J. Physiol. (1987), **386**, *pp.* 455–463
With 3 text-figures
Printed in Great Britain

THE ACTIONS OF PRESYNAPTIC SNAKE TOXINS ON MEMBRANE CURRENTS OF MOUSE MOTOR NERVE TERMINALS

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(*Received 11 August 1986*)

SUMMARY

1. The m. triangularis sterni of the mouse was used to investigate the actions of dendrotoxin, β-bungarotoxin, crotoxin, taipoxin, bee venom phospholipase A_2, aprotinin and apamin on presynaptic currents which flow inside the perineural sheath of nerve bundles upon nerve stimulation.

2. Neither the fast K^+ current ($I_{K,f}$) nor the Ca^{2+}-dependent K^+ current $I_{K(Ca)}$ (unmasked after blockade of $I_{K,f}$ by 3,4-diaminopyridine) was affected by the neurotoxins and drugs mentioned.

3. Inhibition of both $I_{K,f}$ and $I_{K(Ca)}$ by tetraethylammonium (30 mM) prolonged presynaptic depolarization owing to Ca^{2+} influx through fast and slow Ca^{2+} channels. Additional application of dendrotoxin, β-bungarotoxin, crotoxin or taipoxin in the nanomolar range caused further prolongation of Ca^{2+} influx, presumably due to blockade of slowly activating K^+ current ($I_{K,S}$). Onset of toxin effects was immediate and could not be reversed by washing for 60 min.

4. Similar prolongation of slow Ca^{2+} current was effected by 3,4-diaminopyridine, whereas addition of apamin, aprotinin or phospholipase A_2 left the signals unchanged.

5. These data indicate that facilitatory actions of dendrotoxin, β-bungarotoxin, taipoxin and crotoxin are mediated by an increase of Ca^{2+} entry into nerve terminals. The actions of these toxins are discussed in terms of a blockade of presynaptic K^+ channels with slow activation kinetics.

INTRODUCTION

Snake venoms contain a variety of pharmacologically active polypeptides. Amongst them, β bungarotoxin (β-BuTX), crotoxin (CroTX) and taipoxin (TPX) have attracted particular interest as they act presynaptically to impair evoked transmitter release from motor nerve endings (for review see Howard & Gundersen, 1980). This effect is preceded by a facilitatory phase in which the amplitudes of nerve-evoked end-plate potentials are increased (Abe, Limbrick & Miledi, 1976; Livengood, Manalis, Donlon, Masukawa, Tobias & Shain, 1978). Although both actions appear to rely on the toxins' phospholipase A_2 (PLA_2) activity (Abe, Alemà & Miledi, 1977; Livengood *et al.* 1978), little is known about the actual mechanism by which the alterations of transmitter release are brought about.

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Dendrotoxin (DTX) is another snake toxin that facilitates evoked transmitter release but lacks the final depressant phase (Harvey & Karlsson, 1980, 1982). It shares structure homologies and binding sites with β-BuTX (Rehm & Betz, 1984), but is devoid of phospholipase activity. DTX has been reported to inhibit K^+ currents in hippocampal neurones (Dolly, Halliwell, Black, Williams, Pelchen-Matthews, Breeze, Mehraban, Othman & Black, 1984; Halliwell, Othmann, Pelchen-Matthews & Dolly, 1986), in frog motor nerves (Weller, Bernhardt, Siemen, Dreyer, Vogel & Habermann, 1985), visceral ganglion neurones (Stansfeld, Marsh, Halliwell & Brown, 1986) and dorsal root ganglion neurones (Penner, Petersen, Pierau & Dreyer, 1986). Recently, we have shown that β-BuTX acts like DTX as an inhibitor of a slowly activating, non-inactivating K^+ current in guinea-pig dorsal root ganglion neurones (Petersen, Penner, Pierau & Dreyer, 1986).

Reduction of presynaptic K^+ currents in nerve terminals may tentatively account for the facilitatory effects of these so-called presynaptic toxins. We have therefore investigated the effects of DTX and three PLA_2 toxins (β-BuTX, CroTX and TPX) on presynaptic currents of mouse motor nerve endings using the subendothelial recording technique (Gundersen, Katz & Miledi, 1982; Mallart, 1985*a*; Penner & Dreyer, 1986). The results clearly demonstrate that these snake toxins, irrespective of their enzymatic activity, have a common mode of action on presynaptic membrane currents. All of them act to increase Ca^{2+} influx into nerve terminals. The effects observed can satisfactorily be interpreted as an inhibition of a slowly activating K^+ current, thus being consistent with the results obtained with sensory neurones under voltage clamp (Penner *et al.* 1986; Petersen *et al.* 1986). It is therefore suggested that blockade of K^+ channels accounts for the facilitatory effects of DTX and the PLA_2 snake toxins.

METHODS

Experiments were performed on the triangularis sterni nerve–muscle preparation of adult mice (McArdle, Angaut-Petit, Mallart, Bournaud, Faille & Brigant, 1981). Bath solution comprised (mM): NaCl, 115; KCl, 5; $CaCl_2$, 2·5; $MgSO_4$, 1; $NaHCO_3$, 25; Na_2HPO_4, 1; glucose, 11; gassed with 95% O_2–5% CO_2 (pH 7·3). In all experiments *d*-tubocurarine (50 μM) was used to abolish post-synaptic responses and procaine (100 μM) was used to suppress repetitive firing of nerves which occurred in the presence of K^+ channel blockers. Measurements were conducted at room temperature (21–24 °C).

Signals following nerve stimulation through a suction electrode were recorded inside the endothelial tube of nerve bundles containing two to four nerve fibres. Glass micro-electrodes filled with 0·5 M-NaCl (resistance, 4–10 MΩ) were placed close to some of the nerve terminals, while a chlorided silver wire in the recording chamber served as reference electrode. This configuration allows one to record potentials between recording electrode and reference electrode, generated by longitudinal currents flowing along the outside of the axons but within the endothelial tube. The signals recorded reflect currents activated in the nodal membrane as well as conductance changes originating from the presynaptic membrane of distant nerve endings. At the site of the recording electrode, the latter show up with reversed polarity (for details see Mallart, 1985*a*; Penner & Dreyer, 1986).

DTX was isolated by U. Weller (this institute) as described by Harvey & Karlsson (1980), β-BuTX was obtained from Sigma (Lot T 2762), TPX was kindly provided by E. Mebs (University of Frankfurt, F.R.G.), apamin and CroTX were a gift from E. Habermann (this institute). All toxins employed were found free of contaminants when assayed using disk–gel electrophoresis.

RESULTS

Control traces in Fig. 1 show typical nerve signals recorded inside the endothelial tube close to some nerve terminals. While the first negative peak can be attributed to Na^+ influx in nodes of Ranvier (the propagating nerve action potential), the second negativity corresponds to a K^+ outward current generated in the nerve terminals

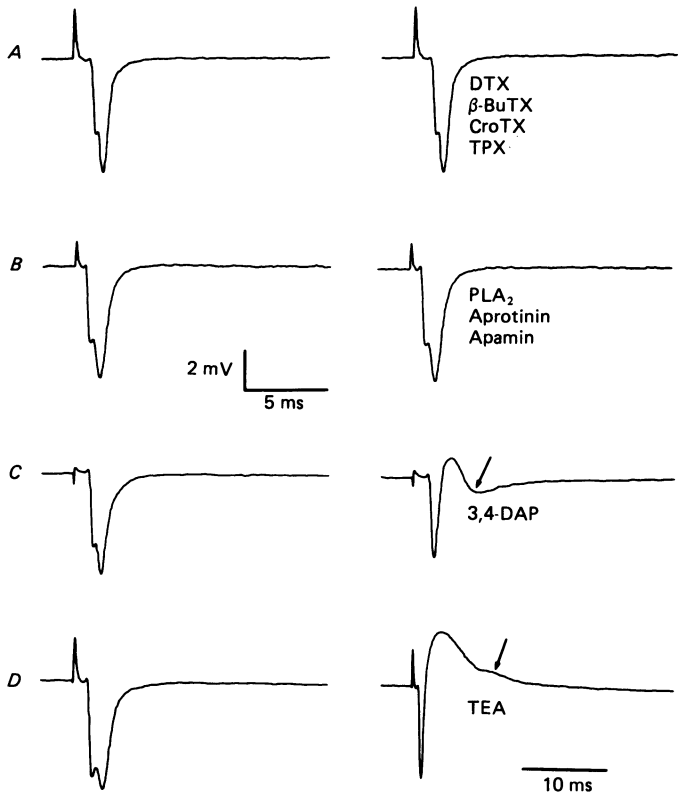

Fig. 1. The effects of toxins and other substances on fast K^+ current. *A*, an example of typical subendothelial signals before and 10 min after addition of DTX (1·5 μM), β-BuTX, CroTX or TPX (500 nM each). *B*, an example of signals before and 10 min after addition of PLA_2 (1 μg/ml), aprotinin (1·5 μM) and apamin (5 μM). *C*, effect of 3,4-DAP (500 μM), arrow indicates $I_{K(Ca)}$. *D*, effect of TEA (30 mM), arrow indicates slow Ca^{2+} current (note the different time scale in this trace).

(Mallart, 1985*b*; Penner & Dreyer, 1986). For reasons outlined in the Discussion, we will refer to this signal component as the 'fast' K^+ current $I_{K,f}$.

Fig. 1 *A* illustrates that the fast K^+ current in this preparation is neither influenced by DTX nor the PLA_2 toxins β-BuTX, CroTX and TPX. For better comparison and evaluation of snake toxin actions we have also investigated aprotinin (a protease inhibitor with structural homologies to DTX), bee venom phospholipase A_2 and apamin (a putative blocker of Ca^{2+}-activated K^+ channels). Fig. 1 *B* demonstrates the lack of effect of these substances on nodal as well as presynaptic membrane currents.

So far, the only pharmacological tools available to affect the second negative deflexion of subendothelial signals in this preparation are the known, well-characterized K^+ channel blockers tetraethylammonium (TEA) and 3,4-diaminopyridine (3,4-DAP; Mallart, 1985*b*; Penner & Dreyer, 1986). Both drugs reduced the fast K^+ current in a concentration-dependent manner, whereby 3,4-DAP was more potent than TEA. In contrast to a previous report (Mallart, 1985*b*) high concentra-

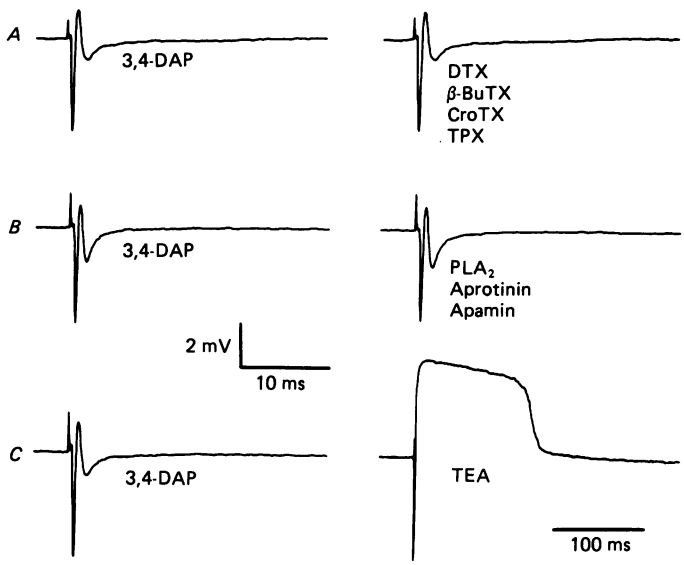

Fig. 2. Effects of toxins and other substances on Ca^{2+}-dependent K^+ current, recorded in the presence of 3,4-DAP (500 μM). *A*, an example of signals before and 10 min after addition of DTX (1·5 μM), β-BuTX, CroTX or TPX (500 nM each). *B*, an example of signals before and 10 min after addition of PLA_2 (1 μg/ml), aprotinin (1·5 μM) and apamin (5 μM). *C*, effect of TEA (10 mM) on $I_{K(Ca)}$ (note the different time scale in this trace).

tions of 3,4-DAP (500 μM) consistently caused replacement of the second negativity by a positive-going wave (Fig. 1*C*). The positive signal component is due to the prolonged depolarization of nerve terminals caused by blockade of the fast-repolarizing K^+ current and corresponds to a Ca^{2+} influx through fast Ca^{2+} channels (Penner & Dreyer, 1986). In turn, the increase in intracellular Ca^{2+} triggers a Ca^{2+}-dependent K^+ current $I_{K(Ca)}$ (Mallart, 1985*b*) which is resistant to 3,4-DAP and shows up as a late negative deflexion (arrow in Fig. 1*C*). Activation of $I_{K(Ca)}$ prevented a further prolongation of Ca^{2+} influx even when further raising 3,4-DAP concentrations.

Fig. 1*D* shows a typical signal recorded after perfusing the preparation with TEA (30 mM). As with 3,4-DAP, the fast presynaptic Ca^{2+} current was prominent but the late negativity was absent, suggesting that TEA had blocked $I_{K(Ca)}$ (Mallart, 1985*b*). Instead, varying with preparations, a more or less pronounced 'slow' positivity became noticeable. Pharmacological evidence has been presented that this slow component is carried by a distinct set of 'slow' Ca^{2+} channels (Penner & Dreyer, 1986).

A different interpretation has been advanced by Mallart (1985*b*), who suggested that fast and slow signal components (which are also seen when using a combination of 150 μM-3,4-DAP and 500 μM-TEA) reflect one Ca^{2+} current which becomes regenerative. According to this view, the current is modulated by the activation of $I_{K(Ca)}$ (incompletely blocked by TEA), and therefore only apparently two components

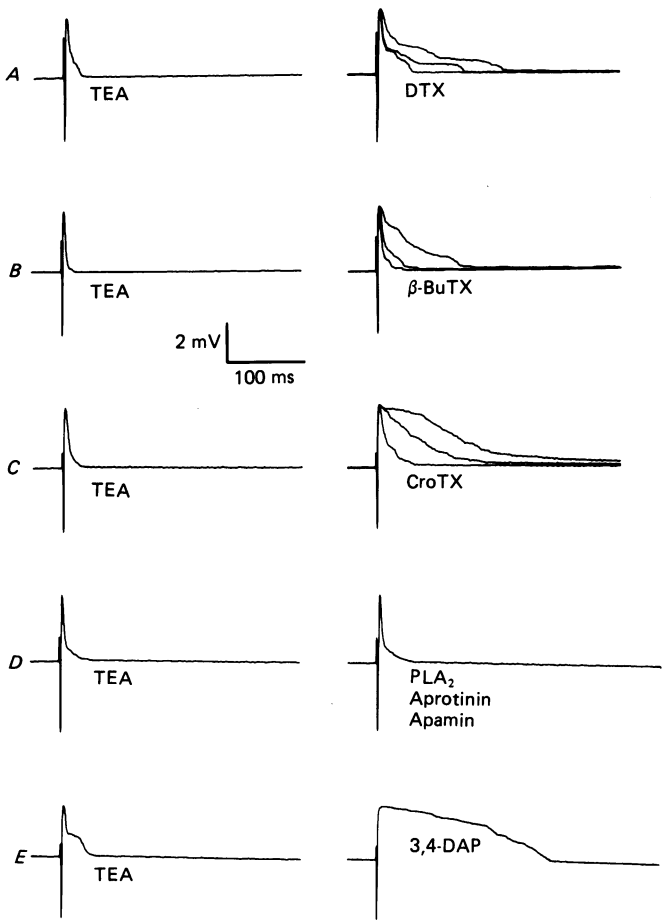

Fig. 3. Effects of toxins and other substances on subendothelial signals, recorded in the presence of TEA (30 mM). *A*, signals before and 10 min after addition of cumulative concentrations of DTX (1·5, 15 and 150 nM). *B* and *C*, signals before and 10 min after addition of cumulative concentrations of β-BuTX and CroTX (5, 50 and 500 nM). *D*, an example of signals before and 10 min after addition of PLA_2 (1 μg/ml), aprotinin (1·5 μM) and apamin (5 μM). *E*, effect of 3,4-DAP (50 μM).

may be distinguished. Elevation of TEA concentration beyond 2 mM (still in the presence of 3,4-DAP) generates large plateau responses (cf. our Fig. 2*C*). From this it was suggested that after complete block of $I_{K(Ca)}$, development of full Ca^{2+} plateau currents was no longer prevented. Our results with high concentrations of TEA alone, confirm a potent block of $I_{K(Ca)}$ by TEA (Fig. 1*D*). But still (in fact, as a result), fast and slow Ca^{2+} components were clearly distinguishable, further supporting

the notion that two different presynaptic Ca^{2+} currents with different activation thresholds are present in mouse motor nerve terminals (Penner & Dreyer, 1986).

For investigations of $I_{K(Ca)}$, 500 μM-3,4-DAP was added to the bath solution. This concentration ensured maximal responses and consistently revealed the typical triphasic signals shown as controls in Fig. 2. Additional application of DTX, β-BuTX, CroTX or TpX did not affect any of the signal components suggesting that $I_{K(Ca)}$, like $I_{K,f}$ is resistant to these toxins. Similarly, aprotinin and the bee venom phospholipase A_2 were found devoid of noticeable effects (Fig. 2*B*). Interestingly, this also applied to apamin which has been reported to block one type of Ca^{2+}-dependent K^+ channel. (Romey & Lazdunski, 1984; Pennefather, Lancaster, Adams & Nicoll, 1985). Amongst the drugs tested, only TEA was able to block $I_{K(Ca)}$ as witnessed by the prolonged positive wave in Fig. 2*C*.

As has been mentioned above, TEA caused concentration-dependent prolongation of presynaptic depolarization. At concentrations of 1–10 mM, the signals obtained were similar to those seen with 3,4-DAP (except for the missing $I_{K(Ca)}$ component), i.e. the fast Ca^{2+} current displayed comparable amplitude and duration (not illustrated). Under these circumstances, no effects of additionally applied snake toxins were ever observed. However, raising TEA concentration to 30 mM resulted in the above-mentioned appearance of a positive 'shoulder' (Fig. 1*D* and control signals in Fig. 3). Now an additional application of DTX caused a concentration-dependent prolongation of Ca^{2+} influx (Fig. 3*A*), starting at concentrations in the nanomolar range. Similar results were obtained with the PLA_2 toxins β-BuTX (Fig. 3*B*), CroTX (Fig. 3*C*) and TPX (not illustrated). With all toxins the onset of action occurred after 2–3 min and was completed within 5-7 min. We failed to obtain significant recovery even with washing periods of 1 h.

The effects of DTX and the PLA_2 toxins appear to be very specific and unrelated to enzymatic activity as aprotinin (possessing structure homology) and bee venom phospholipase A_2 (higher specific enzymatic activity than the toxins) did not influence membrane currents (Fig. 3*D*). An important finding for the interpretation of toxin action was that 3,4-DAP, like the toxins, was able to prolong the positive deflexion. This suggests a similar action of these drugs on K^+ conductances which remained unblocked by concentrations of TEA as high as 30 mM.

DISCUSSION

The results show that the so-called presynaptic toxins DTX, β-BuTX, CroTX and TPX influence presynaptic membrane currents. The method by which these currents were measured seems to be the best available but does not allow an unambiguous characterization of the current actually influenced. This is because a mixture of all currents being activated always contributes to the recorded wave form. Therefore, the interpretation of drug effects has to be based on analogies of their pharmacological actions in other, better-controllable experimental systems such as voltage clamp.

The characterization of the second negative peak of subendothelial signals as a K^+ current is based on the finding that the known K^+ channel blockers 3,4-DAP and TEA block this signal component. Despite the fact that this negative peak has previously been assigned to the delayed rectifier I_K (Mallart, 1985*b*), and although

small contributions from other types of K^+ currents are likely (see below), several lines of evidence indicate that the main current underlying this signal component may rather be classified as 'fast' K^+ current ($I_{K,f}$): (a) in this, as in most other preparations (for review see Rogawski, 1985), 'fast' K^+ currents are more sensitive to aminopyridine block, (b) the actions of the snake toxins presented in this paper are closely analogous to the effects observed in sensory neurones (Penner *et al.* 1986; Petersen *et al.* 1986), where the fast K^+ current remains unaffected by DTX and

TABLE 1. Comparison of the actions of various toxins and other substances on different types of K^+ currents in mouse motor nerve endings and in dorsal root ganglion neurones (Penner *et al.* 1986; Petersen *et al.* 1986)

	Motor nerve terminal			Sensory neurones	
	$I_{K,f}$	$I_{K(Ca)}$	$I_{K,s}$	$I_{K,f}$	$I_{K,s}$
DTX	—	—	*	—	*
β-BuTX	—	—	*	—	*
CroTX	—	—	*	—†	*†
TPX	—	—	*	?	?
3,4-DAP	*	—	*	*	*
TEA	*	*	*	*	*

Classification of currents and evaluation of effects given in this Table is based on the interpretation provided in the Discussion section. Symbols indicate either inhibition (*), no effect (—) or not known (?). † Own unpublished observations.

β-BuTX. It should be noted, however, that DTX has been reported to block selectively a transient K^+ current (I_A) in hippocampal neurones (Halliwell *et al.* 1986) and an outward current in rat visceral sensory neurones which shows slow and incomplete inactivation (Stansfeld *et al.* 1986). The latter current may be closely analogous to the slowly activating K^+ current ($I_{K,s}$) found in dorsal root ganglion neurones, which is also selectively inhibited by DTX (Penner *et al.* 1986). $I_{K,s}$ does not show marked inactivation within 500 ms, although longer depolarizations in the seconds range induce partial inactivation (see Penner *et al.* 1986).

With the use of K^+ channel blockers which inhibit the fast K^+ current, two previously masked currents were revealed due to the delay of membrane repolarization. Now more Ca^{2+} channels were activated and stayed open for prolonged periods. This resulted in pronounced activation of $I_{K(Ca)}$ which is resistant to 3,4-DAP and could therefore be investigated separately. The additional application of snake toxins left $I_{K(Ca)}$ and I_{Ca} unaffected, suggesting that neither of the channels carrying these currents are toxin targets.

The actions of TEA at high concentrations were less specific than those of 3,4-DAP as not only $I_{K,f}$ but also $I_{K(Ca)}$ was inhibited. Under these conditions, where most of the outward current is already blocked by high doses of TEA, the balance between outward and inward currents is very sensitive to any further block of K^+ conductances through channels spared by TEA. Now, additional application of snake toxins (cf. Fig. 3*A–C*) caused extreme prolongation of slow Ca^{2+} currents, presumably, by blocking a slowly activating K^+ current ($I_{K,s}$). Although an influence of the toxins on activation or inactivation kinetics of slow Ca^{2+} channels cannot

completely be ruled out, the striking similarity to the actions of 3,4-DAP (cf. Fig. 3*E*) rather indicates an inhibition of K^+ channels.

In accordance with many other neuronal membranes, the channel types in mouse motor nerve endings may be classified as fast ($I_{K,f}$), slow ($I_{K,s}$) and Ca^{2+} dependent ($I_{K(Ca)}$). Since marked effects of snake toxins were only registered when presynaptic depolarization exceeded a period of several milliseconds, this may indicate that the current affected by the toxins features slow activation kinetics. This is in close analogy to what is known from the action of DTX and β-BuTX on outward currents in guinea-pig dorsal root ganglion neurones, where these toxins selectively inhibit a slowly activating and non-inactivating K^+ current (Penner *et al.* 1986; Petersen *et al.* 1986; but see Halliwell *et al.* 1986). Based on the interpretation provided above, the actions of drugs and toxins on K^+ currents in nerve terminals may be compared with those in sensory neurones (Table 1).

From the results presented we conclude that the presynaptic membrane of motor nerve endings is equipped with at least three different types of K^+ channels, which can be characterized by pharmacological means. Selective inhibition of slowly activating K^+ channels in nerve terminals may account for the facilitatory effects of DTX and the PLA_2 toxins β-BuTx, CroTX and TPX on transmitter release. Although the toxins did not noticeably affect control signals, changes of some sort have to be assumed, if one accepts increases in transmitter release to result from increased Ca^{2+} influx due to blockade of K^+ channels. A subtle increase in Ca^{2+} current is likely to remain hidden, because the second negative peak recorded in control signals reflects a predominant fast K^+ current, which quickly repolarizes the presynaptic membrane. As a result, the fast I_{Ca} (and also other ionic currents such as $I_{K(Ca)}$ or $I_{K,s}$) will rapidly be shut off, hence contributing only to a minor extent to the recorded signal.

From several arguments it may be reasoned that the effects of β-BuTX, CroTX and TPX are not mediated by their enzymatic activity, which, in any case, is low at room temperature: (i) DTX has a similar action although being devoid of PLA_2 activity, (ii) with bee venom phospholipase A_2, which has higher enzymatic activity, no effects on K^+ currents were found. Since facilitatory effects of the PLA_2 toxins are lost upon treatments which inactivate PLA_2 activity, one may assume that these treatments also affect the toxins' binding or channel-blocking features.

We thank Miss J. Schäfer for technical assistance. This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

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