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The facilitatory actions of snake venom phospholipase A_2 neurotoxins at the neuromuscular junction are not mediated through voltage-gated K^+ channels

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Abstract

Electrophysiological investigations have previously suggested that phospholipase A_2 (PLA2) neurotoxins from snake venoms increase the release of acetylcholine (Ach) at the neuromuscular junction by blocking voltage-gated K^+ channels in motor nerve terminals.

We have tested some of the most potent presynaptically-acting neurotoxins from snake venoms, namely β-bungarotoxin (BuTx), taipoxin, notexin, crotoxin, ammodytoxin C and A (Amotx C & A), for effects on several types of cloned voltage-gated K^+ channels (mKv1.1, rKv1.2, mKv1.3, hKv1.5 and mKv3.1) stably expressed in mammalian cell lines. By use of the whole-cell configuration of the patch clamp recording technique and concentrations of toxins greater than those required to affect acetylcholine release, these neurotoxins have been shown not to block any of these voltage-gated K^+ channels. In addition, internal perfusion of the neurotoxins (100 μg/ml) into mouse B82 fibroblast cells that expressed rKv1.2 channels also did not substantially depress K^+ currents. The results of this study suggest that the mechanism by which these neurotoxins increase the release of acetylcholine at the neuromuscular junction is not related to the direct blockage of voltage-activated Kv1.1, Kv1.2, Kv1.3, Kv1.5 and Kv3.1 K^+ channels.

Keywords: β-bungarotoxin; Taipoxin; Notexin; Crotoxin; Ammodytoxin; Phospholipase A_2; Voltage-gated K^+ channels; Neuromuscular junction; Acetylcholine release

1. Introduction

Several snake venoms contain phospholipase A_2 (PLA_2) neurotoxins that affect the neuromuscular junction. These PLA_2 neurotoxins include β-bungarotoxin (from the banded krait *Bungarus multicinctus*) with two dissimilar A and B chains, which are covalently cross-linked; taipoxin from (from the taipan *Oxyuranus scutellatus scutellatus*) with three non-covalent cross-linked, α, β, and γ subunits; crotoxin (from the rattlesnake *Crotalus durissus terrificus*) composed of two non-covalently linked subunits, CA and CB; notexin (from the tiger snake *Notechis scutatus scutatus*), which is a single peptide chain; and ammodytoxin (from the viper *Vipera ammodytes ammodytes*), which is a single *chain* PLA_2.These neurotoxins are primarily characterized by their ability to block the release of acetylcholine (Ach) from motor nerves. However, prior to blocking Ach release these toxins initially depress before transiently increasing Ach release. The mechanism of action responsible for these triphasic effects on Ach release is not clear. The facilitatory effect of these toxins in mammalian nerve-muscle preparations is independent of phospholipase A_2 enzyme activity (Chang et al., 1973, 1997; Landon et al., 1980; Su and Chang, 1981, 1984; Chang and Su, 1982). Previous electrophysiological investigations indicate that the facilitatory stage may be associated with the blockade of some types of nerve terminal K^+ channels (North, 1995; Dreyer and Penner; 1987; Rowan and Harvey, 1988). Blocking K^+ channels at nerve terminals would slow repolarization of the nerve terminal after action potentials. It is then predicted that voltage-dependent Ca^{2+} channels would open for longer than normal, allowing a larger Ca^{2+} influx to the

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nerve terminal to trigger a greater release of transmitter (Penner and Dreyer, 1986). However, there have been few direct demonstrations of the potassium channel blocking actions of PLA_2 neurotoxins and most of the reports are only about effects of β-bungarotoxin.

At the perineural waveforms of mouse motor nerve terminals, β-bungarotoxin, crotoxin, taipoxin and notexin were shown to block a fraction of the waveform thought to be associated with K^+ currents (Rowan and Harvey, 1988). β-Bungarotoxin partly blocks K^+ current in dorsal root ganglion neurons of the guinea pig (Petersen et al., 1986) and blocks rKv1.2 potassium channels expressed in *Xenopus* oocytes (Guillemare et al., 1992)

From chick brain a binding protein for β-bungarotoxin has been purified. This protein binds ^{125}I-labelled β-bungarotoxin, which can be displaced from this protein by the K^+ channel blockers, dendrotoxin I and mast cell degranulating peptide (MCDP). Hence, it was concluded that the β-bungarotoxin binding protein is a member of a family of voltage-gated K^+ channels (possibly an A-type K^+ channel) (Schmidt and Betz, 1988, 1989). Moreover, β-bungarotoxin could partially displace ^{125}I dendrotoxin from its specific binding in rat central nervous system (Dolly et al., 1987). It was concluded that this effect may be related to the B chain of the toxin that is homologous with dendrotoxin (Rehm and Betz, 1982; Othman et al., 1982).

Little is known about the channel blocking activity of the other PLA_2 neurotoxins, crotoxin, notexin, taipoxin and ammodytoxin. Because of their similar pharmacological effect on neuromuscular junction, it is reasonable to ask whether these neurotoxins can also be demonstrated to block voltage-gated potassium channels. Hence, the aim of this study was to examine the effects of several PLA_2 neurotoxins on a family of cloned voltage-gated K^+ channels, namely mKv1.1, rKv1.2, mKv1.3, hKv1.5 and mKv3.1 stably expressed in mammalian cell lines. Since these channels are widely distributed in the nervous system and BuTx has blocking effects on rKv1.2 expressed in *Xenopus* oocyte voltage-gated K^+ channel (Guillemare et al., 1992), we choose these channels for our experiments. The pharmacological characterization of these cloned voltage-gated K^+ channels in mammalian cell lines has been determined previously (Grissmer et al., 1994).

2. Methods and materials

2.1. Twitch tension recording

Biventer cervicis nerve-muscle preparations (Ginsborg and Warriner, 1960) were isolated from 3 to 14 day old chicks killed by exposure to CO_2 and mounted with a resting tension of approximately 1 g in 10 ml tissue baths containing physiological salt solution of the following composition (mM): NaCl 118.5, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_2 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 11.1. The solution was continuously bubbled with 95% O_2 plus 5% CO_2, maintained at 33 ± 1°C and muscles were indirectly stimulated at 0.1 Hz using ring electrodes with pulses of 0.2 ms duration and a voltage greater than that which produced a maximal twitch. To detect any change in postsynaptic sensitivity, in absence of nerve stimulation, responses to acetylcholine (1 mM), carbachol (20 μM) and KCl (40 mM) were recorded prior to the addition of toxin and at the end of experiment. The muscles were exposed to acetylcholine and KCl for 30 s and to carbachol for 60 s, and heights of contractures were measured at these times. The preparations were washed free of these drugs and allowed 20–30 min to stabilise before the application of toxin. Twitches and contractures were recorded isometrically using Grass Model 79 and Grass Model 7D polygraphs, and Grass Force-Displacement Transducers FTO3.

2.2. Electrophysiology

Experiments were carried out with the whole-cell configuration of the patch clamp technique at the room temperature (20–24°C) with an EPC-7 patch clamp amplifier (List Electronic, Darmstadt, Germany). Cells were superfused at 1 ml/min with filtered (0.45 μm) external solution (290–320 mOsM) containing (in mM): glucose 10, NaCl 145, KCl 5, MgCl 1, NaH_2PO_4 0.06, $CaCl_2$ 2 and HEPES 10, adjusted to pH 7.4 with NaOH. Pipettes were manufactured from borosilicate glass capillaries (Clark Electromedical Instruments), fire-polished and coated with Sigmacoat. Pipettes had resistance of about 2–3 MΩ when filled with the following internal solution (pipette solution) (in mM): EGTA 1, glucose 10, NaCl 5, KCl 140, $MgCl_2$ 1, and HEPES 10, adjusted to pH 7.3 with KOH (290–320 mOsM). This solution was filtered and divided into 1 ml aliquots and stored at −20°C until used on the day of experiments. Toxin stock solutions were prepared in distilled water and were diluted to the appropriate concentration and added to a 1 ml bath that contained external solution. All experiments were done in triplicate unless otherwise stated in the text with at least two different concentrations of toxins, first with a low concentration (3–10 μg/ml) and then with a high concentration (100 μg/ml).

Cells were depolarized by applying either families of 250–600 ms, depolarizing voltage steps in 10 mV increments ranging from −60 to +50 mV or single steps with a 100 mV pulse from −60 to +40 from a holding potential of −60 mV. Cells were depolarized every 20 s before, during and after drug or toxin application. Voltage errors in membrane potential (Vm) were minimized by applying more than 70% series resistance (Rs) compensation without introducing oscillations into the current output of the clamp amplifier. For recording intracellular application of toxins whole cell clamp was established with an electrode filled with intracellular solution containing toxin. Because of time required to establish the whole cell recording, we filled the tips of the electrodes with a small amount of normal

intracellular solution and than back filled with solution containing toxins or drugs. The 100% level was taken as the current in response to the first recorded voltage step. Currents were recorded via a National Instrument Lab's PC analog-to-digital convertor. The data was stored on hard disk and displayed and analysed on an IBM PC-compatible microcomputer, equipped with Strathclyde Electrophysiology Software WCP version 2.3 (supplied by Dr J. Dempster).

2.3. Cell culture

B82 mouse fibroblasts stably transfected with rKv1.2, mouse erythroleukemia (MEL) cells stably transfected with hKv1.5, and L929 mouse fibroblasts cells transfected with Kv1.1, Kv1.3 and 3.1 channels were obtained from Professor Stephan Grissmer, University of Ulm, Albert-Einstein-Alee ll, Ulm, Germany. They were cultured routinely in 50 ml flasks containing media supplemented with 2 mM L-glutamine, penicillin 30 units/streptomycin 30 μg/ml, glucose 0.45 g/ml, geneticin 0.3 mg/ml, sodium bicarbonate 25 mM in Dulbecco's Modified Eagles Medium (DMEM) with 10% foetal calf serum (FCS). pH was adjusted to 7.3 by addition of sodium hydroxide (NaOH).

The flasks of cells were kept in an incubator at 37°C in a moist atmosphere of air with 5% CO_2. Cells were regularly maintained by splitting them after confluence. On the day of experiment, the cells were removed by trypsin-EDTA and a small number of cells was added to two wells of a 24-well plate containing glass coverslips. The coverslips with attached cells were placed in a small (1 ml) experimental chamber and perfused (1 ml/min) with external solution.

MEL cells were differentiated by adding 1% dimethyl sulfoxide (DMSO) into the medium 48–72 h before use for patch clamp experiments.

2.4. Source of toxins and chemicals

β-Bungarotoxin (T-5644, Lots 124H40081, 33H40141 and 68H4003) was from Sigma Chemical Co. Ltd., Poole, Dorset, England and Latoxan, 20 Rue Leon Blum, 2600 Valence, France. Crotoxin was a gift from Dr Grazyna Faure from Institut Pasteur, Paris and notexin was kindly provided by Dr André Ménez from DIEP, CEN Saclay, France and was purchased from Latoxan. Taipoxin was a gift from Dr David Eaker, Biochemistry Department, Uppsala University, Sweden. Dendrotoxin I (DpI) was purchased from Ventoxin (Frederick, MD, USA). Two PLA_2 toxins from the long-nosed viper (*Vipera ammodytes ammodytes*) ammodytoxin A and C were gifts from Dr Igor Krizaj from Department of Biochemistry and Molecular Biology, J. Stefan Institute, University of Ljubljana, Slovenia.

Tetraethylammonium (TEA) and diaminopyridine (3,4-DAP) and materials required for cell cultures were purchased from Sigma Chemical Co. Ltd. Other materials and media required for cell culture were purchased from Gibco Brl., Life Technologies Ltd., Renfrewshire, Scotland.

3. Results

3.1. Twitch tension experiments

The chick biventer cervicis nerve-muscle preparation was used as a convenient test of the biological activity of the phospholipase A_2 neurotoxins, prior to patch clamp experiments. All neurotoxins were tested on indirectly stimulated chick biventer cervicis preparations and equieffective concentrations were determined (data not shown). The toxins caused a slow, progressive decrease in twitch responses to nerve stimulation. Time taken to 50% block was about 100 min with different concentrations of them: β-bungarotoxin (0.47 nM), taipoxin (6.3 nM), notexin (0.2 μM), crotoxin (7.2 nM) and Amtx (3.6 μM). Among these neurotoxins, β-bungarotoxin was more potent than others on the chick preparation (in agreement with results of Chang et al., 1977). β-Bungarotoxin abolished twitch responses in 60 min at 47 nM ($n = 7$), in 120 min at 4.7 nM ($n = 4$), and in 180 min at 0.47 nM ($n = 4$) whereas at least ten times higher concentrations were needed with the other toxins to block twitch responses to nerve stimulation completely in similar times.

3.2. Electrophysiology

Whole-cell K^+ currents were evoked by applying families of 250–600 ms long depolarizing voltage steps in 10 mV increments ranging from −60 to +50 mV or single step depolarizations with a 100 mV pulse from −60 to +40 mV. After control recordings had stabilized with time, the toxin was added to the bath and K^+ currents recorded for 20–30 min. In control experiments, the sensitivity of the K^+ currents to the well-known K^+ channel blockers 3,4-diaminopyridine (3,4-DAP), tetraethylammonium (TEA) and dendrotoxin I (DpI) was tested. Our results are consistent with previous results reported by Grissmer et al. (1994), although we used 3,4-DAP instead of 4-AP (data not shown).

3.3. Effect of β-bungarotoxin, taipoxin, notexin, crotoxin and ammodytoxin

Because of extensive studies on β-bungarotoxin and the potency of this toxin in the chick biventer cervicis preparation, we tested several concentrations (220 nM to 4.7 μM) of β-bungarotoxin on cloned voltage-gated potassium channels, especially on rKv1.2. There was no reduction of K^+ currents compared with control even at high concentrations (up to 100 μg/ml = 4.7 μM) (Fig. 1). Furthermore, to confirm these results we tested different batches of β-bungarotoxin from of different sources (Sigma, UK and

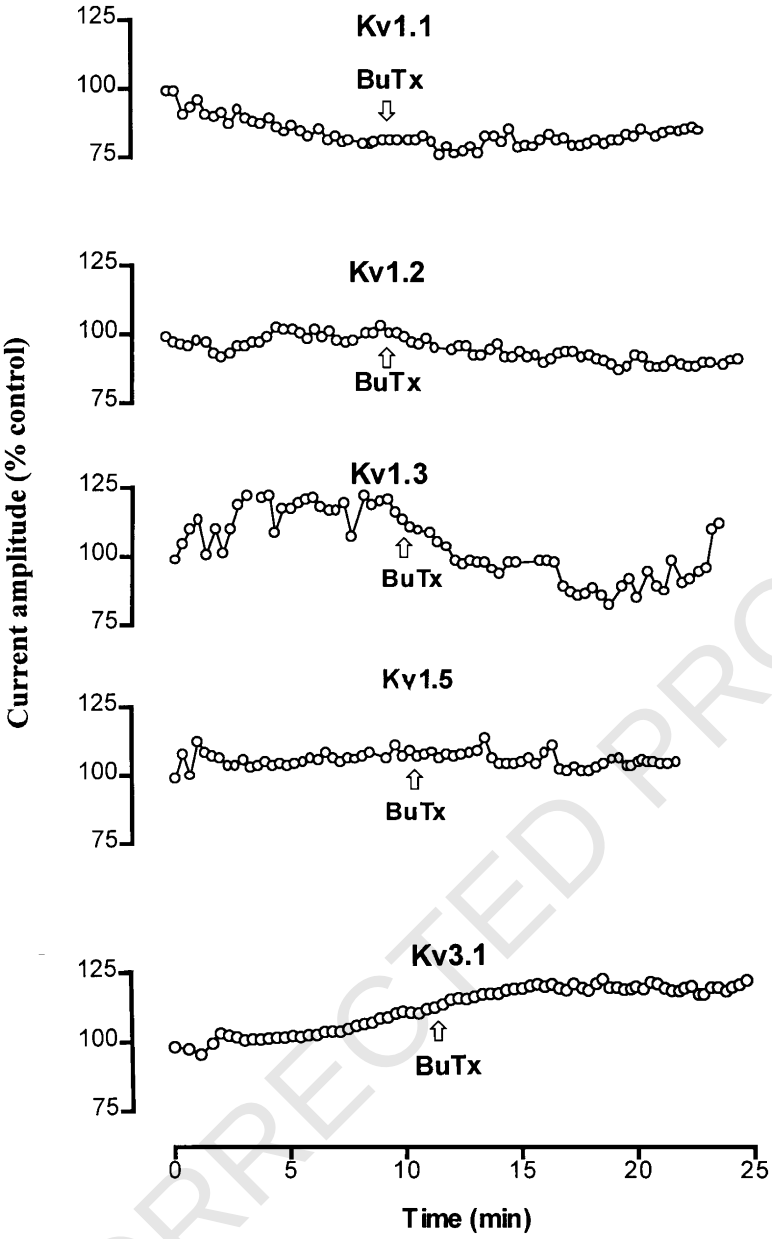


Fig. 1. Lack of effect of β-bungarotoxin (100 μg/ml = 4.7 μM) on K^+ currents through different cloned voltage-gated K^+ channels, Kv1.1, Kv1.2, Kv1.3, Kv1.5 and Kv3.1. stably expressed in different cell lines. Currents were evoked by single depolarizing voltage step from −60 to +40 mV for 250–600 ms duration every 20 s from a −60 mV holding potential.

Latoxan, France). All batches of β-bungarotoxin were shown to lack effect on the K^+ channels studied.

As with BuTx, taipoxin at 20 μM had no effect on any of the cloned voltage-gated K^+ channels tested (Fig. 2). Notexin at 7.3 μM did not affect K^+ currents through Kv1.1, Kv1.2, Kv1.3, Kv1.5 and Kv3.1 (Fig. 3). Crotoxin at 2.4 μM (Fig. 4) and ammodytoxin C at 2 μM (Fig. 5) did not change the K^+ currents through all cloned voltage-gated K^+ channels tested. Because of a shortage of Amotx C, we did not test it on Kv1.3 and used Amtox A on Kv1.1 (Fig. 5), but there was no effect on the currents tested.

3.4. Internal aplication of toxins

To clarify whether these neurotoxins can affect K^+ currents from an intracellular site, internal application of the neurotoxins was tested in B82 fibroblast cells that expressed rKv1.2 K^+ channels. Whole cell recording was

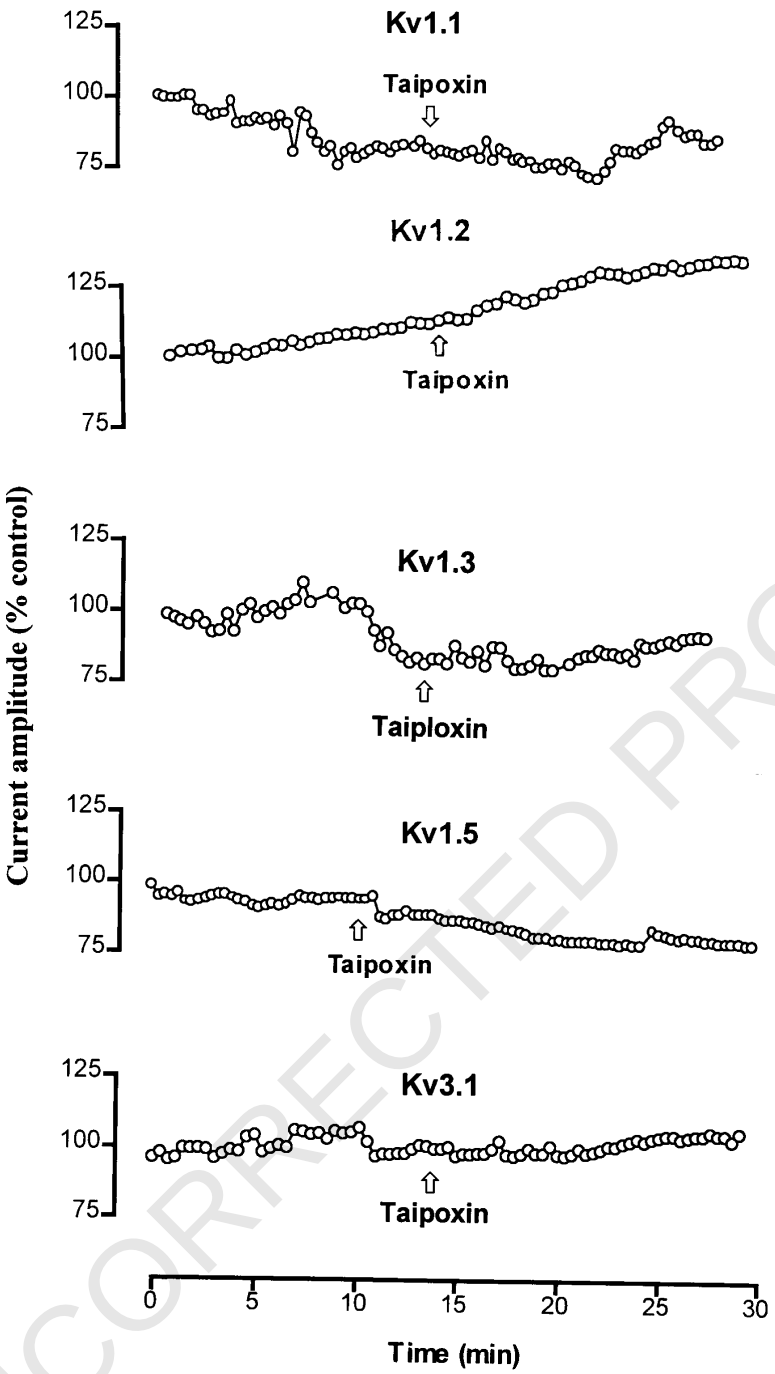


Fig. 2. Lack of effect of taipoxin (100 μg/ml = 20 μM) on K^+ currents through different cloned voltage-gated K^+ channels, Kv1.1, Kv1.2, Kv1.3, Kv1.5 and Kv3.1. stably expressed in different cell lines. Currents were evoked by single depolarizing voltage step from −60 to +40 mV for 250–600 ms duration every 20 s from a −60 mV holding potential.

established with an electrode filled with intracellular solution containing a high concentration (100 μg/ml) of the toxins. Initiation of whole cell recording was taken as time 0. The 100% level was taken as the current in response to the first voltage step. The current was evoked by one-step depolarizing voltage steps from −60 mV to +50 mV at 20 s intervals. As shown in Fig. 6, there was considerable variablity in the currents recorded over a 25 min period. However

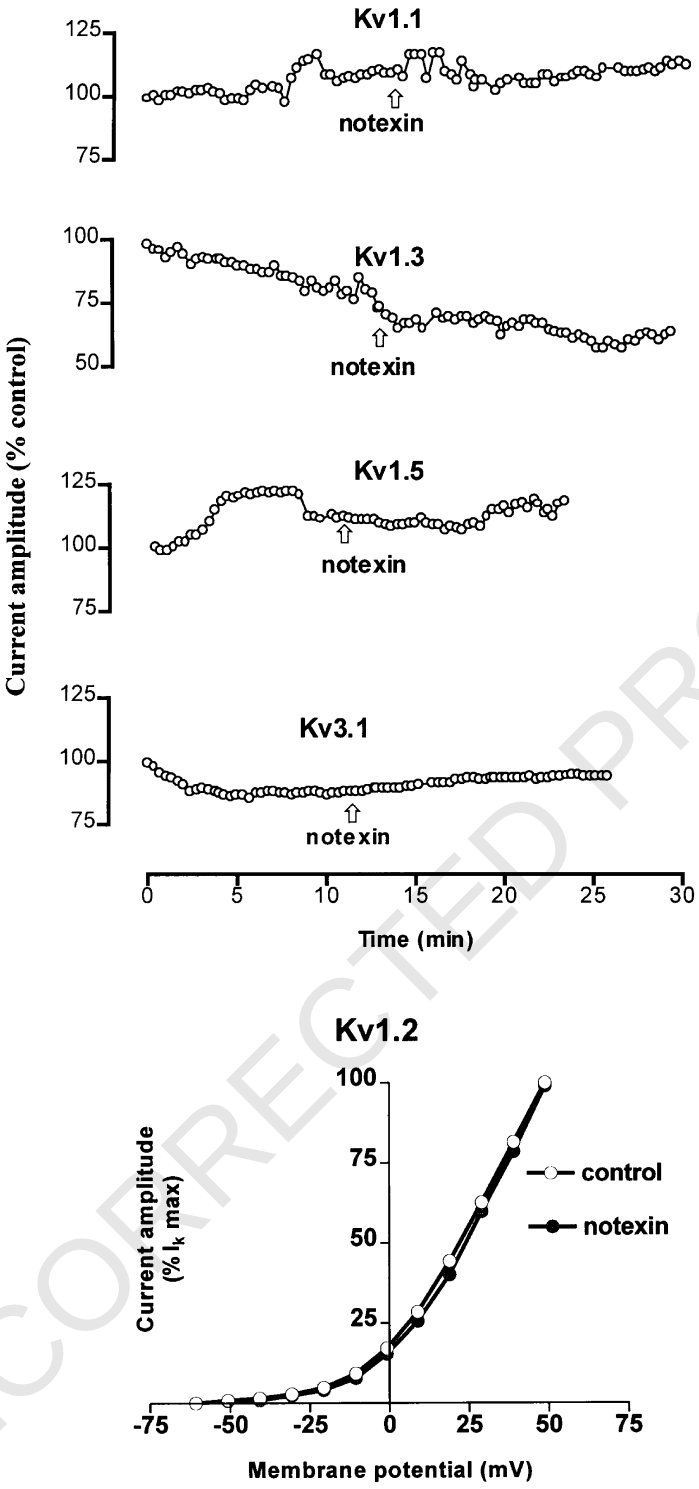


Fig. 3. Lack of effect of notexin (100 μg/ml = 7.3 μM) on K^+ currents through different cloned voltage-gated K^+ channels, Kv1.1, Kv1.2, Kv1.3, Kv1.5 and Kv3.1. stably expressed in different cell lines. Currents were evoked by single depolarizing voltage step from −60 to +40 mV for 250–600 ms duration every 20 s from a −60 mV holding potential and (for effect of notexin on Kv1.2) family of voltage steps from −60 to +50 mV in 10 mV steps for 250 ms duration every 20 s from a −60 mV holding potential.

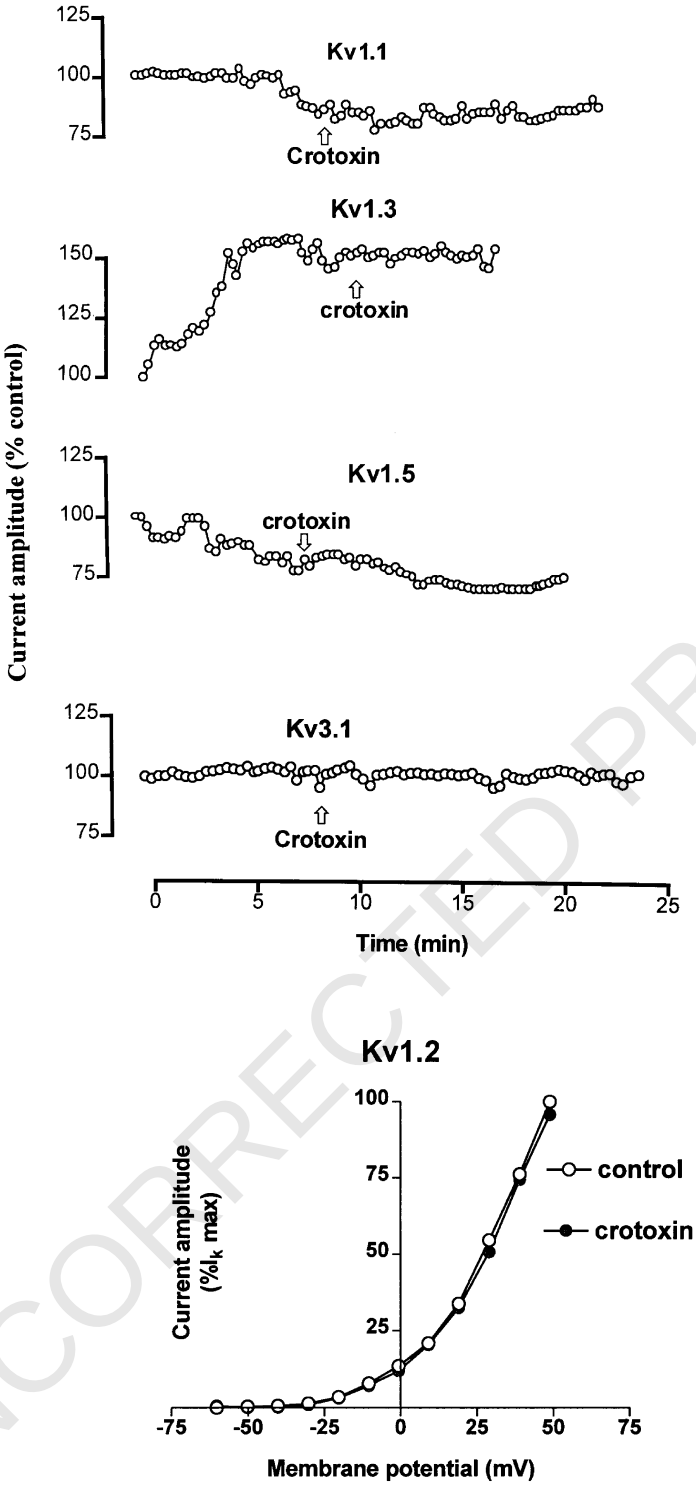
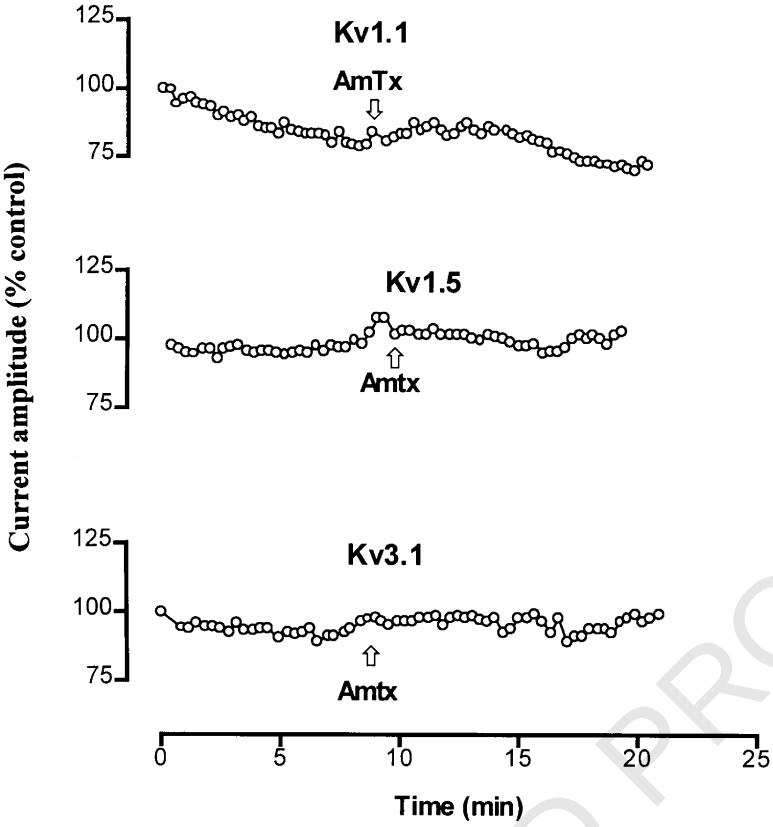


Fig. 4. Lack of effect of crotoxin (100 μg/ml = 2.4 μM) on K^+ currents through different cloned voltage-gated K^+ channels, Kv1.1, Kv1.2, Kv1.3, Kv1.5 and Kv3.1. stably expressed in different cell lines. Currents were evoked by single depolarizing voltage step from −60 to +40 mV for 250–600 ms duration every 20 s from a −60 mV holding potential and (for effect of crotoxin on Kv1.2) family of voltage steps from −60 to +50 mV in 10 mV steps for 250 ms duration every 20 s from a holding potential −60 mV.



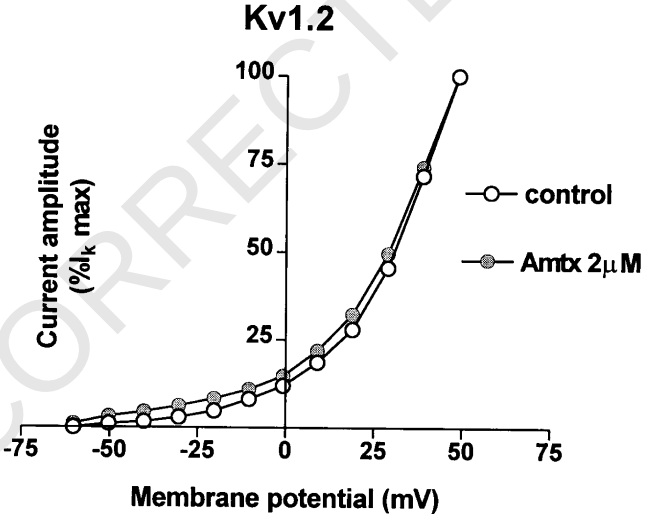


Fig. 5. Lack of effect of ammodytoxin (100 μg/ml = 2 μM) on K^+ currents through different cloned voltage-gated K^+ channels, Kv1.1, Kv1.2, Kv1.3, Kv1.5 and Kv3.1. stably expressed in different cell lines. Currents were evoked by single depolarizing voltage step from −60 to +40 mV for 250–600 ms duration every 20 s from a holding potential −60 mV and (for effect of ammodytoxin on Kv1.2) family of voltage steps from −60 to +50 mV in 10 mV steps for 250 ms duration every 20 s from a holding potential −60 mV.

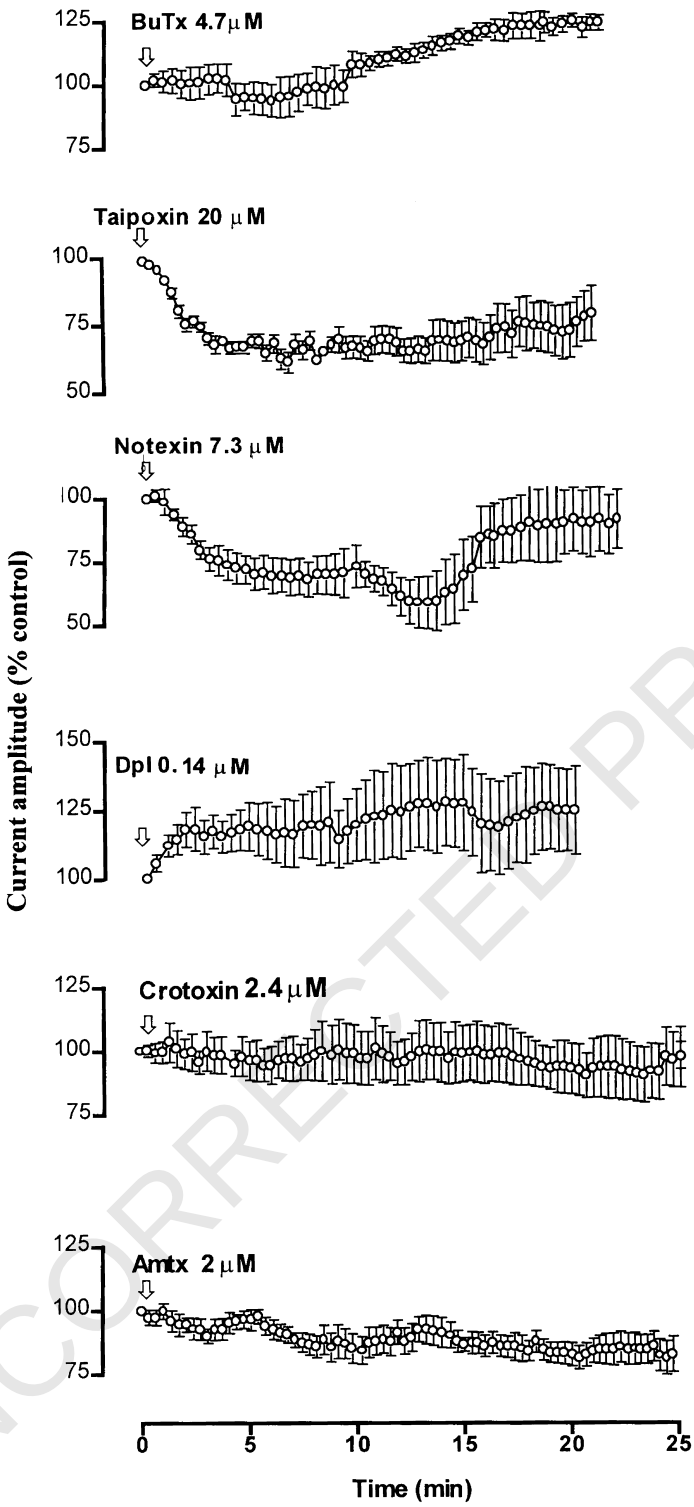


Fig. 6. Lack of effect of internal application of β-bungarotoxin ($n = 5$), notexin ($n = 5$), crotoxin ($n = 4$), ammodytoxin C ($n = 4$) (all at 100 μg/ml), dendrotoxin I (DpI) (1 μg/ml = 0.14 μM) ($n = 4$) and weak effect of taipoxin ($n = 4$) on K^+ currents through cloned voltage-gated Kv1.2 potassium channel stably expressed in B82 mouse fibroblast cells. Currents were evoked by depolarizing voltage steps from −60 to +40 mV for 250 ms duration every 20 s. Each point represents the mean ± S.E.M. of four to five different cells.

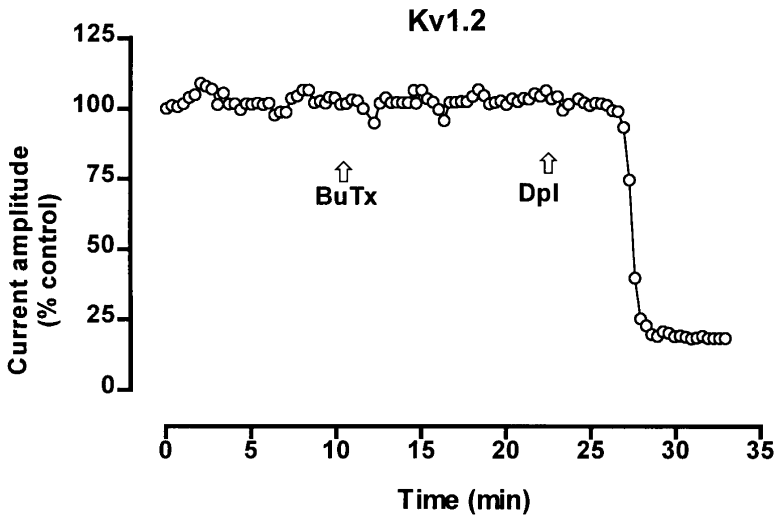


Fig. 7. Effect of external application of dendrotoxin I (1 μg/ml = 0.14 μM) on K^+ currents through cloned voltage-gated K^+ channel Kv1.2 stably expressed in B82 mouse fibroblast cell compared with external application of β-bungarotoxin (100 μg/ml = 4.7 μM). Currents were evoked by single depolarizing voltage step from −60 to +40 mV for 250–600 ms duration every 20 s from a holding potential −60 mV.

β-bungarotoxin (4.7 μM), crotoxin (2.4 μM) and ammodytoxin A (2 μM) did not substantially alter the amplitude of the K^+ current. With taipoxin (20 μM) and notexin (7.3 μM) the results were less clear: there was an initial apparent fall in current amplitude of about 20–30% followed by a partial recovery in three out of five experiments. Overall, it seems unlikely that any of the toxins were directly blocking the rKv1.2 channels from the internal site of the membrane, particularly when it was observed that the cells become rather unstable during the internal application of toxins.

Internal application of dendrotoxin I (1 μg/ml = 0.14 μM) did not affect K^+ current through rKv1.2 K^+ channels while the same concentration was enough to block this channel externally (Fig. 7). Hence, the lack of activity of the PLA_2 neurotoxins is not due to the inability of toxic polypeptides to move from the pipette into the cell cytosole.

4. Discussion

The pharmacological effects of PLA_2 neurotoxins from snake venoms have been extensively studied. These neurotoxins act at the neuromuscular junction to cause an increase of acetylcholine release prior to blockade of release. The mechanism of this facilitatory effect is not known, although several hypotheses have been suggested. In particular (as described in the Introduction), previous reports showed that some of these neurotoxins can decrease K^+ currents. We investigated more directly the possible potassium channel-blocking action of these neurotoxins on cloned potassium channels by use of patch-clamp recording.

In this study, we found that β-bungarotoxin, taipoxin, notexin, crotoxin, and ammodytoxin do not block voltage-activated K^+ currents through cloned Kv1.1, Kv1.2, Kv1.3, Kv1.5 and Kv3.1 potassium channels expressed in mammalian cells. In addition, internal application of these neurotoxins into cells expressing rKv1.2 potassium channels did not affect K^+ current through this channel. These results strongly indicate that the mechanism of the facilitatory effect of these PLA_2 neurotoxins at the neuromuscular junction is not associated with direct block of these K^+ channels. However, it should be pointed out that β-bungarotoxin was found to block Kv1.2 channels expressed in oocytes (Guillemare et al., 1992), implying that the different expression systems may affect properties of the channels. Additionally, it is important to realise that the current recorded from a cloned channel after expression may not always exactly reflect the behaviour of the channel in the native cell. This is because the subunit structure of native and expressed channels may not always be the same, and because expressed channels may sometimes lack certain regulatory proteins even though they are still functional. This is particularly true of voltage-activated K^+ channels which form as tetramers of individual subunits (Hille, 1992; Pongs, 1992a). It has been shown that multiple voltage-gated K^+ channels subtypes containing several α and β subunits coexist in native cells such as brain, heart, myelinated nerves, myocytes and single dorsal root ganglion (DRG) neurons, and forming heteromultimeric channels (Parcej et al., 1992; Wang et al., 1993; Sheng et al., 1993; Scott et al., 1994; Accili et al., 1997a,b; Jan and Jan, 1997; Ishikawa et al., 1999) which have properties different to those of cloned channels (Pongs, 1992b; Sheng et al., 1993; North, 1995). We still do not know the subunit composition of native

channels in motor nerve terminals affected by these PLA_2 neurotoxins. Many details of differences between the behaviour of expressed channels in vitro and native channels in vivo still remain to be discovered (Robertson, 1997).

Further evidence dissociating facilitatory effects of PLA_2 neurotoxins from potassium channel blockade comes from experiments with suramin. Suramin is an anti-trypanosomiasis drug which reverses the effects of non-depolarizing neuromuscular blockers (Henning et al., 1993) and inhibits the prejunctional Ca^{2+} channels lead to decrease Ach release (Henning et al., 1996). Suramin also antagonises effects of β-bungarotoxin by prolonging time to paralysis in vivo and delaying the block of transmitter release in vitro (Lin-Shiau and Lin, 1999). In mouse hemi-diaphragm nerve-muscle preparations that are partly paralysed by high Mg^{2+}, suramin alters the triphasic action of β-bungarotoxin and taipoxin and inhibits the facilitatory effect of these PLA_2 neurotoxins (unpublished results), while pretreatment of mouse nerve-triangularis sterni muscles with 0.3 mM suramin does not change the blocking effect of β-bungarotoxin on the nerve terminal K^+ current (Lin-Shiau and Lin, 1999).

Based on these results we conclude that the mechanism underlying the facilitation of acetylcholine release at mammalian neuromuscular junctions is not due to direct blockade of voltage-gated K^+ channels. However, we cannot exclude the possibility that the cloned voltage-gated K^+ channels used in the present study may not express the binding site for PLA_2 neurotoxins. In addition, it is possible that the decrease of K^+ current at perineural waveforms from mouse nerve-triangularis sterni muscle preparations induced by these neurotoxins is an indirect effect whose mechanism is not yet clear.

4. Uncited References

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