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PROTEASE INHIBITOR HOMOLOGUES FROM MAMBA VENOMS: FACILITATION OF ACETYLCHOLINE RELEASE AND INTERACTIONS WITH PREJUNCTIONAL BLOCKING TOXINS

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1 Five polypeptides, which were isolated from elapid snake venoms and which are structurally related to protease inhibitors, were tested for action on isolated biventer cervicis nerve-muscle preparations of the chick.

2 Dendrotoxin from the Eastern green mamba (*Dendroaspis angusticeps*) and toxins K and I from the black mamba (*Dendroaspis polylepis polylepis*) increased responses to indirect stimulation without affecting responses to exogenous acetylcholine, carbachol or KCl.

3 The two other protease inhibitor homologues, HHV-II from Ringhals cobra (*Hemachatus haemachatus*) and NNV-II from Cape cobra (*Naja nivea*) did not increase responses to nerve stimulation. Trypsin inhibitor from bovine pancreas also had no facilitatory effects on neuromuscular transmission.

4 The facilitatory toxins from mamba venoms interacted with the prejunctional blocking toxins, β-bungarotoxin, crotoxin and notexin, but not with taipoxin. The blocking effects of β-bungarotoxin were reduced by pretreatment with the mamba toxins, whereas the blocking actions of crotoxin and notexin were enhanced.

5 The results indicate that protease inhibitor homologues from mamba venoms form a new class of neurotoxin, which acts to increase the release of acetylcholine in response to motor nerve stimulation.

6 From the interaction studies it is concluded that the facilitatory toxins bind to motor nerve terminals at sites related to those occupied by the prejunctional blocking toxins. However, differences in interactions with individual toxins suggest that there must be several related binding sites on the nerve terminals.

Introduction

Snake venoms contain many polypeptides which can be used to further the study of neuromuscular transmission. Postjunctional neurotoxins, such as α-bungarotoxin, have been instrumental in acetylcholine receptor isolation. Since some venoms also contain neurotoxins, e.g. β-bungarotoxin, notexin and taipoxin, that inhibit acetylcholine release, it was hoped that these prejunctional toxins would provide labels for the structures involved in transmitter release (Kelly, Deutsch, Carlson & Wagner, 1979; Howard & Gundersen, 1980). However, all these prejunctionally-acting venom components are phospholipases and they destroy the nerve terminal. More recently, green mamba venom was shown to augment acetylcholine release (Barrett & Harvey, 1979), an action produced by one polypeptide called dendrotoxin and not associated with enzyme activity (Harvey & Karlsson, 1980; Harvey & Gage, 1981). Since dendrotoxin does not break down motor nerve terminals (Pécot-Dechavassine & Harvey, unpublished observations), it should be useful in further studies on prejunctional function.

Dendrotoxin contains 59 amino acids in a single chain which is cross-linked by three disulphide bonds; its amino acid sequence (Karlsson, unpublished observations) appears to be identical to that recently reported for polypeptide $C_{13}S_2C_3$ (Joubert & Taljaard, 1980). As noted previously (Harvey & Karlsson, 1980), dendrotoxin is chemically similar to toxin I, a protease inhibitor homologue from the venom of the black mamba, *Dendroaspis polylepis polylepis* (Strydom, 1973). However, the pharmacological activities of toxin I and other similar polypeptides are not known, apart from their relatively low toxicity *in vivo*. In the present study, we have compared the ability of a number of protease inhibitor homologues with that of dendrotoxin to enhance neuromuscular transmission. Additionally,

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we have attempted to characterize the binding sites for the prejunctional facilitatory toxins in terms of their interactions with the prejunctional blocking toxins, β-bungarotoxin, crotoxin, notexin and taipoxin. Some of these results were presented to the 4th Symposium of the International Society on Toxinology European Section, Marseilles, 1981.

Methods

Chick biventer cervicis nerve-muscle preparations

Biventer cervicis nerve-muscle preparations (Ginsborg & Warriner, 1960) were isolated from chicks aged 4–16 days and mounted in 7 ml organ baths with a resting tension of approximately 0.5 g in physiological salt solution (NaCl 118.4, KCl 4.7, $MgSO_4.7H_2O$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 11.1 mM) which was maintained at 32°C and bubbled with O_2 containing 5% CO_2. For indirect stimulation, contractions were elicited by stimulating the motor nerve in the tendon at a frequency of 0.1 Hz by square wave pulses of 0.2 ms and a strength greater than that required for maximal contractions. Responses similar in size to the twitches were obtained to submaximal concentrations of acetylcholine (0.2–1 mM), carbachol (10–20 μM) and KCl (20–30 mM) in the absence of nerve stimulation. Acetylcholine and KCl were allowed to remain in contact with the tissue for 30 s and carbachol for 60 s; preparations were washed by overflow for 15 s, the rate of overflow being 5–7 ml/s.

In some experiments, $CaCl_2$ in the physiological salt solution was replaced by 2.5 mM $SrCl_2$. Preparations were equilibrated for at least 60 min in Sr^{2+}-containing solution before the addition of test compounds.

Contractions and contractures were recorded isometrically on Grass 79B Polygraphs using Grass FT03 force displacement transducers.

Toxins and drugs

Dendrotoxin was isolated from the venom of *Dendroaspis angusticeps* by gel filtration followed by ion exchange chromatography as described previously (Harvey & Karlsson, 1980). Toxins I and K from the venom of *Dendroaspis polyepis polyepis* (Strydom, 1976) were isolated by the same procedure as used for dendrotoxin; the amino acid compositions of the toxins agreed with those published by Strydom (1976).

Protease inhibitor NNV-II from Cape cobra (*Naja nivea*) venom and protease inhibitor HHV-II from Ringhal's cobra (*Hemachatus haemachatus*) venom (Hokama, Iwanaga, Tatsuki & Suzuki, 1976) were gifts from Prof. S. Iwanaga. Taipoxin was isolated from the venom of *Oxyuranus scutellatus scutellatus* as described by Fohlman, Eaker, Karlsson & Thesleff (1976). β-Bungarotoxin from *Bungarus multicinctus* venom was obtained from the Boehringer Corporation (London) Ltd, notexin from the venom of *Notechis scutatus scutatus* was a gift from Prof. J.B. Harris, and crotoxin CA and CB were gifts from Dr C. Bon. Other drugs were obtained from the Sigma Chemical Co.

Results

Effect of protease inhibitor homologues on neuromuscular transmission

The effects of dendrotoxin, toxin I, toxin K, HHV-II, NNV-II and bovine pancreatic trypsin inhibitor were tested on chick biventer cervicis nerve-muscle preparations in order to distinguish between prejunctional and postjunctional actions. Augmentation of responses to indirect stimulation, without corresponding increases in responses to exogenous actylcholine, carbachol or KCl, was taken to reflect an increase in the evoked release of acetylcholine. In addition, comparative experiments were performed with 3,4-diaminopyridine, a drug known to increase acetylcholine release at the neuromuscular junction (Harvey & Marshall, 1977; Durant & Marshall, 1980).

As found previously (Harvey & Karlsson, 1980), dendrotoxin causes a slow increase in responses to indirect stimulation without affecting responses to exogenous acetylcholine, carbachol or KCl (Figure 1). Concentrations of dendrotoxin of 70 nM (0.5 μg/ml) and above produced twitch augmentation, there being little difference in the extent of the augmentation produced by concentrations from 0.14–11 μM (Figure 2). Similar activity was found with toxin I and toxin K from black mamba venom (Figure 2). These toxins did not affect responses to acetylcholine, carbachol, KCl or direct electrical stimulation.

Protease inhibitor HHV-II and trypsin inhibitor from bovine pancreas in concentrations of 1–30 μM had no effect on responses to indirect stimulation (Figure 2), acetylcholine or KCl. Protease inhibitor NNV-II did not augment responses to nerve stimulation but produced a transient contracture followed by a block of responses to indirect stimulation. These effects were concentration-dependent: at 0.8 μM the contracture was 33 ± 5% (mean ± s.e.mean, $n = 4$) of the control twitch response and responses to indirect stimulation were abolished in 96 ± 6 min, and at 3 μM the contracture was 69 ± 9% of twitch height and the time to complete block of responses to indirect stimulation was 45 ± 4 min. Responses to acetylcholine and, to a lesser extent, KCl were also reduced. For

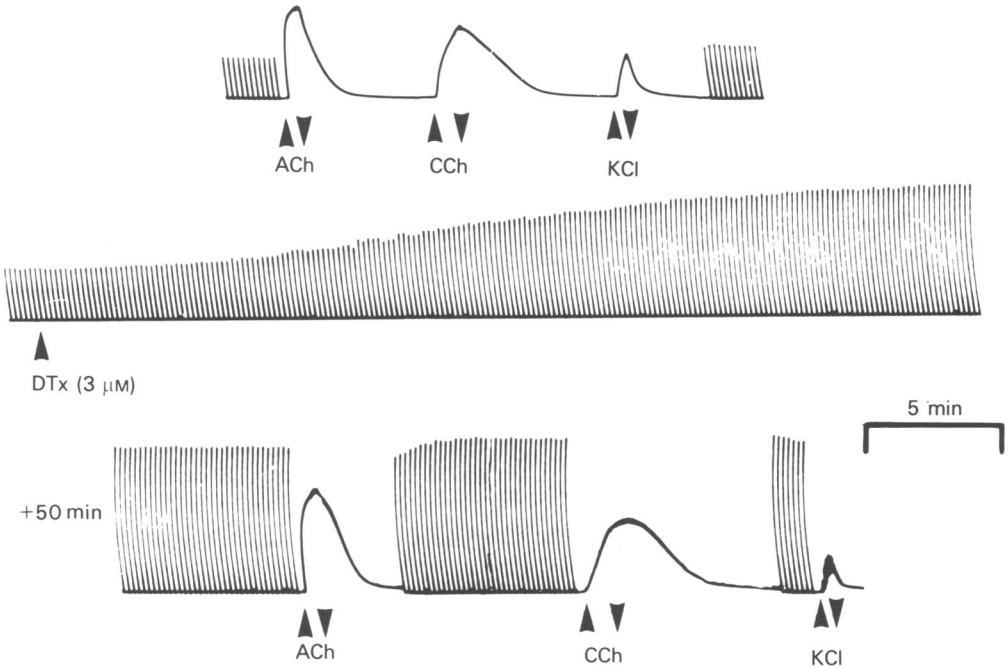

Figure 1 Effect of dendrotoxin (DTx, 3 μM) on responses of a chick biventer cervicis nerve-muscle preparation to indirect stimulation (at 0.1 Hz), acetylcholine (ACh, 0.2 mM), carbachol (CCh, 20 μM) and KCl (20 mM). Drugs were injected and washed out at the points marked with the upward and downward arrowheads, respectively. Between the middle and lower panels, there is a break in the record of 50 min.

example, after 3 μM NNV-II the responses to acetylcholine were 6 ± 2% of control values and responses to KCl were 50 ± 12% of control values.

As previously reported (Harvey & Marshall, 1977), 3,4-diaminopyridine also augmented responses to indirect stimulation without affecting responses to acetylcholine, carbachol or KCl. However, the concentrations of 3,4-diaminopyridine required to produce twitch augmentation were considerably greater than those of the mamba toxins (Figure 2). The maximum increase in twitch height (about 300% augmentation with 1 mM) produced by 3,4-diaminopyridine was greater than that produced by dendrotoxin and the other facilitatory mamba toxins.

Interactions of the facilitatory neurotoxins with prejunctional blocking toxins

The interactions of the facilitatory neurotoxins, dendrotoxin, toxin I and toxin K, with the prejunctional blocking toxins, β-bungarotoxin, notexin, crotoxin and taipoxin, were examined in two types of experiments. In the first, preparations were pretreated with one of the facilitatory toxins and then the time taken for one of the blocking toxins to inhibit responses to indirect stimulation was measured. In the second, preparations were exposed to one of the blocking toxins before the addition of a facilitatory mamba toxin; these experiments were performed in physiological salt solution in which Ca^{2+} was replaced by 2.5 mM Sr^{2+} as Sr^{2+} inhibits the phospholipase A_2-dependent blocking activity, but not the binding, of the prejunctional blocking toxins (Chang, Su, Lee & Eaker, 1977; Caratsch, Maranda, Miledi & Strong, 1981; Harris & MacDonell, 1981). To show that any interaction was not simply a consequence of the increased transmitter release caused by the mamba toxins, parallel experiments were performed with 3,4-diaminopyridine.

In normal solution Pretreatment with dendrotoxin (1.4 μM), toxin I (0.4 μM) or toxin K (80 nM) significantly reduced the blocking activity of β-bungarotoxin (50 nM), as revealed by an increased time taken to produce twitch blockade (Table 1). Addition of β-bungarotoxin after pretreatment with dendrotoxin enhanced the initial increase in twitch height that is often observed with prejunctional blocking toxins (Figure 3). The reduction in blocking activity of β-bungarotoxin after treatment of the preparations to one of the mamba toxins did not appear to be a direct consequence of the increased transmitter release caused by the mamba toxins because the effective-

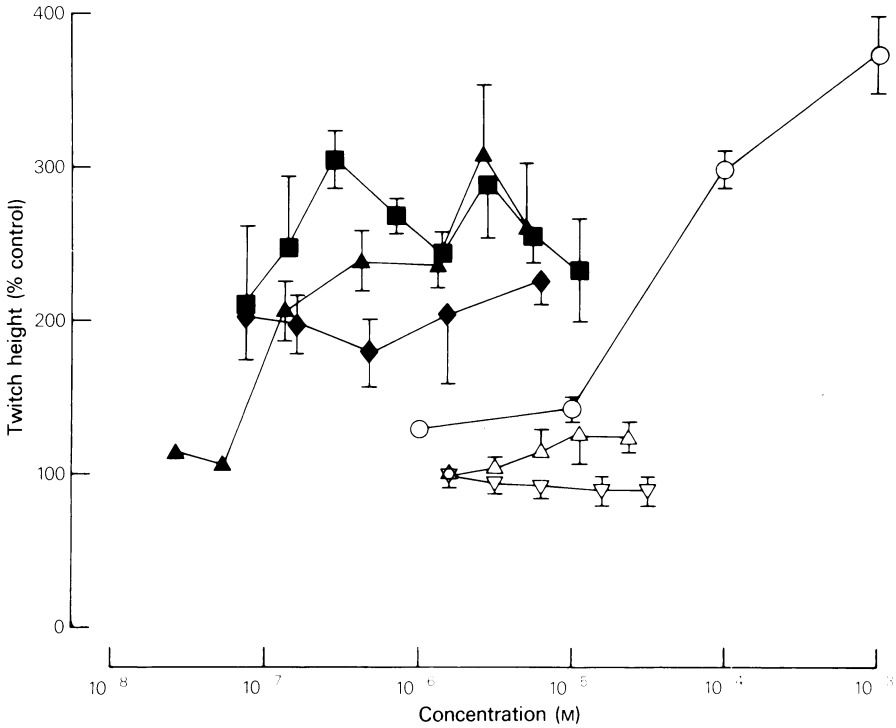

Figure 2 Effect of dendrotoxin, protease inhibitor homologues and 3,4-diaminopyridine on the response of chick biventer cervicis preparations to indirect stimulation. Each point represents the mean of 4-8 determinations; standard errors are shown by the bars, unless smaller than the symbols. (■) Dendrotoxin; (▲) toxin I from *Dendroaspis polylepis polylepis*; (◆) toxin K from *Dendroaspis polylepis polylepis*; (△) bovine pancreatic trypsin inhibitor; (▽) protease inhibitor HHV-II from *Hemachatus haemachatus*; (○) 3,4-diaminopyridine.

Table 1 Effects of pretreatment with facilitatory toxins on the blockade produced by β-bungarotoxin, notexin, crotoxin and taipoxin

Treatment	*Time to 50% twitch blockade* (min)	*Time to 100% twitch blockade* (min)	n
β-Bungarotoxin (50 nM)	35 ± 2	65 ± 3	8
β-Bungarotoxin (50 nM) after dendrotoxin (1.4 μM)	68 ± 8*	135 ± 11*	8
β-Bungarotoxin (50 nM) after toxin K (80 nM)	69 ± 6*	148 ± 16*	8
β-Bungarotoxin (50 nM) after toxin I (0.4 μM)	46 ± 2*	126 ± 7*	4
β-Bungarotoxin (50 nM) plus 3,4-diaminopyridine (0.1 mM)	24 ± 1*	69 ± 4	5
Notexin (0.15 μM)	109 ± 8	190 ± 12	8
Notexin (0.15 μM) after dendrotoxin (1.4 μM)	60 ± 12*	156 ± 18	6
Crotoxin (74 nM)	49 ± 4	75 ± 7	10
Crotoxin (74 nM) after toxin I (0.4 μM)	29 ± 1*	46 ± 3*	8
Taipoxin (22 nM)	92 ± 15	159 ± 12	8
Taipoxin (22 nM) after toxin I (1.3 μM)	83 ± 8	145 ± 15	5

Values are given as means ± s.e.mean

*Significantly different from the values with the appropriate blocking toxin alone (Student's *t* test, $P < 0.01$)

ness of β-bungarotoxin was not decreased by the presence of 0.1 mM 3,4-diaminopyridine (Table 1, Figure 3). Indeed, the initial development of the blockade induced by β-bungarotoxin was significantly faster (Table 1) in the presence of 3,4-diaminopyridine, an effect also seen in mouse phrenic nerve-hemidiaphragm preparations (Chang & Su, 1980a).

However, the blocking activity of all prejunctional toxins was not inhibited by pretreatment with facilitatory mamba toxins. Notexin (0.15 μM) reduced responses to nerve stimulation faster in preparations that had been pretreated with dendrotoxin (1.4 μM) than in control experiments (Table 1, Figure 4). Dendrotoxin pretreatment appeared to reduce markedly the lag phase of the action of notexin (Figure 4).

The blocking activity of crotoxin (45 nM) was also enhanced by pretreatment with one of the facilitatory neurotoxins (Table 1). As shown in Figure 5, addition of crotoxin after pretreatment with 0.4 μM toxin I led to a transient increase in the responses to indirect stimulation followed by a more rapid onset of blockade than found in control experiments.

Crotoxin is a complex of two polypeptides: an acidic subunit CA of very low toxicity and a basic subunit CB which has phospholipase A_2 activity (Hendon & Fraenkel-Conrat, 1971). In control experiments, 74 nM crotoxin abolished responses to indirect stimulation in 75 ± 7 min ($n = 10$) whereas 120 nM component CA took 366 ± 45 min ($n = 4$) to abolish twitches. Preparations were pretreated with 120 nM component CA and then 150 nM toxin I was added. Under these conditions Toxin I produced only 31 ± 8% ($n = 8$) increase in the response to indirect stimulation compared to the 143 ± 27% ($n = 4$) increase produced by the same concentration of toxin in control preparations.

In contrast to the results with β-bungarotoxin, notexin and crotoxin, there was no significant interaction between taipoxin and toxin I. When 22 nM taipoxin was administered after pretreatment with 1.3 μM toxin I, the time taken to twitch blockade was not significantly different from the time taken in control experiments with taipoxin (Table 1).

In Sr^{2+}-containing solution Pretreatment with β-bungarotoxin (50 nM) in the presence of Sr^{2+} abolished the facilitatory effects of dendrotoxin (1.4 μM) (Figure 6). After 40 min in the presence of 50 nM β-bungarotoxin, the responses to indirect stimulation were 101 ± 7% ($n = 7$) of control values.

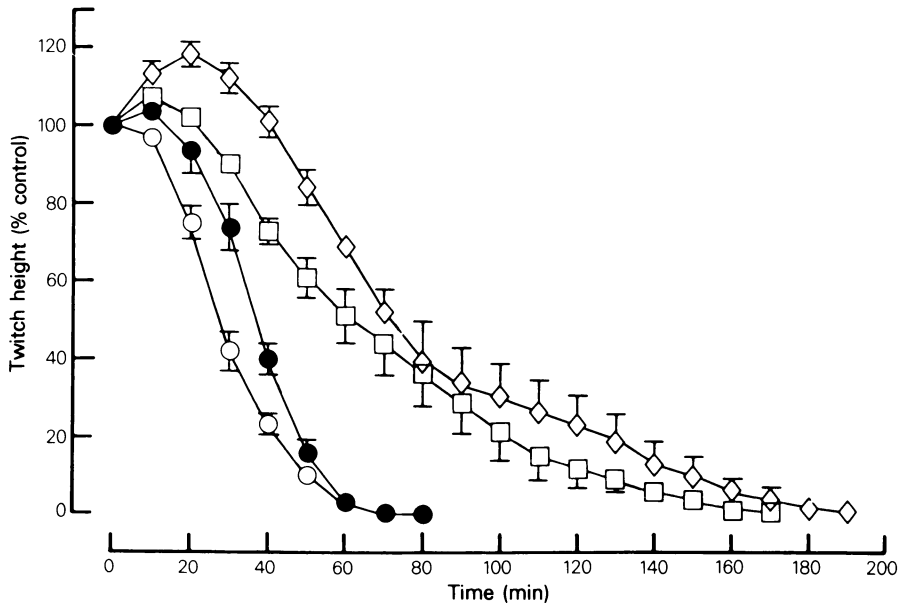

Figure 3 Effect of pretreatment with facilitatory mamba toxins on the blocking action of β-bungarotoxin on indirectly stimulated chick biventer cervicis preparations. Each point represents the mean of 4–8 preparations; standard errors are shown by the vertical bars, unless smaller than the symbols. (●) β-Bungarotoxin (50 nM); (□) 50 nM β-bungarotoxin after 60–70 min exposure to 1.4 μM dendrotoxin; (◇) 50 nM β-bungarotoxin after 60–70 min exposure to 80 nM toxin K; (○) 50 nM β-bungarotoxin in the presence of 0.1 mM 3,4-diaminopyridine.

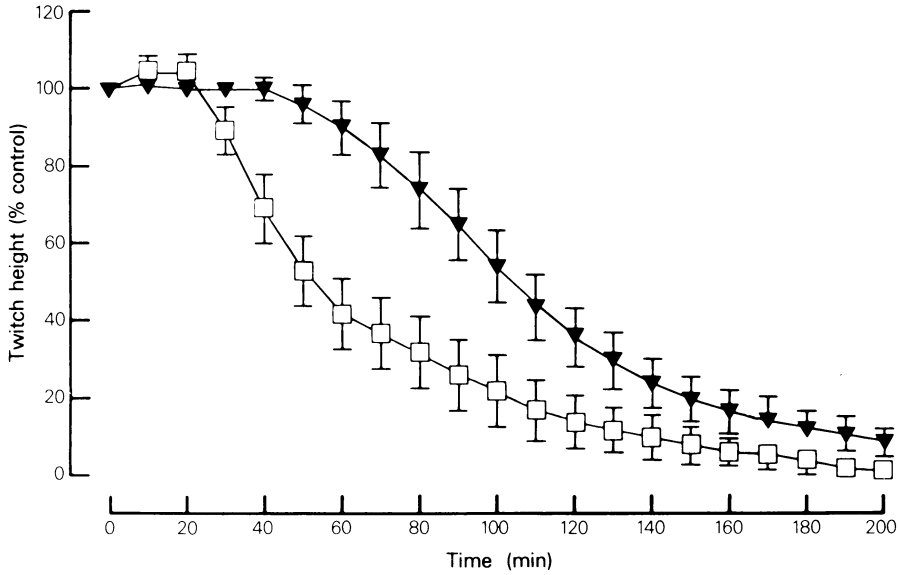

Figure 4 Effect of dendrotoxin on the blocking action of notexin on indirectly stimulated chick biventer cervicis preparations. Each point represents the mean of 8 experiments; standard errors are shown by vertical bars. (▼) Notexin (0.15 μM); (□) notexin (0.15 μM) after the preparations had been exposed for 60–70 min to dendrotoxin (1.4 μM).

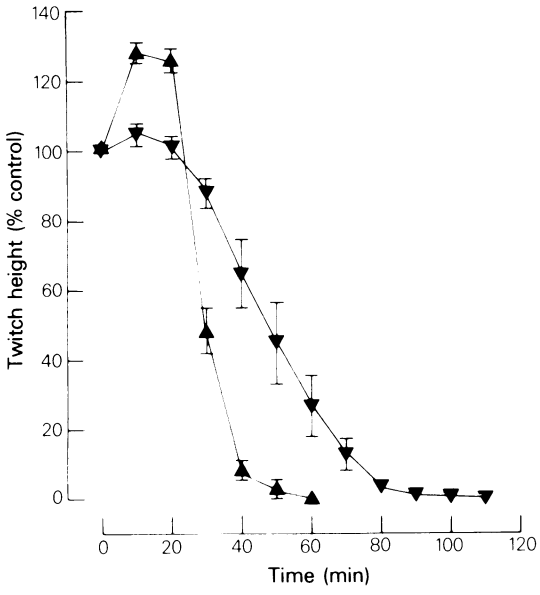

Figure 5 Effect of toxin I on the blocking action of crotoxin on indirectly stimulated chick biventer cervicis preparations. Each point represents the mean of 7–10 experiments; s.e.mean are shown by vertical bars. (▼) Crotoxin (74 nM); (▲) crotoxin (74 nM) after the preparations had been exposed for 60–70 min to toxin I 0.4 μM).

Dendrotoxin (1.4 μM) was added but there was no increase in the size of the responses, rather the twitches decreased to about 90% of control values after a further 50 min (Figure 6a) which is similar to the effects of β-bungarotoxin on its own in Sr^{2+}-solution (Figure 6b). When the preparations were returned to solutions containing 2.5 mM Ca^{2+}, the responses to indirect stimulation were abolished in 81 ± 15 min, indicating that the binding of β-bungarotoxin had not been reversed by dendrotoxin. Substitution of Sr^{2+} for Ca^{2+} did not by itself prevent the action of dendrotoxin: twitch responses were almost doubled after 90 min in 1.4 μM dendrotoxin in Sr^{2+} (Figure 6b).

Treatment with β-bungarotoxin in Sr^{2+}-containing solution did not prevent all increases in transmitter release. As shown in Figure 6a, 1 mM 3,4-diaminopyridine was as effective after 40 min exposure to β-bungarotoxin as in preparations that had not been exposed to the toxin.

Because of the limited amounts of toxins available, few experiments could be performed with notexin, crotoxin, and taipoxin in Sr^{2+}-containing solution. Notexin pretreatment (either at 37 or 150 nM) did not block the facilitatory actions of dendrotoxin. In contrast, dendrotoxin acted more rapidly in preparations that had been exposed to notexin (Figure 7). Crotoxin apparently had similar, though weaker, effects on the actions of mamba toxins in Sr^{2+}-containing solutions. Taipoxin was not tested under these conditions.

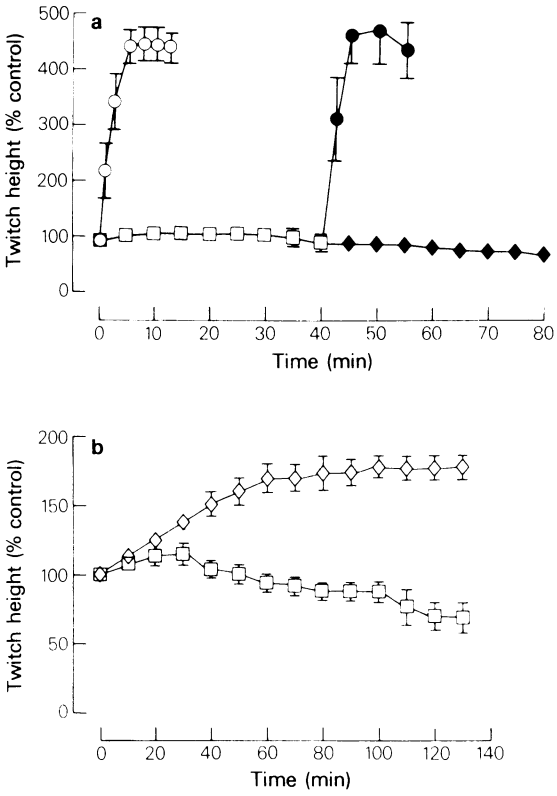

Figure 6 Interactions of β-bungarotoxin, dendrotoxin and 3,4-diaminopyridine in Sr^{2+}-containing physiological salt solution. (a) (□) β-Bungarotoxin (50 nM); (♦) dendrotoxin (1.4 μM) after 40 min pretreatment with β-bungarotoxin (50 nM); (○) 3,4-diaminopyridine (0.1 mM); (●) 3,4-diaminopyridine (0.1 mM) after 40 min pretreatment with β-bungarotoxin (50 nM). (b) (◇) Dendrotoxin (1.4 μM); (□) β-bungarotoxin (50 nM). Each point represents the mean of 4–8 experiments; s.e.means are shown by vertical bars.

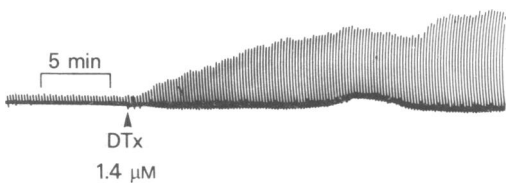

Figure 7 Effect of dendrotoxin (1.4 μM, DTx) on responses of the chick biventer cervicis preparation to indirect stimulation after 140 min pretreatment with notexin (0.15 μM). Twitches had been reduced by notexin to 60% of control values.

Discussion

A new class of neurotoxin

The results indicate that protease inhibitor homologues isolated from mamba venoms can act like dendrotoxin to facilitate the release of acetylcholine at the neuromuscular junction. However, the components must be closely homologous with dendrotoxin for activity. As shown in Figure 8, all the protease inhibitors that were tested in this study share common residues in almost half their sequences. However, the active facilitatory toxins I and K from black mamba venom only differ from dendrotoxin in 7 and 20 amino acids, respectively, whereas the inactive components HHV-II, NNV-II and bovine pancreas trypsin inhibitor have considerably more differences from dendrotoxin (30, 31 and 36 residues, respectively). Some other venom constituents could be predicted to have facilitatory activity on the basis of structural similarity to dendrotoxin. These would include toxin E from black mamba venom (Joubert & Strydom, 1978), and possibly fractions 4-13-2 and 4-14 from the venom of the Western green mamba, *Dendroaspis viridis* (Shipolini, Bailey, Edwardson & Banks, 1973).

Although compounds such as α-latrotoxin from black widow spider venom accelerate the spontaneous release of acetylcholine (see Howard & Gundersen, 1980), there do not appear to be any reports on naturally-occurring molecules that can augment the amount of acetylcholine released in response to motor nerve stimulation. Hence, the facilitatory compounds from mamba venoms appear to form a new class of venom neurotoxin. Since they are extremely potent and long-lasting, they should be useful in further studies on the mechanisms of neurotransmitter release.

Multiple binding sites on motor nerve terminals

Although the prejunctional blocking toxins from snake venoms have similar pharmacological effects, they differ in molecular structure. Their common feature is that they all have phospholipase A_2 activity but their structures vary from the single polypeptide chain of notexin to taipoxin with its three subunits (Fohlman *et al.*, 1976). β-Bungarotoxin has two polypeptide chains that are held together by a disulphide bridge (Kondo, Narita & Lee, 1978a, b) whereas the two components of crotoxin form a non-covalent complex that is readily dissociable (Hendon & Fraenkel-Conrat, 1971; Breithaupt, Rübsamen & Habermann, 1971). It is thought that the phospholipase activity is responsible for the toxicity of these molecules and that the additional subunits, where present, may help to increase the

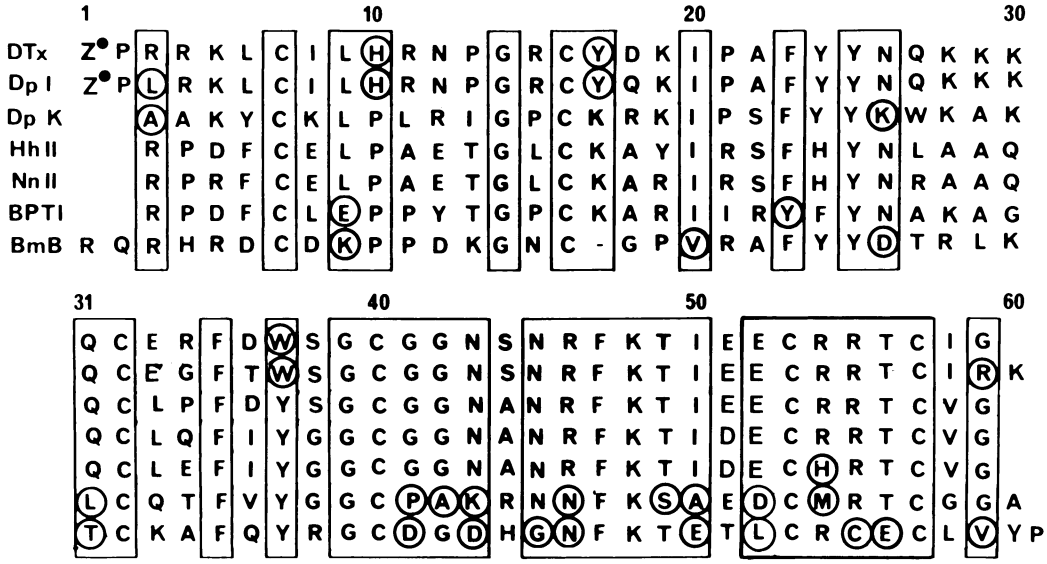

Figure 8 The alignment of dendrotoxin (DTx) with protease inhibitor homologues. The sequences have been aligned with respect to the position of the cystine residues. DpI = toxin I from *Dendroaspis polylepis polylepis*. DpK = toxin K from *Dendroaspis polylepis polylepis*. HhII = protease inhibitor HHV-II from *Hemachatus haemachatus*. NnII = protease inhibitor NNV-II from *Naja nivea*. BPTI = bovine pancreatic trypsin inhibitor. BmB = the B chain of β-bungarotoxin. Blocks drawn around the residues indicate invariant amino acids, with variant residues being indicated by circles. The IUPAC one-letter notation for amino acids is used (*Eur. J. Biochem.*, **5**, 151–153, 1968). ● The first residue in dendrotoxin and probably in toxin I is pyroglutamic acid.

specificity of binding. With such a variety of structures, it is perhaps to be expected that all of these toxins would not bind to identical sites on the motor nerve terminals.

The B chain of β-bungarotoxin has 60 amino acid residues and its sequence (Kondo *et al.*, 1978b) shows a high degree of similarity with dendrotoxin and the other protease inhibitors (Figure 8). The results of the interaction studies suggest that the facilitatory toxins can compete with β-bungarotoxin for a prejunctional binding site. The function of the B chain of β-bungarotoxin may be to bind to the nerve terminal and hence direct the phospolipase activity of the A chain to suitable substrates. It might be expected that the facilitatory toxins could play a similar role for a phospholipase in mamba venoms. However, the venom of *Dendroaspis angusticeps*, at least, has no detectable phospholipase A_2 activity (R.C Hider, personal communication, 1981).

Dendrotoxin did not appear to inhibit the binding of notexin; in fact, the blocking activity of notexin was enhanced by dendrotoxin pretreatment. Qualitatively similar results were obtained with crotoxin. Notexin and crotoxin presumably bind to the nerve terminal at sites distinct from the dendrotoxin and β-bungarotoxin sites, although these seem to be capable of interactions. The relatively nontoxic CA component of crotoxin could reduce the facilitation caused by one of the mamba toxins. Since the CA component appears to direct the enzymic CB component to a separate binding site and then to dissociate and unbind (Bon, Changeux, Jeng & Fraenkel-Conrat, 1979), it is possible that the CA component binds transiently to the dendrotoxin site while the CB component binds to a site similar to the one for notexin.

These results can be interpreted to provide evidence for at least two separate binding sites for the prejunctional neurotoxins: one for dendrotoxin, β-bungarotoxin and component CA from crotoxin, and another for notexin and component CB from crotoxin. Since no interactions could be demonstrated between taipoxin and the facilitatory mamba toxins, it would appear that taipoxin acts independently through a third site. Additional evidence for the existence of separate binding sites comes from work which demonstrates synergistic interactions between prejunctional blocking toxins (Chang & Su, 1980b; Ho & Lee, 1981).

Note added in proof: The sample of crotoxin CA used in this study had significant phospholipase activity.

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