JPET Fast Forward. Published on August 31, 2005 as DOI: 10.1124/jpet.105.093641
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TITLE PAGE

Isolation and pharmacological characterisation of cannitoxin, a presynaptic neurotoxin from the venom of the Papuan taipan (*Oxyuranus scutellatus canni*)

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RUNNING TITLE PAGE

a) **Cannitoxin: a β-neurotoxin from Papuan taipan venom.**

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c) Number of text pages: 24

Number of tables: 2

Number of figures: 5

Number of references: 21

Number of words in abstract: 176

Number of words in introduction: 485

Number of words in discussion: 963

d) Chick biventer cervicis nerve muscle (CBCNM); Phospholipase A_2 (PLA_2); 4-Bromophenacyl bromide (4-BPB); Matrix assisted laser desorption - time of flight (MALDI-TOF).

e) Section assignment: Neuropharmacology

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ABSTRACT

The Papuan taipan (*Oxyuranus scutellatus canni*) is widely distributed throughout much of Papua New Guinea. Although neurotoxicity is a major symptom of envenomation, no neurotoxins have been isolated from this venom. Using a series of size exclusion chromatography steps, we report the isolation of cannitoxin, a presynaptic neurotoxin (44,848 Da) which represents approximately 16% of the whole venom. The toxin displayed high PLA_2 activity (330 ± 5 µmol/min/mg) and caused concentration-dependent (11-66 nM) inhibition of indirect (0.2 ms, 0.1 Hz, supramaximal V) twitches of the chick biventer cervicis nerve-muscle preparation (CBCNM) without effecting nicotinic receptor agonists. Prior addition of CSL Taipan antivenom (5 U/ml), or inhibition of PLA_2 activity by incubation with 4-bromophenacyl bromide (4-BPB), prevented the inhibition of twitches. Cannitoxin is composed of three different subunits, α, β and γ with the possibility of two β isomers. However, only the α subunit displayed in vitro neurotoxic activity of its own. Thus, cannitoxin is similar in structure and pharmacology to taipoxin which has been isolated from the closely related Australian species *O. scutellatus scutellatus* (coastal taipan).

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INTRODUCTION

Three species of taipans (genus *Oxyuranus*) have been identified, and are considered to be the world's most venomous snakes (Sutherland and Tibballs, 2001). These are the Australian coastal taipan (*O. s. scutellatus*), inland taipan (*O. microlepidotus*) and Papuan taipan (*O. s. canni*), which are found throughout the northern coastal region of Australia (Worrell, 1970), central Australia (Sutherland and Tibballs, 2001), and in the low lying and mountainous terrains of Papua New Guinea (O'Shea, 1996), respectively. The rank order of potency of the venoms based on murine LD_{50} (s.c.) data are as follows: *O. microlepidotus* (0.025 mg/kg) (Broad et al., 1979) > *O. s. canni* (0.0505 mg/kg) (Sutherland and Tibballs, 2001) > *O. s. scutellatus* (0.099 mg/kg) (Broad et al., 1979). However, t_{90} (i.e. time to produce 90% inhibition of indirect twitches) data obtained in the chick biventer cervicis nerve muscle preparation, indicated that *O. s. scutellatus* venom is more neurotoxic than that of *O. s. canni* venom (Crachi et al., 1999b). They also showed that CSL taipan antivenom was markedly more effective in neutralising the neurotoxic effects of the Papuan or coastal taipan venoms than that of the inland taipan (Crachi et al., 1999b).

Presynaptic neurotoxins act at the motor nerve terminal to either facilitate (e.g. dendrotoxin) or inhibit (e.g. β-bungarotoxin, taipoxin and paradoxin) the release of neurotransmitter. All the presynaptic inhibitors have phospholipase A_2 activity (Harris, 1991). Taipoxin and paradoxin were isolated from the venoms of *O. s. scutellatus* (Fohlman et al., 1976) and *O. microlepidotus*, respectively. Taipoxin is a ternary complex of three subunits, α-, β- and γ-taipoxin which exist in a stoichiometry of 1:1:1 held together by non-covalent interactions. The α subunit is the only subunit with toxic activity on its own. The β subunit is neutral and exists in two isoforms, i.e. β_1 and β_2, which are interchangeable in the complex. The γ subunit is the largest of the three and appears to be glycosylated (Fohlman et al., 1976). Paradoxin has

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a similar structure to taipoxin (Fohlman, 1979), however this neurotoxin has been less well studied and no reports of the presence of two β isoforms have been located in the literature.

Envenoming by the Papuan taipan is a significant health problem in Papua New Guinea, with 70-80 patients being admitted to the Port Moresby General Hospital alone each year (Connolly et al., 1995). Symptoms of envenoming include local tender lymphadenopathy, abdominal pain, coagulopathy and neurotoxicity (Connolly et al., 1995). However, research on the venom of the Papuan taipan has been limited to a basic pharmacological examination (Crachi et al., 1999a; Crachi et al., 1999b) and no neurotoxins have been isolated so far. In particular, it is not known if the venom contains a presynaptic neurotoxin similar to paradoxin and taipoxin which have been isolated from the two closely related species.

This study reports the isolation and characterisation of cannitoxin, a presynaptic neurotoxin from the venom of the Papuan taipan.

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MATERIALS AND METHODS

All chromatography separations were performed using a Shimadzu High Performance Liquid Chromatography system (LC-10ATvp pump and SPD-10AVP detector).

Size exclusion chromatography.

Freeze-dried venom was dissolved in ammonium acetate buffer (0.1M; pH 5.0) and insoluble material removed by centrifugation at 5000 x g for 5 min. The supernatant was applied to a Superdex G-75 column (13 µM, 10 x 300 mm) equilibrated with ammonium acetate buffer, and the sample was eluted at a flow rate of 0.5 ml/min. The purified component was rerun under the same conditions to ensure purity. The eluant was monitored at 280 nm.

Separation of cannitoxin subunits using reverse phase-high performance liquid chromatography (RP-HPLC).

The freeze-dried purified component was reconstituted in MilliQ water and applied to a Phenomenex Jupiter analytical (150 mm x 2 mm, 5µ, 300Å) C18 column after equilibrating with solvent A (0.1% TFA). The sample was eluted with the following gradient conditions of solvent B at a flow rate of 0.2 ml/min: 0-20% over 5 min, 20-60% in 40 min and then 60-80% over 5 min (Wickramaratna et al., 2004). The eluant was monitored at 280 and 214 nm.

Molecular mass determination.

Molecular mass was determined by both size exclusion chromatography and mass spectrometry.

(a) Size exclusion chromatography on non denaturing media – The molecular weight of the complex was determined by gel filtration on a Superdex G75 column equilibrated with

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ammonium acetate buffer (0.1M; pH 5.0). The column was calibrated with the series of known standards (6,500 – 66,000 Da) using the molecular weight marker kit for gel filtration chromatography (Sigma; Lot # 093K9307; MW-GF-70). The eluant was monitored at 280 nM and a flow rate of 0.6 ml/min was used. Void volume (V_o) of the column was determined by running blue dextran and the elution volume (V_e) was calculated for each molecular weight marker, prior to injecting the purified component (0.5 mg). The molecular weight of the toxin was determined from a plot of Log (MW) Vs V_e/V_o ratio.

(b) Mass spectrometry – MALDI-TOF MS analysis was performed with an Applied Biosystems Voyager-DE STR BioSpectrometry Workstation. The instrument was operated in positive polarity in linear mode using sinapinic acid matrix (Agilent Technologies) for low-resolution protein analysis. Matrix (1 µl) was spotted on the sample plate and allowed to air dry; sample (1 µl) diluted in acetonitrile/water (1:1) containing 0.1% (v/v) formic acid was subsequently spotted on dried matrix and allowed to air dry. Data from 500 laser shots (337 nm nitrogen laser) were collected, signal averaged, and processed with the instrument manufacturer's Data Explorer software.

Chick biventer cervicis nerve-muscle (CBCNM) preparation.

Chickens (4-10 day old male) were sacrificed with CO_2 and both biventer cervicis nerve-muscle preparations were dissected. These were mounted under 1 g resting tension in 5 ml organ baths containing physiological salt solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $CaCl_2$, 2.5; $NaHCO_3$, 25 and glucose, 11.1. The solution was maintained at 34°C bubbled with carbogen (95% O_2 and 5% CO_2).

Motor nerves were stimulated every 10 s (0.2 ms duration) at supramaximal voltage using a Grass S88 stimulator (Harvey et al., 1994). d-Tubocurarine (dTC) was added (10 µM)

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and the subsequent abolition of twitches confirmed the selective stimulation of nerves. Responses to nerve stimulation were re-established by thorough washing. Contractile responses to acetylcholine (ACh; 1 mM for 30 s), carbachol (CCh; 20 μM for 60 s) and potassium chloride (KCl; 40 mM for 30 s) were obtained in the absence of stimulation (Harvey et al., 1994). The preparations were then equilibrated for at least 30 min with continuous nerve stimulation (as above) before addition of toxin. In all experiments toxin (11–66 nM), subunits (100 nM) or venom (10 μg/ml) were left in contact with the preparations until responses to nerve stimulation were abolished or for a maximum of 5 h if total twitch blockade did not occur. At the conclusion of the experiment, responses to ACh, CCh and KCl were obtained as previously described. Time taken to reduce the amplitude of the indirect twitches by 90% (t_{90}) was calculated in order to provide a quantitative measure of neurotoxicity (Crachi et al., 1999a).

Where indicated, CSL Taipan antivenom (TPAV; 5 U/ml) was added 10 min prior to the addition of toxin. Reversibility of the venom was tested by adding TPAV (5 U/ml) at t_{90} after the addition of venom (10 μg/ml). Antivenom was left in contact with the tissue for 2 h.

Determination of PLA_2 activity.

PLA_2 activity of the venom, cannitoxin and cannitoxin subunits was determined using a colourimetric assay kit (Cayman Chemical, USA) designed to test the activity of secretory PLA_2s. This assay uses the 1,2-dithio analog of diheptanoyl phosphatidylcholine which serves as a substrate for PLA_2 enzymes. Free thiols generated following the hydrolysis of the thio ester bond at the *sn*-2 position by PLA_2 are detected using DTNB (5,5'-dithio-*bis*-(2-nitrobenzoic acid)). Colour changes were monitored using a CERES900C micro-plate reader (Bio-Tek Instruments, USA) at 405 nm, sampling every minute for a 5 min period. PLA_2

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activity was expressed as micromoles of phosphatidylcholine hydrolysed per min per milligram of enzyme.

Phospholipase A_2 (PLA_2) inhibition with 4-bromophenacyl bromide (4-BPB).

PLA_2 activity of venom was inhibited by alkylation with 4-BPB. Toxin (11 nM) made up in sodium cacodylate-HCl buffer (0.1 M, pH 6.0), and 4-BPB made up in acetone were added to produce a final concentration of 1.8 mM (Abe et al., 1977; Bell et al., 1998; Crachi et al., 1999a). Vials containing the above mixture were incubated for 16 h at 30°C. Where indicated, toxin made up in sodium cacodylate-HCl buffer incubated with acetone was used as a vehicle control for 4-BPB (Wickramaratna et al., 2003).

N-terminal amino acid sequence determination.

Purified peptides were loaded into the sequencing chamber of a Procise N-Terminal amino acid sequencer (Applied Biosystems, Foster City CA. USA) and the amino acid sequence determined (Edman degradation, PTH derivatisation chemistry and the separation of derivatised amino acids by RP HPLC), using the manufacturers recommended methods and reagents.

Chemicals and drugs.

The following drugs were used: acetylcholine chloride, bovine serum albumin, carbamylcholine chloride (carbachol), d-tubocurarine chloride (dTC), 4-bromophenacyl bromide, ammonium acetate, molecular weight marker kit (6,500-66,000), Cacodylic acid (Sigma Chemical Co., St. Loius, MO, USA), potassium chloride (Ajax chemicals, Sydney Australia), trifluoroacetic acid (Auspep, Melbourne, Australia), acetonitrile (Ajax Finechem,

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Australia) and Taipan antivenom (CSL Ltd, Melbourne, Australia), Acetone (BDH chemicals, Victoria, Australia).

Analysis of results and statistics.

In isolated tissue experiments, responses were measured via a Grass force displacement transducer (FTO3) and recorded on a Powerlab system. Twitch height and contractile responses to agonists were expressed as a percentage of the corresponding value prior to the addition of toxin. Statistical difference was determined by a one-way analysis of variance (ANOVA) on the twitch height at the 300 min time point and on the contractile responses to exogenous agonists. All ANOVAs were followed by a Bonferroni *post hoc* test, and statistical significance was indicated where $P<0.05$.

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RESULTS

Chick biventer studies - whole venom.

Venom from the Papuan taipan displays postsynaptic neurotoxic activity (Crachi et al., 1999a) which is prevented by CSL taipan antivenom (Crachi et al., 1999b). In the current study, taipan antivenom (5 U/ml) added at t_{90} (44 ± 8.2 min) failed to reverse, or prevent further, inhibition of indirect twitches over a period of 2 h (Fig. 1a). However, antivenom restored the response of the tissue to exogenous ACh and CCh (Fig. 1b). This confirmed the presence of a presynaptic neurotoxin and indicated that only the postsynaptic neurotoxic effects of the venom can be reversed by taipan antivenom. Therefore we decided to purify and characterize the presynaptic neurotoxin.

Size exclusion chromatography.

Cannitoxin was isolated from the venom of the Papuan taipan following successive separations by size exclusion chromatography. Initial fractionation on a Superdex G-75 column produced six main peaks (Fig. 2a). Screening in the chick biventer cervicis nerve-muscle preparation indicated presynaptic neurotoxic activity in the second peak. The third peak appeared to contain postsynaptic neurotoxins. The elution profile of the venom displayed a similar profile to coastal taipan venom with the second and third peaks showing presynaptic and postsynaptic neurotoxic effects, respectively (Fohlman et al., 1976). Therefore the second peak was chosen for further purification of the presynaptic toxin using size exclusion chromatography and RP-HPLC. Cannitoxin elutes as clean peak with an approximate retention time of 20 min (Fig. 2b) and accounts for approximately 16 % of the whole venom.

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Reverse phase–high performance liquid chromatography (RP-HPLC).

The subunits of cannitoxin were further fractionated using a RP-HPLC (Phenomenex Jupiter analytical) column. This produced four main peaks (Fig. 2c) which were analysed based on the N-terminal amino acid sequences, as well as molecular masses, and assigned as the following subunits (see below): α (13824 Da), β_1 (13242 Da), β_2 (13276 Da), and γ (17762 Da). The molecular weights were determined by MALDI-TOF (see below) analysis and confirmed by ESI-MS.

Molecular mass determination.

Size exclusion chromatography in non denaturing media.

Molecular mass was determined by size exclusion chromatography on a Superdex G-75 column, calibrated with the following protein standards: BSA (66,000 Da), Carbonic anhydrase (29,000 Da), Cytochrome C (12,400 Da) and Aprotinin (6,500 Da). The void volume (Vo) of the column was determined as 7.56 ml using blue dextran (2,000,000 Da), and the elution volume (Ve) of cannitoxin was calculated as 10.15 ml. The molecular weight of cannitoxin was determined from a plot of Log MW Vs corresponding Ve/Vo ratio (of the protein standards described above) to be 45,000 Da (Fig. 3).

Mass spectrometry.

Initial MALDI-TOF analysis of the complex protein indicated the presence of four subunits, two of which are likely to represent isomers as evidenced by similar molecular mass resemblance as well as the split peaks in MALDI spectra (data not shown). MALDI analysis also indicated a possible glycosylation in one of the subunits.

In addition, individual subunits purified by RP-HPLC were analysed by MALDI-TOF. The subunits were found to be of the following molecular masses: 13,242 (β_2), 13,276 (β_1),

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13,824 (α) and 17,762 (γ). MALDI-TOF analysis showed the γ subunit (17,762 Da) to be heterogeneously glycosylated (data not shown), confirming the data mentioned above. These masses are in agreement with those observed upon initial MALDI-TOF analysis of the protein complex, and were also confirmed by ESI-MS. The sum of the molecular masses of α, γ and β_1 or β_2 subunits results in an average mass of 44,848 Da, which is in agreement with that determined by size exclusion chromatography.

N-terminal amino acid sequence.

Partial N-terminal amino acid sequence of the isolated subunits of cannitoxin was determined using Edman degration (Table 1). These were compared with the protein sequences of the corresponding subunits of taipoxin at the National Center for Biotechnology Information (NCBI) database using the BLAST service. The subunits showed sequence identity in the order β_1 (100 %) > γ (86 %) > α (85 %), based on the partial N-terminal sequence. Since the other subunit is structurally similar to β_1 we identified this subunit as β_2. The N-terminal sequence of β_2 taipoxin was not listed in the database or literature, hence comparisons cannot be made.

Chick biventer studies.

(a) Cannitoxin - cannitoxin (11-66 nM) caused concentration-dependent inhibition of indirect twitches in the CBCNM preparation (Fig. 4a), but had no effect on the response of the tissue to ACh, CCh or KCl (Fig. 4b) indicating its action at the presynaptic terminal. Canitoxin incubated with 4-BPB, or TPAV added prior to the addition of cannitoxin, resulted in prevention of the toxin induced inhibition of indirect twitches (Fig. 4a), but had no significant effect on the response of the tissue to exogenous agonists (Fig. 4b). Vehicle (i.e.

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BSA) and cannitoxin in the presence of vehicle (i.e. acetone) had no effect on the agonist responses (data not shown).

(b) Subunits of cannitoxin - The γ and β_2 subunits (100 nM) of cannitoxin had no significant effect on indirect twitches of the CBCNM preparation. However, the α subunit (100 nM) caused a slight but significant inhibition of indirect twitches compared with the vehicle (Fig. 5a). None of the subunits had any significant effect on the response of the tissue to agonists (Fig. 5b).

Phospholipase A_2 activity.

Venom had a specific activity of 330 ± 5 µmol/min/mg while that of the positive control (i.e., bee venom) was 344 ± 26 µmol/min/mg. 4-BPB significantly inhibited the PLA_2 activity of cannitoxin (Table 2; n=3-6). The PLA_2 activity of taipoxin, determined previously (Fohlman, 1979), is included for comparison. cannitoxin and taipoxin The α-subunit appears to be the only subunit with a significant level of activity in (Table 2).

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DISCUSSION

This study describes the isolation and the pharmacological characterization of the first presynaptic neurotoxin, cannitoxin, from the venom of *O. s. canni.*

The venom from the Papuan taipan contains post synaptic neurotoxic activity (Crachi et al., 1999a), which is neutralised by the prior addition of taipan antivenom (Crachi et al., 1999b). However, in the current study antivenom added at the t_{90} time point failed to reverse the indirect twitches indicating the presence of a presynaptic neurotoxin(s). Such neurotoxins are unable to be reversed by the addition of antivenom once they have been bound and internalised (Fohlman et al., 1976) due to the physical damage to the presynaptic membrane by phospholipid hydrolysis.

The molecular weight of cannitoxin was determined as 45,000 Da using of a series of known molecular weight standards on non denaturing media. RP-HPLC of cannitoxin produced four main peaks, the molecular weights of which do not add up to 45,000 Da. The similar molecular mass of the β_1 and β_2 components suggest the possibility of isomerism, which is confirmed by the high sequence similarity (90 %) between these two peptides. Further to this, a combined molecular mass of 44,848 Da (consisting of the sum of α, γ, plus β_1 or β_2 subunits) is in agreement with the molecular weight estimated from size exclusion chromatography. The average molecular weight of cannitoxin can thus be calculated as 44,912 Da. MALDI-TOF analysis revealed the γ subunit of cannitoxin to be a glycoprotein as evidenced by the areas of heterogenous glycosylation observed in the MALDI spectra. High similarity of the partial N-terminal sequences was observed between the corresponding subunits of taipoxin and cannitoxin. Therefore, it appears that cannitoxin is similar in structure to that of taipoxin, isolated from the coastal taipan, which consists of an α subunit, two isomers (see comments above) of the β subunit and a glycosylated γ subunit (Fohlman et al., 1977).

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Cannitoxin was examined for in vitro neurotoxic effects using the CBCNM preparation. Cannitoxin caused concentration-dependent inhibition of the indirect twitches, with a lack of effect on contractile responses to exogenous nicotinic agonists confirming its pre synaptic activity. Reduction of twitches by cannitoxin is triphasic with an initial decrease, a transient increase followed by the complete inhibition of indirect twitches. This triphasic effect is commonly associated with other presynaptic neurotoxins such as taipoxin, notexin and β-bungarotoxin (Harris, 1991). The initial two phases appear to be independent of PLA_2 activity (Harvey, 1990) and are particularly evident when the safety factor of transmission is lowered by reducing the Ca^{2+} or increasing the Mg^{2+} content of the bathing medium (Chang et al., 1977). Therefore, cannitoxin is similar in pharmacology and toxicology to taipoxin, which causes the inhibition of nerve-mediated twitches in the CBCNM with a lack of effect on contractile responses to exogenous nicotinic agonists (Crachi et al., 1999).

PLA_2 enzymes are found in the venoms of snakes of all families (Harris, 1991), hence the whole venom, cannitoxin and its subunits were examined for PLA_2 activity. The level of activity displayed by cannitoxin was approximately three fold less than that of the whole venom suggesting the presence of other venom components with PLA_2 activity. Although there is no quantitative relationship between the potency and PLA_2 activity of presynaptic neurotoxins, inhibition of enzymatic activity is known to prevent their toxic effects (Yang, 1997). Thus in order to test whether the PLA_2 activity of cannitoxin is essential for its toxic effects, it was subjected to 4-BPB modification. Previous studies have shown that PLA_2 activity can be inhibited by selective acylation of His-48 residue using 4-BPB (Volwerk et al., 1974; Abe et al., 1977). When cannitoxin was incubated with 4-BPB, PLA_2 activity as well as the neurotoxic effects were abolished. This suggests that PLA_2 activity is essential for the neurotoxic effects of cannitoxin. Similarly, modification by 4-BPB inhibits the effects of other presynaptic neurotoxins such as taipoxin (Fohlman et al., 1979) and β-bungarotoxin

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(Abe et al., 1977). Further similarity between cannitoxin and taipoxin is highlighted by the PLA_2 activity being largely confined to the α-subunit of cannitoxin.

Given the medical importance of this species, it was important to assess the efficacy of the commercially available taipan antivenom in neutralising the effects of cannitoxin. Prior addition of antivenom prevented the toxin-induced inhibition of indirect twitches in the CBCNM preparation. Addition of antivenom after the addition of toxin was not undertaken in this study since a similar experiment involving whole venom confirmed the irreversible nature of presynaptic neurotoxins.

Presynaptic neurotoxins with PLA_2 activity can be classified based on structure as being single, dimeric or multichain complexes (Yang, 1997). Multichain neurotoxins consist of several different polypeptide chains held together by non-covalent interactions, with at least one of the subunits having toxic activity on its own (Yang, 1997). However, previous studies have shown that the activity of the toxic subunit is far less in comparison to the native toxin (Fohlman et al., 1976; Francis et al., 1993). Therefore, the subunits of cannitoxin were tested for their neurotoxic effects. Despite having a high level of PLA_2 activity, only the α subunit, at a much higher concentration (100 nM), caused a slight but statistically significant decrease in the indirect twitches. This further proves the lack of any quantitative relationship between enzymatic and toxic activity, as well as the possibility of the other subunits acting as chaperones (Fohlman et al., 1976). The toxic effects and PLA_2 activity of the β_1 isosubunit could not be tested due its low abundance in the venom, and hence, the small amounts isolated. However, since the β_1 and β_2 subunits of cannitoxin are isomers, and given the high sequence identity between β_1 taipoxin and β_1 cannitoxin, it is unlikely that the latter subunit has any neurotoxic activity. Neither of the β subunits of taipoxin are known to have any toxic activity (Fohlman et al., 1976).

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In conclusion, cannitoxin is the first neurotoxin to be isolated from the venom of the Papuan taipan. Cannitoxin is a presynaptic neurotoxin consisting of 3 different subunits held together by non covalent interactions. The toxic effects of cannitoxin can be neutralised by commercially available TPAV and are dependant on its PLA_2 activity. Cannitoxin is a major neurotoxic component of the venom, thus is likely to be responsible for much of the neurotoxic effects observed in envenomed patients.

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ACKNOWLEDGEMENTS

We would like to thank Dr Simon Harris (Green Chemistry, Department of Chemistry, Monash University, Australia) for performing the mass spectrometry analysis, and Associate Professor Mibel Aguilar (Department of Biochemistry & Molecular Biology, Monash University, Australia) and Dr Evert Karlsson (Sweden) for advice on protein purification.

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REFERENCES

Abe T, Alema S, and Miledi R (1977) Isolation and characterization of presynaptically acting neurotoxins from the venom of Bungarus snakes. *Eur J Biochem* **80**:1-12.

Bell KL, Sutherland SK, and Hodgson WC (1998) Some pharmacological studies of venom from the inland taipan (*Oxyuranus microlepidotus*). *Toxicon* **36**:63-74.

Broad AJ, Sutherland SK, and Coulter AR (1979) The lethality in mice of dangerous Australian and other snake venom. *Toxicon* **17**:661-664.

Chang CC, Lee JD, Eaker D, and Fohlman J (1977) The presynaptic neuromuscular blocking action of taipoxin. A comparison with β-bugarotoxin and crotoxin. *Toxicon* **15**:571-576.

Connolly S, Trevett AJ, Nwokolo NC, Lalloo DG, Naraqi S, Mantle D, Schofield IS, Fawcett PR, Harris JB, and Warrell DA (1995) Neuromuscular effects of Papuan Taipan snake venom. *Ann Neurol* **38**:916-920.

Crachi MT, Hammer LW, and Hodgson WC (1999a) A pharmacological examination of venom from the Papuan taipan (*Oxyuranus scutellatus canni*). *Toxicon* **37**:1721-1734.

Crachi MT, Hammer LW, and Hodgson WC (1999b) The effects of antivenom on the in vitro neurotoxicity of venoms from the taipans *Oxyuranus scutellatus*, *Oxyuranus microlepidotus* and *Oxyuranus scutellatus canni*. *Toxicon* **37**:1771-1778.

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JPET Fast Forward. Published on August 31, 2005 as DOI: 10.1124/jpet.105.093641
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Fohlman J (1979) Comparison of two highly toxic Australian snake venoms: the taipan (*Oxyuranus s. scutellatus*) and the fierce snake (*Parademansia microlepidotus*). *Toxicon* **17**:170-172.

Fohlman J, Eaker D, Dowdall MJ, Lullmann-Rauch R, Sjodin T, and Leander S (1979) Chemical modification of taipoxin and the consequences for phospholipase activity, pathophysiology, and inhibition of high-affinity choline uptake. *Eur J Biochem* **94**:531-540.

Fohlman J, Eaker D, Karlsson E, and Thesleff S (1976) Taipoxin, an extremely potent presynaptic neurotoxin from the venom of the australian snake taipan (*Oxyuranus s. scutellatus*). Isolation, characterization, quaternary structure and pharmacological properties. *Eur J Biochem* **68**:457-469.

Fohlman J, Lind P, and Eaker D (1977) Taipoxin, an extremely potent presynaptic snake venom neurotoxin. Elucidation of the primary structure of the acidic carbohydrate-containing taipoxin-subunit, a prophospholipase homolog. *FEBS Lett* **84**:367-371.

Francis B, Williams ES, Seebart C, and Kaiser, II (1993) Proteins isolated from the venom of the common tiger snake (*Notechis scutatus scutatus*) promote hypotension and hemorrhage. *Toxicon* **31**:447-458.

Harris JB (1991) Phospholipases in snake venoms and their effects on nerve and muscle, in *Snake toxins* (Harvey AL ed) pp 91-129, Pergamon Press, New York.

Harvey AL (1990) Presynaptic effects of toxins. *Int Rev Neurobiol* **32:**201-239.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 20, 2024

JPET Fast Forward. Published on August 31, 2005 as DOI: 10.1124/jpet.105.093641
This article has not been copyedited and formatted. The final version may differ from this version.

Harvey AL, Barfaraz A, Thomson E, Faiz A, Preston S, and Harris JB (1994) Screening of snake venoms for neurotoxic and myotoxic effects using simple in vitro preparations from rodents and chicks. *Toxicon* **32**:257-265.

O'Shea M (1996) *A Guide to the snakes of Papua New Guinea*. Independent Group Pty Ltd, Singapore.

Sutherland S, and Tibballs J (2001) *Australian Animal Toxins, the creature, their toxins and care of the poisoned patient.* Oxford University Press.

Volwerk JJ, Pieterson WA, and de Haas GH (1974) Histidine at the active site of phospholipase A_2. *Biochemistry* **13**:1446-1454.

Wickramaratna JC, Fry BG, Aguilar MI, Kini RM, and Hodgson WC (2003) Isolation and pharmacological characterization of a phospholipase A_2 myotoxin from the venom of the Irian Jayan death adder (*Acanthophis rugosus*). *Br J Pharmacol* **138**:333-342.

Wickramaratna JC, Fry BG, Loiacono RE, Aguilar MI, Alewood PF, and Hodgson WC (2004) Isolation and characterization at cholinergic nicotinic receptors of a neurotoxin from the venom of the *Acanthophis sp*. Seram death adder. *Biochem Pharmacol* **68**:383-394.

Worrell E (1970) *Reptiles of Australia.* Angus and Robertson, Sydney.

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JPET Fast Forward. Published on August 31, 2005 as DOI: 10.1124/jpet.105.093641
This article has not been copyedited and formatted. The final version may differ from this version.

Yang CC (1997) Chemical modification and functional sites of phospholipases A_2, in *Venom Phospholipase A_2 Enzymes: Structure Function and Mechanism.* (Kini RM ed) p 509, Wiley, Chichester.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 20, 2024

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LEGENDS FOR FIGURES

Fig. 1. The effect of taipan antivenom (5 U/ml) after the addition of venom (10 µg/ml) or vehicle at t_{90} (arrow) on (a) indirect twitches and (b) contractile responses of the CBCNM preparation. **P*<0.05, significantly different compared to vehicle control, one-way ANOVA, n=4.

Fig. 2. Size exclusion chromatograph of (a) Papuan taipan venom and (b) cannitoxin run on a Superdex G-75 column, equilibrated with ammonium acetate (0.1 M, pH 5.0) at a flow rate of 0.5 ml/min. RP-HPLC chromatograph of (c) cannitoxin, run on a Jupiter analytical C18 column, equilibrated with solvent A (0.1 % TFA) and the gradient conditions of solvent B (Wickramaratna et al., 2004). Molecular mass indicated above peaks.

Fig. 3. A plot of Log MW Vs Ve/Vo of a series of molecular weight standards, run on a Superdex G-75 column equilibrated with ammonium acetate (0.1M, pH 5.0) at a flow rate of 0.6 ml/min. Vo of the column was 10.15ml. X indicates: cannitoxin.

Fig. 4. The effect of cannitoxin (11-66 nM) alone, cannitoxin in the presence of taipan antivenom, 4-BPB or vehicle (i.e. acetone) on (a) indirect twitches and (b) response to exogenous agonists. **P*<0.05, significantly different compared to cannitoxin 1 in the presence of antivenom, one-way ANOVA. ***P*<0.05, significantly different compared to cannitoxin in the presence of 4-BPB, one-way ANOVA, n=4.

Fig. 5. The effect of α, β, γ subunits (100 nM) and vehicle (i.e. 0.1% BSA) on (a) nerve mediated twitches and (b) response to exogenous agonists of the CBCNM preparation. **P*<0.05, significantly different compared to vehicle, one-way ANOVA, n=3-6.

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TABLES

Table 1
Partial N-terminal sequences and molecular masses of the subunits of cannitoxin and taipoxin

Species	Toxin	Subunit	Molecular Mass (Da)	N-terminal sequence
O. s. scutellatus	Taipoxin	α	13,750[1]	NLLQF GFMIR CANRR SPPVW
O. s. canni	Cannitoxin	α	13,824	NLLQF GYMIR CANGR SRPVW*
O. s. scutellatus	Taipoxin	γ	18,354[1]	SELPQ PSIDF EQFSN MIQCT IPCGS ECIAY
O. s. canni	Cannitoxin	γ	17,762	SEIPQ PSLDF EQFSN MIQCT IPPGE E*C*LAY
O. s. scutellatus	Taipoxin	β_1	13,457[1]	NLVQF GKMIE CAIRN RRPAL DFMNY GCYCG KGGSG TPVDD
O. s. canni	Cannitoxin	β_2	13,242	NLVQF GFMIE CAIRN RQPAL DFMNY GCYCG TVGRG TPVDD
O. s. canni	Cannitoxin	β_1	13,276	NLVQF GKMIE

[1] Fohlman et al., 1976

* Residue predicted on sequence homology
Underlined: Low level sequence (within signal to noise).

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Table 2
PLA_2 activities

Toxin/Subunit	PLA_2 activity (µmol/min/mg)
Taipoxin	0.4 [1]
α-taipoxin	3.8 [1]
β-taipoxin	0 [1]
γ-taipoxin	0.7 [1]
Cannitoxin	90.5 ± 1.1
Cannitoxin + 4-BPB	2.1 ± 0.1
α-cannitoxin	129.7 ± 12.4
β-cannitoxin	4.8 ± 3.8
γ-cannitoxin	23.4 ± 7.6

[1] Fohlman et al., 1979 NB. taipoxin data was obtained using a different assay so caution should be exercised when comparing activity with cannitoxin

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Fig 1

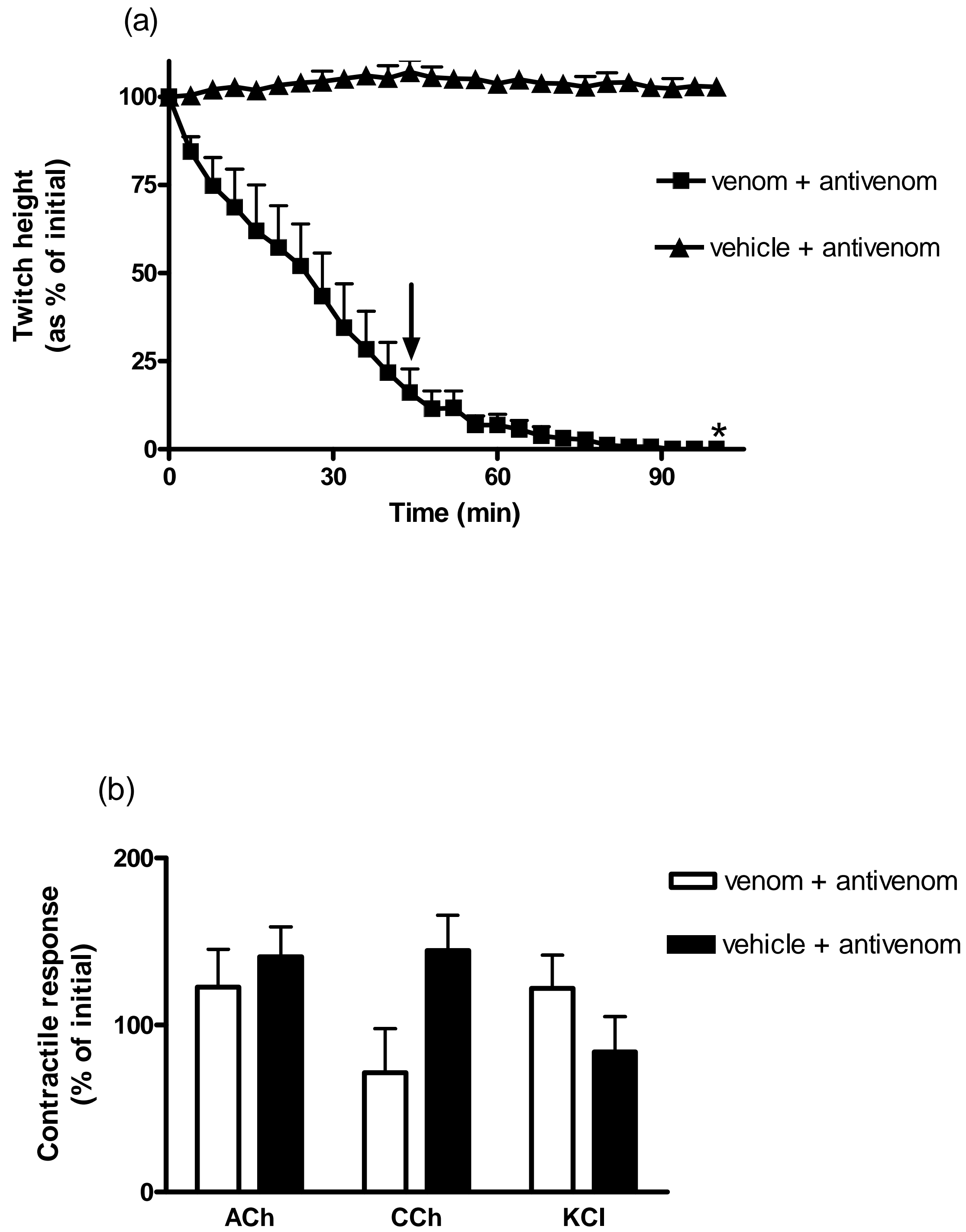

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Fig 2

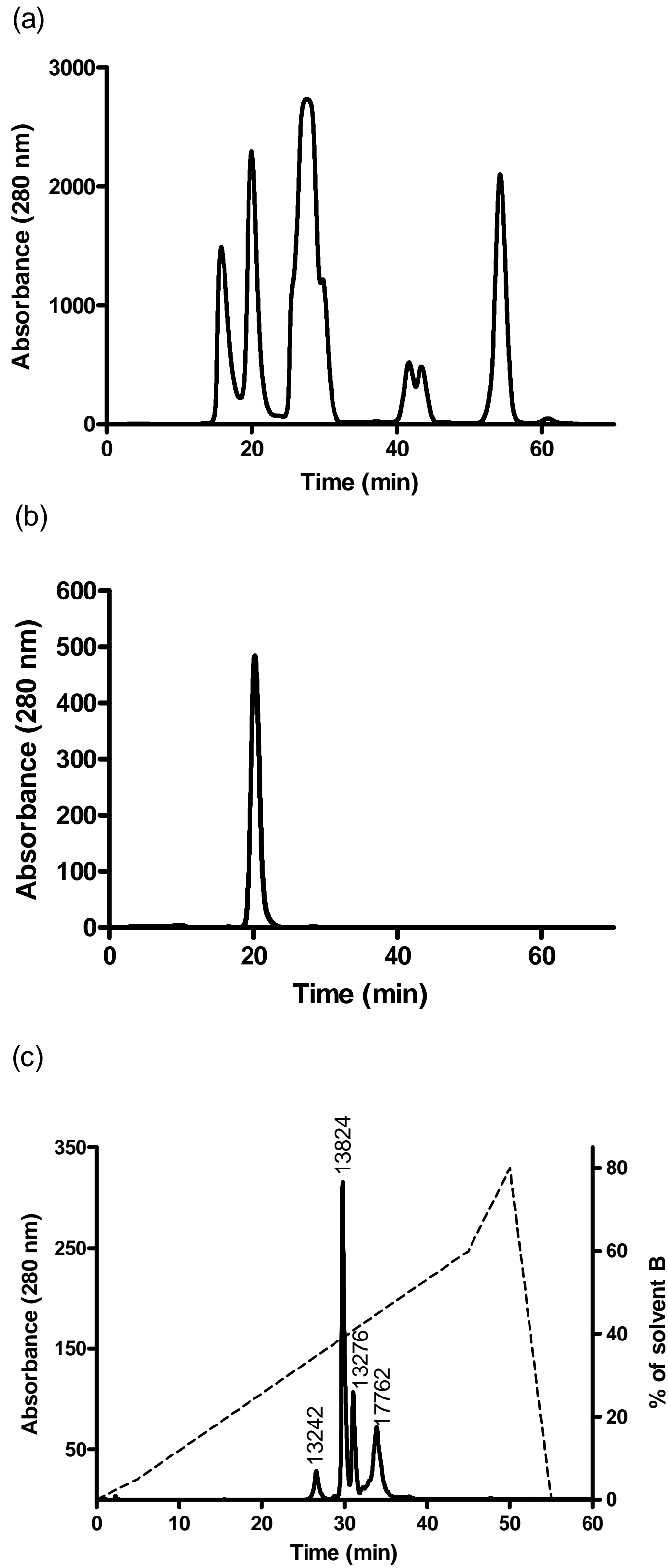

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Fig 3

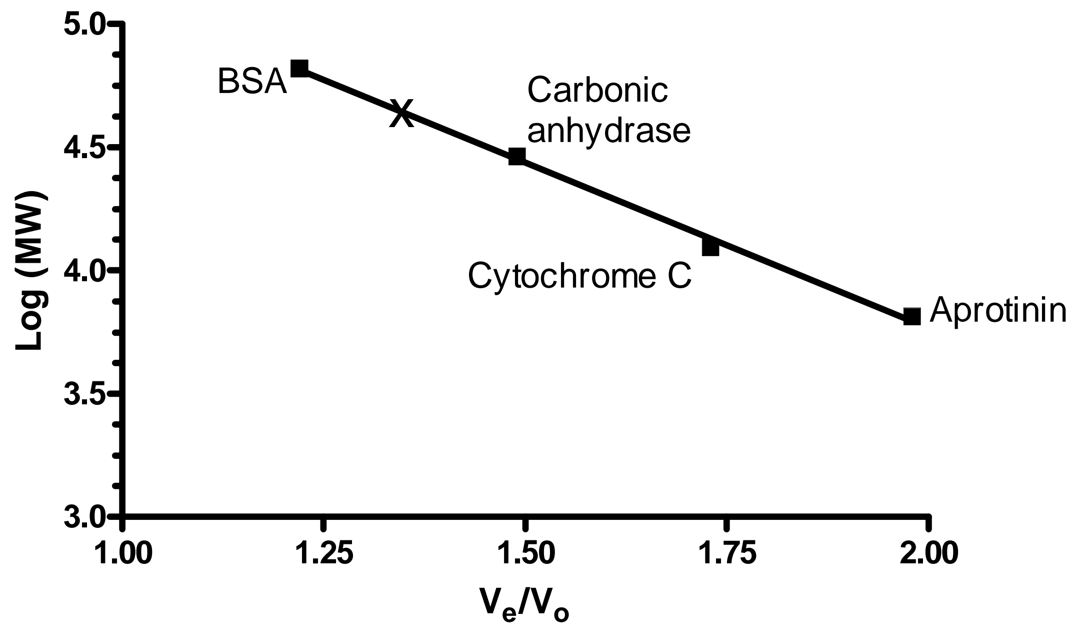

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Fig 4

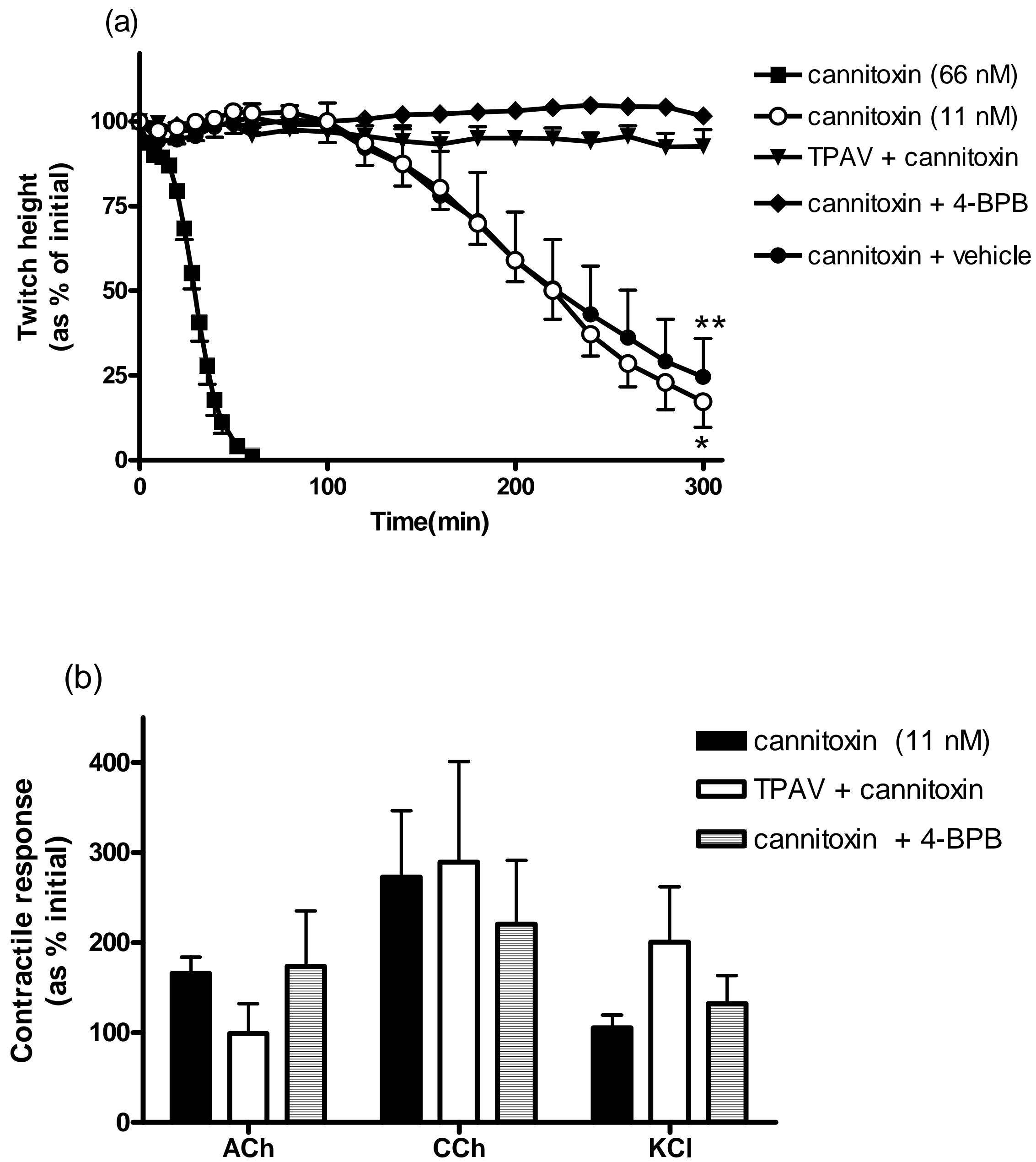

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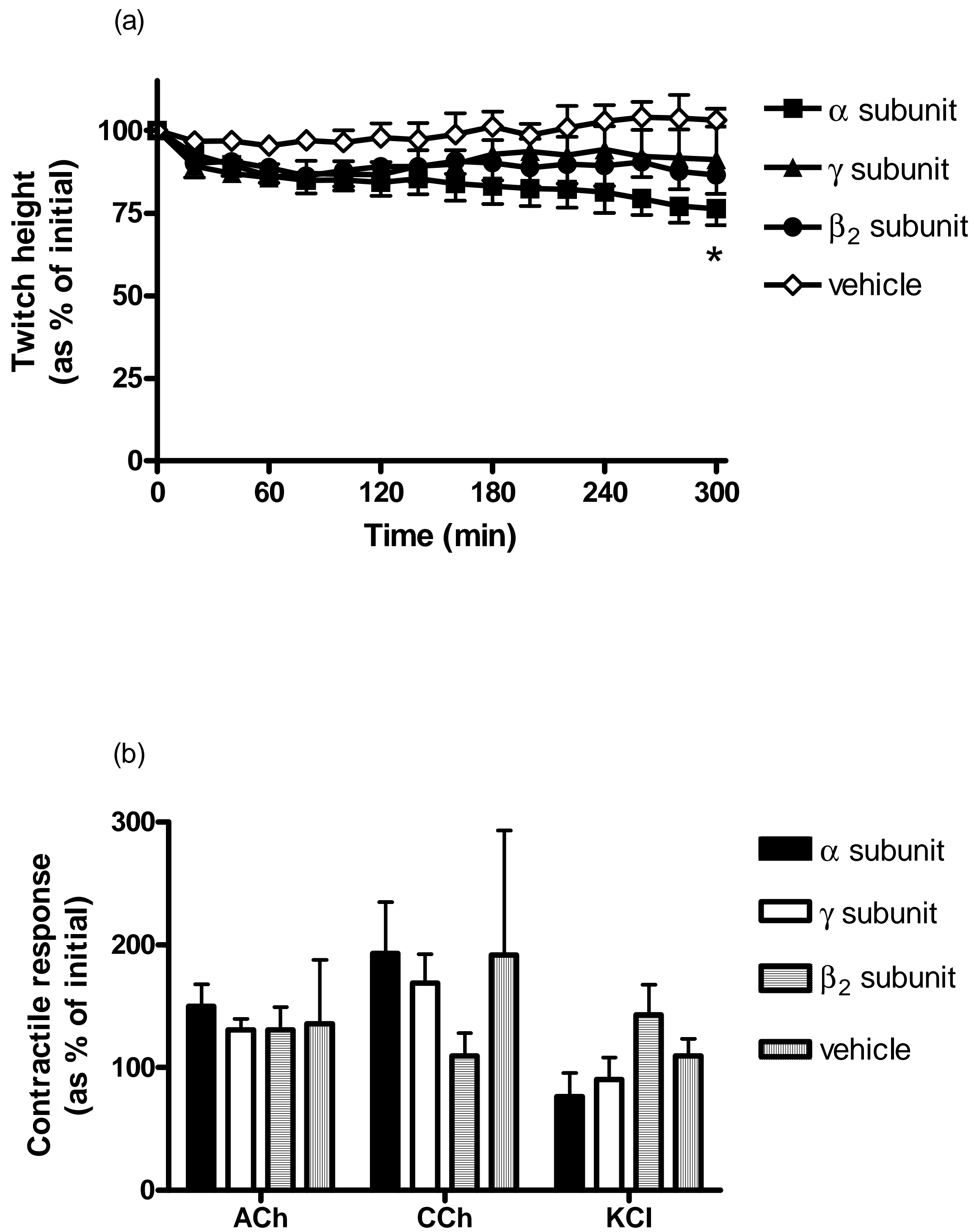
Fig 5



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