independent mechanisms, such as an inhibition of myosin light chain kinase leading to a decrease in the sensitivity of the contractile apparatus to Ca^{2+} or an increased uptake or extrusion of Ca^{2+}, contribute to nerve-evoked inhibition of contractions [31]. The absence of effect of apamin and the inhibition of the MIT_1-induced relaxation by the NO synthase inhibitor L-NAME suggest that MIT_1 indirectly relaxes proximal colonic muscle by stimulating the release of NO from inhibitory enteric neurones.

Although the absence of effect of TTX on the contracting effect of MIT_1 in ileum suggests that the toxin might act directly on smooth muscle cells in this part of the gut, TTX inhibition of the MIT_1-induced contraction in distal colon clearly indicates the involvement of an excitatory neural component. The excitatory neurotransmitter involved remains to be identified as antagonists of muscarinic, serotoninergic or substance P receptors did not affect the MIT_1-induced contraction (not shown).

The identification of ^{125}I-MIT_1 binding sites in both ileum and brain membranes supports the idea that these toxin receptor sites are located both pre- and postsynaptically. Differences in affinities measured by binding experiments with ^{125}I-MIT_1 on the one hand, and evaluated from contraction studies on the other hand, are quite usual in the toxin field. They are probably due to the fact that binding is made on non-polarised membranes which have their two faces exposed to the same ionic medium, whereas pharmacological data are obtained in intact tissue and relate to toxin action on a receptor being part of a cell with a polarised membrane potential and with different ionic conditions on both faces of the membrane. In addition we know very little about the diffusion properties of the toxins to reach their target in ileum longitudinal muscle and distal colon.

A particularly interesting property of animal toxins is that they often have endogenous equivalents. The best known case is that of sarafotoxins for which the endogenous equivalents are the endothelins [32]. However, there are many other cases. For instance, the atrial natriuretic factor has an equivalent in the green mamba venom [33] and conversely the bee venom K^+ channel toxins apamin and mast cell degranulating peptide seem to have endogenous equivalents in the brain [34,35]. Therefore, there might also be an endogenous equivalent of MIT_1 in mammals. None of the intestinal effectors (or their antagonists) which have been tested has an action like MIT_1 and none of them binds to the MIT_1 receptor. Therefore it is to be expected that if such an endogenous compound exists it will be a new important peptide for the control of intestinal motility. Tools which have been developed in this work should facilitate the search of such new and physiologically important molecules.

The physiological data which show that MIT_1 in the presence of TTX has the same effects as increasing the external K^+ concentrations lead to the idea that the high affinity binding site through which MIT_1 exerts its action might be a K^+ channel, although the opening of non-selective cation channels or voltage-gated Ca^{2+} channels cannot be completely eliminated. The putative K^+ conductance involved in MIT_1 action would be a K^+ channel with a very high affinity for the toxin. This particular channel would be in a relatively low concentration in brain membranes ($\sim$25 fmol/mg). Electrophysiological experiments have shown that MIT_1 does not act on the following K^+ channels at low concentration: Kv1.1, Kv1.2, Kv1.3, Kv1.4, Kv1.5, Kv2.1, Kv3.4, Kv4.2, TREK-1, HERG, human KCNQ1 coexpressed with IsK, KCNQ2, KCNQ3, IRK1, IRK2, ROMK1, GIRK1,2 and GIRK1,4. However, there are more than 60 different genes for K^+ channels that have now been cloned and many more are probably to come. Therefore, the search should continue for the molecular identification of the MIT_1 target. It might have a particularly high importance for the physiology and physiopathology of the intestinal system since MIT_1, this new peptide with a new structure, has limited action when injected into the central nervous system and essentially no action on vascular smooth muscle or cardiac systems.

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