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The in vitro neuromuscular activity of Indo-Pacific sea-snake venoms: efficacy of two commercially available antivenoms

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Abstract

We examined the neurotoxicity of the following sea snake venoms: *Enhydrina schistosa* (geographical variants from Weipa and Malaysia), *Lapemis curtus* (Weipa and Malaysia), *Laticauda colubrina*, *Aipysurus laevis*, *Aipysurus fuscus* and *Aipysurus foliosquamatus*. Venom from a terrestrial snake, *Notechis scutatus* (tiger snake), was used as a reference. All venoms (1 and 3 μg/ml) abolished indirect twitches of the chick biventer cervicis muscle and significantly inhibited responses to ACh (1 mM) and CCh (20 μM), but not KCl (40 mM), indicating the presence of post-synaptic toxins. Prior administration (10 min) of CSL sea snake antivenom (1 unit/ml) attenuated the twitch blockade produced by *N. scutatus* venom and all sea snake venoms (1 μg/ml). Prior administration (10 min) of CSL tiger snake antivenom (1 unit/ml) attenuated the twitch blockade of all venoms except those produced by *E. schistosa* (Malaysia and Weipa) and *A. foliosquamatus*. Administration of CSL sea snake antivenom (1 unit/ml) at t_{90} (i.e. time at which 90% inhibition of initial twitch height occurred) reversed the inhibition of twitches (20–50%) produced by the sea snake venoms (1 μg/ml) but not by *N. scutatus* venom (1 μg/ml). CSL tiger snake antivenom (1 unit/ml) administered at t_{90} produced only minor reversal (i.e. 15–25%) of the twitch blockade caused by *L. curtus* (Weipa), *A. foliosquamatus*, *L. colubrina* and *A. laevis* venoms (1 μg/ml). Differences in the rate of reversal of the neurotoxicity produced by the two geographical variants of *E. schistosa* venom, after addition of CSL sea snake antivenom, indicate possible differences in venom components. This study shows that sea snake venoms contain potent post-synaptic activity that, despite the significant genetic distances between the lineages, can be neutralised with CSL sea snake antivenom. However, the effects of CSL tiger snake antivenom are more variable.

Keywords: Sea snake; Tiger snake; Venom; Antivenom; Neurotoxicity

1. Introduction

The advanced snakes (superfamily Colubroidea) make up over 80% of the approximately 2900 species of snake currently described, and contain all the known venomous forms (Greene, 1997; Vidal, 2002; Vidal and Hedges, 2002). Only about 20% of the advanced snakes (Atractaspididae, Elapidae and Viperidae) have front fanged delivery systems, and are typically regarded as of major medical interest. Within the Elapidae family, two independent colonisations of the oceanic niche have occurred, the sea kraits (*Laticauda* genus) and the diverse true sea snakes comprising a large number of genera (e.g. *Enhydris*, *Lapemis, Hydrophis* and *Pelamis*) (Fig. 1). Previous work by us demonstrated a remarkable streamlining of sea snake venoms (Fry et al., 2003a). The streamlined pattern of sea snake venoms is all the more striking due to its parallel occurrence in the two independent marine radiations; sea kraits and the true sea snakes evolved their marine, ichthyophagous habits, and their simple venom structures, independently from

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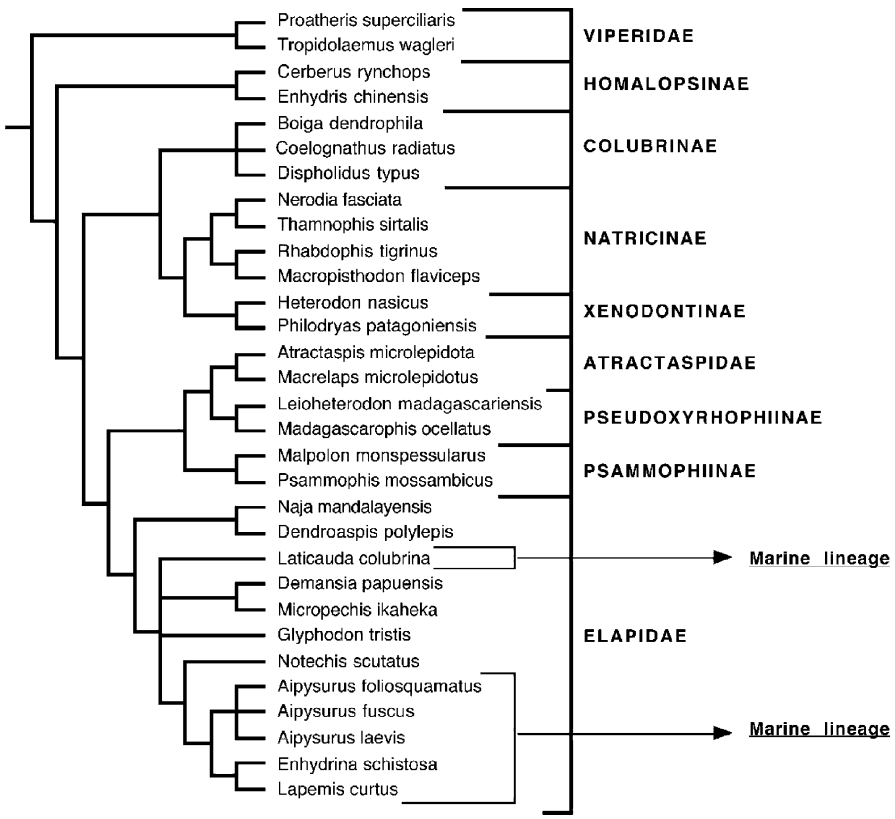

Fig. 1. Colubroidea snake taxonomy and the relative taxonomical positions of the marine lineages examined in this study (Slowinski and Lawson, 2002; Vidal and Hedges, 2002; Scanlon and Lee, 2003).

within the Australasian elapid radiation (Fig. 1). Low diversity within toxin classes is also the case in terms of gene phylogeny. Extensive previous sequencing of sea snake three-finger toxins (3FTx) did not reveal any proteins that were not phylogenetically aligned with the Type I or Type II α-neurotoxins, even from the rigorously studied *Laticauda* venoms (Fry et al., 2003b).

Despite the diverse range of sea snakes that inhabit the Indo-Pacific region, there is only one antivenom available for the treatment of systemic envenomation, which manifests as neurotoxicity and/or myotoxicity (Reid, 1975). Sea snake antivenom, made by CSL Limited, Parkville Australia, is a bivalent antivenom raised against the venom of *Enhydrina schistosa* and *Notechis scutatus* (CSL Antivenom Handbook, White, 2001) *E. schistosa* is responsible for most clinically significant evenomations as it is an estuarine species that is the most commonly encountered true sea snake (Sutherland and Tibballs, 2001). In the absence of this antivenom it has been suggested that CSL tiger snake antivenom be used as a substitute, although at a higher starting dosage (four ampoules) than that recommended for sea snake antivenom itself (Sutherland and Tibballs, 2001). This recommendation appears to have been based largely on experiments where antivenom was administered to mice after venom injection or experiments where venom and antivenom were mixed prior to injection (Baxter and Gallichio, 1974, 1976). These studies indicated that tiger snake antivenom was equivalent, or in some cases more effective, in neutralising sea snake venom, than sea snake antivenom.

In the present study, venoms from the following were examined: the true sea snakes *Aipysurus foliosquamatus* (Leaf-scaled sea snake), *Aipysurus laevis* (Olive sea snake), *Aipysurus fuscus* (Dusky sea snake), *E. schistosa* (Beaked sea snake) and *Lapemis curtus* (Spine-bellied sea snake), and the sea kraits *Laticauda colubrina* (Yellow-lipped sea krait). As previous work has documented regional variation in the venoms of the true sea snakes *E. schistosa* and *L. curtus* (Fry et al., 2003a), the same geographical variants were included to allow for a preliminary investigation into the regional relative neutralisation by antivenom. Tiger snake (*Notechis scutatus*) venom was used as a reference in order to compare the in vitro neuromuscular activity of the sea snake venoms to this extensively studied terrestrial snake venom and to allow for a comparison of the efficacy of sea snake and tiger snake antivenoms.

2. Materials and methods

2.1. Venom collection

Specimens were collected and milked from the following localities: *A. foliosquamatus* (Ashmore Reef, Australia); *A. fuscus* (Ashmore Reef, Australia); *A. laevis* (Ashmore Reef, Australia), *E. schistosa* (Peninsular Malaysia and Albatross Bay, Queensland, Australia), *L. curtus* (Peninsular Malaysia and Albatross Bay, Queensland, Australia) and *Laticauda colubrina* (Bali, Indonesia). Pooled venom from at least six unrelated adult specimens was used to minimise the effect of individual variation (Chippaux et al., 1991).

2.2. Venom storage

Freeze dried venoms were stored at −20 °C. Stock solutions of venoms were prepared in 0.1% bovine serum albumin in 0.9% saline, aliquoted into Eppendorf tubes and kept frozen until required.

2.3. Chick isolated biventer cervicis nerve–muscle preparation

Chicks (4–10 days old) were killed by CO_2 and exsanguination, the biventer cervicis muscles removed and mounted in tissue baths under a resting tension of 1 g. Tissues were bathed in physiological salt solution (34 °C), bubbled with 95% O_2 and 5% CO_2, of the following composition (mM): NaCl, 118.4; $NaHCO_3$, 25; glucose, 11; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2 and $CaCl_2$, 2.5. Twitches were evoked by stimulating the motor nerve at a voltage greater than that required to evoke a maximal twitch (0.1 Hz, 0.2 ms). Tubocurarine (10 μM) was added to the bath and complete abolition of twitches confirmed indirect stimulation. In the absence of nerve stimulation, submaximal responses to ACh (1 mM, 30 s), CCh (20 μM, 60 s) and KCl (40 mM, 30 s) were obtained before and after the addition of venom (Harvey et al., 1994).

2.4. Antivenom studies

Sea snake (1 unit/ml) or tiger snake (1 unit/ml) antivenom was added to the organ bath 10 min prior to the addition of venoms (1 μg/ml) (Wickramaratna and Hodgson, 2001; Hodgson et al., 2003)). These experiments were undertaken to see if the antivenoms possessed any neutralisation capabilities (i.e. if they were unable to prevent the neurotoxic effects of the venoms they were unlikely to be able to reverse the neurotoxic effects). Additional experiments were undertaken to examine the reversibility of the inhibitory effects of the venoms. In these experiments sea snake (1 unit/ml) or tiger snake (1 unit/ml) antivenom was added after the venoms had produced a 90% inhibition of twitches (i.e. at t_{90}) (Wickramaratna and Hodgson, 2001; Hodgson et al., 2003).

2.5. Drugs

The following were used: acetylcholine chloride (Sigma), carbamylcholine chloride (carbachol; Sigma) and *d*-tubocurarine chloride (Sigma). Stock solutions of the drugs were made in distilled water. Sea snake antivenom (batch 0549-06501), obtained from CSL Ltd (Parkville, Australia), was raised in horses hyperimmunised with the venom of *E. schistosa* and *N. scutatus* (Sutherland and Tibballs, 2001). Tiger snake antivenom (batch 0550-09101), obtained from CSL Ltd (Australia), was raised in horses hyperimmunised with the venom of *N. scutatus*.

2.6. Statistical analysis

Responses were measured by a Grass force displacement transducer (FT03) and recorded on a Grass Polygraph (Model 79D) or MacLab System. Statistical analysis of results was performed using SIGMASTAT. Two way ANOVA's were performed for a comparison of t_{90} values between venoms and doses, and for a comparison of contractile responses to ACh, CCh and KCl. All ANOVA's were followed by a Bonferroni post hoc test. Statistical significance was indicated when $P < 0.05$.

3. Results

3.1. Neurotoxicity studies

Both sea krait and true sea snake venoms produced inhibition of indirect twitches in the chick biventer cervicis nerve–muscle preparation at both 1 (data not shown) and 3 μg/ml (Fig. 2a). In addition, the time taken to produce 90% inhibition of twitch height (i.e. t_{90}) was determined (Table 1). *N. scutatus* (tiger snake) venom also abolished twitches at both 1 and 3 μg/ml (see Table 1 for t_{90} values). All marine venoms were significantly more potent than *N. scutatus* venom at 1 μg/ml. However, the venoms of two geographical variants of *L. curtus* were not significantly different from *N. scutatus* venom at 3 μg/ml (Table 1). Contractile responses to exogenous ACh and CCh, but not KCl, were significantly inhibited by all sea snake venoms and *N. scutatus* at both concentrations (i.e. 1 μg/ml, data not shown, and 3 μg/ml, Fig. 2b).

3.2. Antivenom: prior addition

Addition of sea snake antivenom (1 unit/ml), 10 min prior to the addition of venoms, markedly attenuated the blockade of the indirect twitches of the chick biventer cervicis nerve–muscle preparation, caused by both sea krait and true sea snake venoms (Figs. 3–6). In addition, the twitch blockade caused by the venom from *N. scutatus* was also attenuated by sea snake antivenom (Fig. 7).

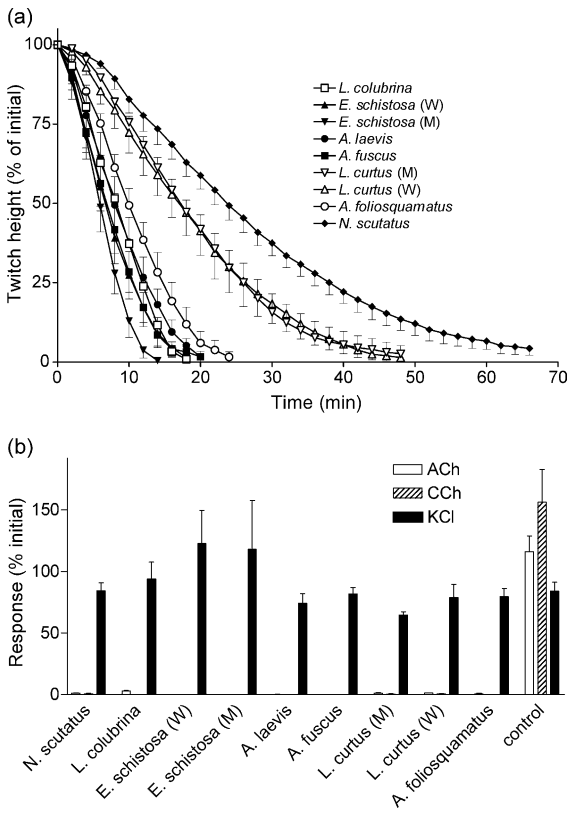

Fig. 2. The effect of venoms (3 μg/ml, $n = 5–6$) on: (a) indirectly mediated twitches in the chick biventer cervicis nerve–muscle preparation and (b) responses to acetylcholine (ACh), carbachol (CCh) and potassium chloride (KCl). All venoms abolished indirect twitches and responses to nicotinic receptor agonists indicating post-synaptic activity.

Addition of tiger snake antivenom (1 unit/ml), 10 min prior to the addition of venoms, markedly attenuated the blockade of indirect twitches produced by *N. scutatus* venom, sea krait (*L. colubrina*) venom as well as the venoms

Table 1
T_{90} values (min) of sea snake venoms

Snake venom	3 μg/ml	1 μg/ml
E. schistosa (Malaysia)	10.5 ± 0.7*	31.2 ± 4.0*
A. fuscus	13.0 ± 1.4*	33.1 ± 6.9*
E. schistosa (Weipa)	13.1 ± 1.6*	39.9 ± 8.0*
L. colubrina	14.6 ± 0.5*	44.5 ± 5.9*
A. laevis	15.5 ± 1.6*	44.0 ± 6.9*
A. foliosquamatus	18.0 ± 1.4*	27.5 ± 3.1*
L. curtus (Weipa)	33.5 ± 3.6	64.9 ± 10.9*
L. curtus (Malaysia)	36.5 ± 4.7	45.4 ± 3.4*
N. scutatus	53.3 ± 4.7	144.3 ± 13.6

N. scutatus (tiger snake) venom was used as a terrestrial snake comparison. Data shown are mean ± SEM. $*P < 0.05$, significantly different from *N. scutatus* at same concentration.

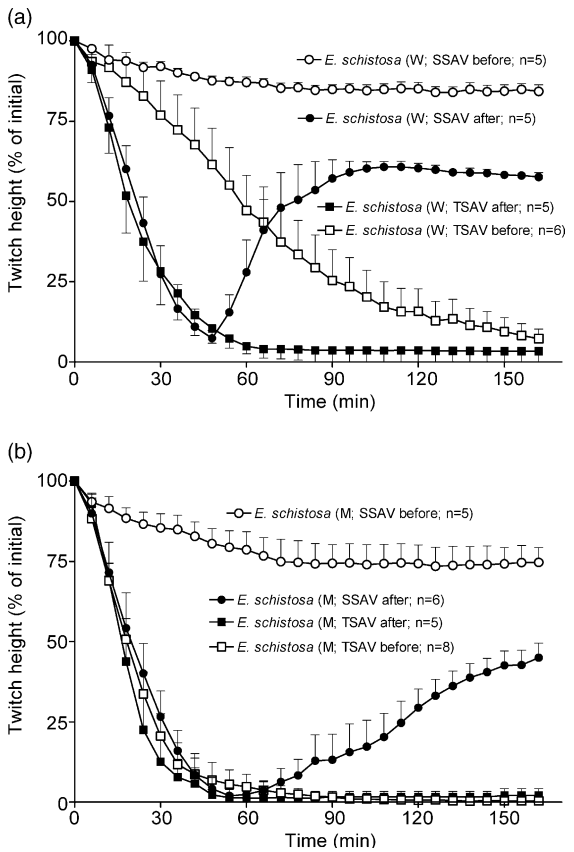

Fig. 3. The effect of prior administration (10 min) of sea snake (SSAV) or tiger snake antivenom (TSAV) (1 unit/ml), addition of sea snake or tiger snake antivenom (1 unit/ml) at t_{90} or venom alone on indirect twitches in the chick biventer cervicis nerve–muscle preparation: (a) *E. schistosa* (Weipa) (1 μg/ml, $n = 5–6$); (b) *E. schistosa* (Malaysia) (1 μg/ml, $n = 5–8$).

of the true sea snakes *A. laevis* and *L. curtus* (both populations) (Figs. 7, 5b, 6b, and 4, respectively). However, only partial attenuation was observed for the venoms of the true sea snakes *E. schistosa* (Weipa) and *A. foliosquamatus* (Figs. 3a and 5a, respectively). Prior administration of tiger snake antivenom had no significant effect on *E. schistosa* (Malaysia) venom (Fig. 3b).

3.3. Antivenom: reversal studies

Sea snake antivenom (1 unit/ml), added at t_{90}, produced reversal of the twitch blockade by venoms in the following rank order by the amounts indicated: *E. schistosa* (Weipa) (~61%), *A. foliosquamatus* (~57%), *A. laevis* (~51%), *L. colubrina* (~47%), *E. schistosa* (Malaysia) (~46%), *L. curtus* (Malaysia) (~40%), *A. fuscus* (~26%) and *L. curtus* (Weipa) (~21%) (Figs. 3–6). However, it had no significant effect on *N. scutatus* venom as complete twitch blockade was still evident (Fig. 7).

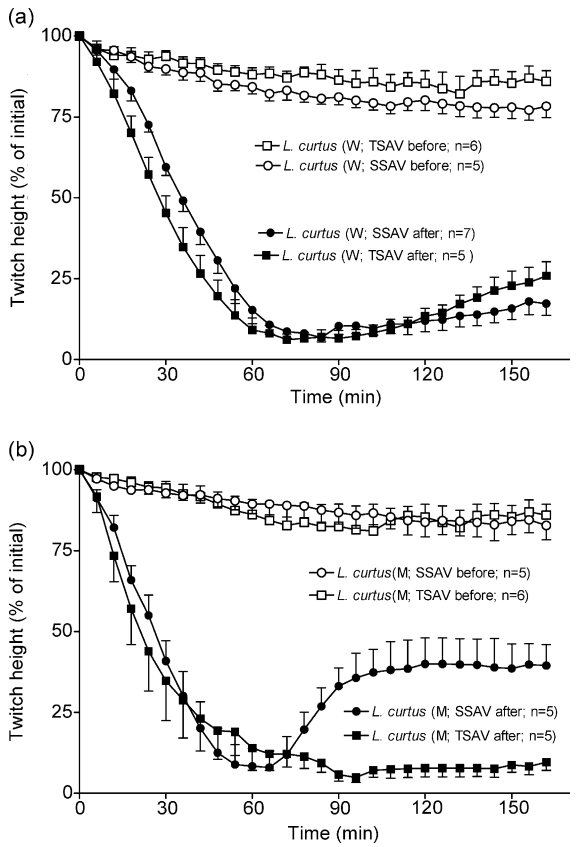

Fig. 4. The effect of prior administration (10 min) of sea snake (SSAV) or tiger snake antivenom (TSAV) (1 unit/ml), addition of sea snake or tiger snake antivenom (1 unit/ml) at t_{90} or venom alone on indirect twitches in the chick biventer cervicis nerve–muscle preparation: (a) *L. curtus* (Weipa) (1 μg/ml, $n = 5–7$); (b) *L. curtus* (Malaysia) (1 μg/ml, $n = 5–6$).

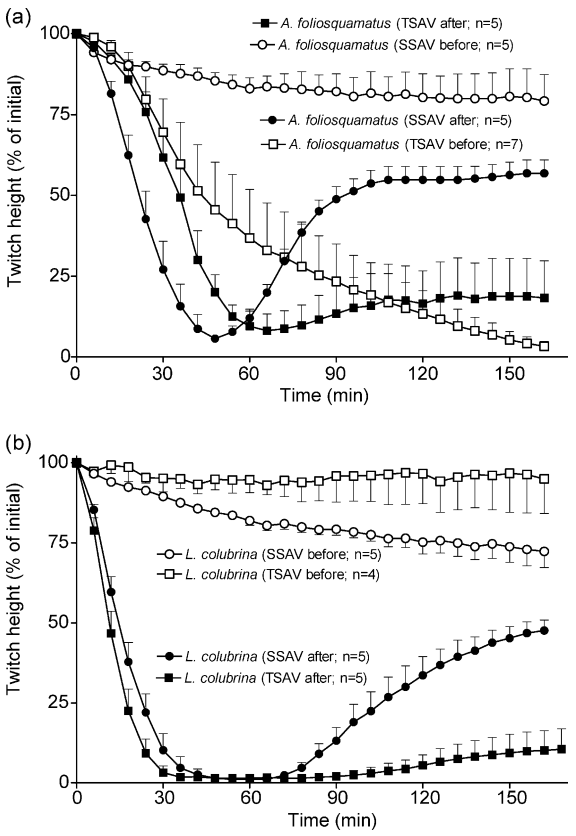

Fig. 5. The effect of prior administration (10 min) of sea snake (SSAV) or tiger snake antivenom (TSAV) (1 unit/ml), addition of sea snake or tiger snake antivenom (1 unit/ml) at t_{90} or venom alone on indirect twitches in the chick biventer cervicis nerve–muscle preparation: (a) *A. foliosquamatus* (1 μg/ml, $n = 5–7$); (b) *L. colubrina* (1 μg/ml, $n = 4–5$).

Tiger snake antivenom (1 unit/ml), added at t_{90}, was generally less effective than sea snake antivenom but produced reversal of the twitch blockade by venoms in the following rank order by the amounts indicated: *L. curtus* (Weipa) (~26%), *A. foliosquamatus* (~19%), *A. laevis* (~16%) and *L. colubrina* (~11%). However, tiger snake antivenom (1 unit/ml), added at t_{90}, had no significant effect on *E. schistosa* (Weipa), *E. schistosa* (Malaysia), *L. curtus* (Malaysia), *A. fuscus* or *N. scutatus* venoms (Figs. 3–7).

4. Discussion

The lethality of some sea snake venoms has previously been determined using the LD_{50} method (e.g. Tu and Ganthavorn, 1969; Broad et al., 1979). This has allowed a limited comparison of sea snake venom LD_{50} values with those of terrestrial species. Our laboratory has utilised the t_{90} method to compare the neurotoxicity in vitro neurotoxicity of terrestrial snake venoms. Based on t_{90} values, *Acanthophis* (Death adder) venoms were the most potent of the venoms examined (Wickramaratna and Hodgson, 2001). All the sea krait and true sea snake venoms examined in the present study, with the exception of both geographical variants of *L. curtus*, have lower t_{90} values than these terrestrial snakes. Contractile responses of the chick biventer cervicis nerve muscle preparation to the exogenous nicotinic agonists were abolished or markedly diminished following the addition of all venoms indicating an inhibitory effect at post-synaptic nicotinic receptors. In a previous study, isolated toxins from *E. schistosa* venom also showed similar neuromuscular blocking effects and abolition of responses to ACh in the chick biventer cervicis muscle (Gawade and Gaitonde, 1982). The results of the present study also confirm earlier reports that the venom of both lineages display highly potent post-synaptic neurotoxic activity (Geh and Toh, 1978; Endo and Tamiya, 1991; Ryan and Yong, 1997; Fry et al., 2003a,b).

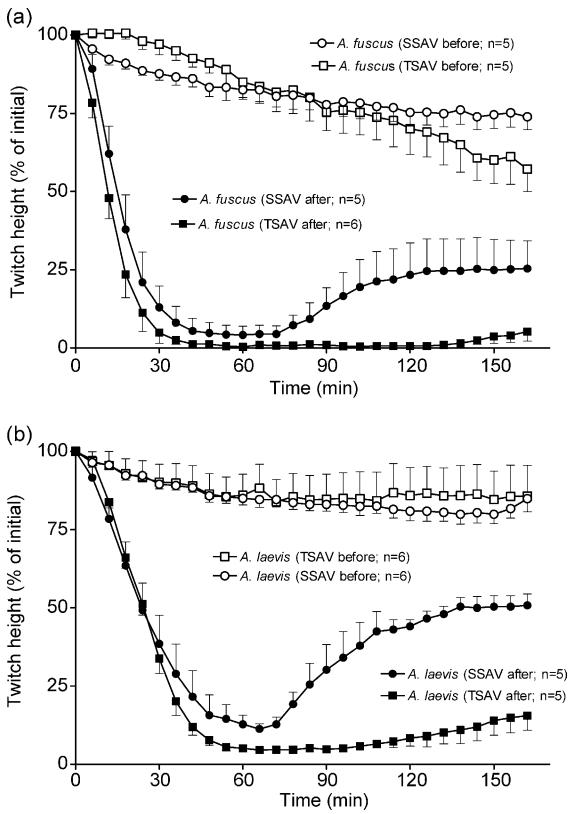

Fig. 6. The effect of prior administration (10 min) of sea snake (SSAV) or tiger snake antivenom (TSAV) (1 unit/ml), addition of sea snake or tiger snake antivenom (1 unit/ml) at t_{90} or venom alone on indirect twitches in the chick biventer cervicis nerve–muscle preparation: (a) *A. fuscus* (1 μg/ml, $n = 5–6$); (b) *A. laevis* (1 μg/ml, $n = 5–6$).

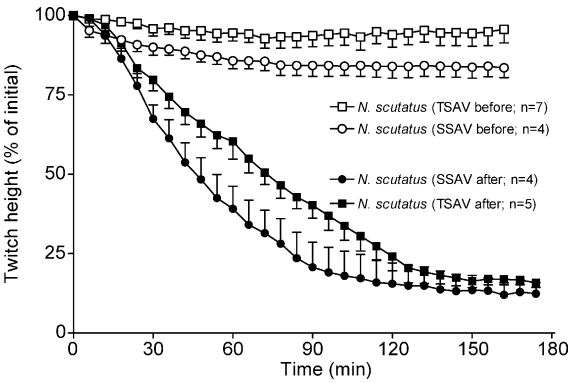

Fig. 7. The effect of prior administration (10 min) of sea snake (SSAV) or tiger snake antivenom (TSAV) (1 unit/ml), addition of sea snake or tiger snake antivenom (1 unit/ml) at t_{90} or venom alone on indirect twitches in the chick biventer cervicis nerve–muscle preparation. *N. scutatus* venom (1 μg/ml, $n = 4–7$).

Since the first protype sea snake antivenom was described in 1960 (Carey and Wright, 1960), a variety of institutions, using a variety of immunising venoms, have developed additional sea snake antivenoms (Barme et al., 1962; Gawade and Gaitonde, 1980). However, whilst products from Vietnam, Japan and India were briefly available in the past, the only remaining commercially available sea snake antivenom is that made since the early 1960s by CSL in Melbourne, Australia using *E. schistosa* venom (Tu and Ganthavorn, 1969; Reid, 1975). Early studies by Baxter and Gallichio (1974, 1976) indicated that tiger snake antivenom was effective in neutralising the toxicity of the venoms of true sea snake as well as of sea kraits. Since these studies were conducted, tiger snake antivenom has been recommended for sea snake envenomation if sea snake antivenom is unavailable (Sutherland and Tibballs, 2001).

To examine the effectiveness of the two antivenoms used clinically in the treatment of sea snake envenomation, pre-incubation studies were conducted. Prior addition (10 min) of sea snake antivenom markedly attenuated twitch inhibition caused by all the marine venoms examined. This was particularly notable in regards to the venom of the taxonomically divergent *Laticauda*—as these snakes represent an independent marine lineage (Fig. 1). These data confirm earlier work using *Laticauda semifaciata* venom (Baxter and Gallichio, 1974, 1976). This cross-reactivity may be due to the remarkable streamlining of the sea snake venoms that occurred independently within these two lineages (Fry et al., 2003a) and the low level of phylogenetic variation of the marine 3FTx (Fry et al., 2003b). A similar neutralisation result was noted for tiger snake venom suggesting that sea-snake antivenom has the ability to neutralise the neurotoxins in tiger snake venom. However, the immunisation protocol for CSL sea snake antivenom includes exposure to tiger snake (*N. Scutatus*) as well as *E. schistosa* venom (White, 2001). This procedure was developed out of Baxter and Gallichio's findings and the relative scarcity of *E. schistoa* venom (Sutherland and Tibballs, 2001). Therefore, the sea snake antivenom contains anti-tiger snake venom antibodies.

Pre-incubation with tiger snake antivenom attenuated twitch blockade caused by *N. scutatus* venom. Twitch blockade caused by the venoms of the true sea snake venoms *A. laevis*, *A. fuscus*, and the two geographical variants of *L. curtus* were also attenuated by the tiger snake antivenom. Only partial attenuation was seen in the twitch blockade caused by venoms of *A. foliosquamatus* and *E. schistosa* (Weipa) and tiger snake antivenom was ineffective against *E. schistosa* (Malaysia). These results conflict with past research that suggested that tiger snake antivenom could be used to neutralise all true sea snake venoms (Minton, 1967; Baxter and Gallichio, 1974, 1976).

To further investigate the efficacy of the antivenoms, in a more clinically relevant situation, reversal studies were conducted. Whilst the twitch inhibition caused by all

venoms was partially reversed when sea snake antivenom was added at the t_{90}, there was a large variation in this effect. In addition, the rate at which the reversal of twitch blockade produced by the two geographical variants of *E. schistosa* venom occurred was markedly different, although both eventually recovered to approximately 50% of the initial twitch height. The difference in the rate of recovery of twitch inhibition may be due to differences in venom composition as previously documented (Fry et al., 2003a), although it is inconsistent with the use of the Malaysian variant of *E. schistosa* as the immunising venom for sea snake antivenom production (Mr Mick Kornitschuk, CSL Limited, personal comm.). It may therefore reflect regional variations in *E. schistosa* venom composition even within the Malaysian peninsula.

Sea snake antivenom did not exhibit any neutralising effects against the twitch inhibition caused by *N. scutatus*, when added at the t_{90}. This is in contrast to the results in the pre-incubation study which indicated some neutralisation of *N. scutatus* venom by the sea snake antivenom. As previously indicated, sea snake antivenom is raised in horses treated with both *E. schistosa* and *N. scutatus* venoms. However, while being effective in pre-incubation experiments, once inhibition of twitch blockade occurs, perhaps there are insufficient *N. scutatus* venom antibodies present to reverse the neurotoxic effects. In addition, the myotoxic effects of *N. scutatus* venom, most likely contribute to the inhibition of twitches, are unlikely to be reversed. Twitch blockade caused by *N. scutatus* venom was not reversed by the addition of tiger snake antivenom at t_{90}. This may be due to the presence of pre-synaptic neurotoxins (eg Notexin) in the venom and the myotoxic effects of the whole venom. Neither of these effects are likely to be effectively reversed by antivenom administration. In unpublished preliminary experiments, examining direct twitches of the chick biventer cervicis muscle, we have shown that the sea snake venoms at 10 μg/ml (i.e. compared to 3 μg/ml for used these neurotoxicity studies) display only minor myotoxic effects within the time frame of the complete abolition of indirect twitches observed in the current study suggesting that myotoxins are unlikely to play a role in this effect.

In vitro and in vivo neutralisation and immunodiffusion studies performed by Baxter and Gallichio (1974, 1976) found that tiger snake antivenom was more effective than sea snake antivenom in neutralising sea snake venom. In the present study, reversal of twitch inhibition caused by *E. schistosa* (Malaysia) was not observed when tiger snake antivenom was added at the t_{90}. However, the immunisation protocol for the batch of antivenom used in the study by Baxter and Gallichio (1974, 1976) has been somewhat controversial (Sutherland and Tibballs, 2001). Baxter and Gallichio (1974, 1976) used tiger snake antivenom that was stated to be derived from a pool of plasma obtained from horses that may have been hyperimmunized against other snake venoms. They did not state which other venoms may have been used for the hyperimmunization. However, if the horses had been previously hyperimmunized with sea snake venoms, it is possible that low levels of sea snake antibodies would still be present. In this case, the neutralisation they observed after increasing the dose of tiger snake antivenom may have actually been due to the sea snake antibodies that were now at an adequate level for neutralisation of sea snake venom.

In the present study, we have confirmed past research by finding that sea snake venoms possess potent post-synaptic neurotoxic activity. Indeed, these venoms appear much more potent, at least in a chick skeletal neuromuscular preparation, than Australian terrestrial snake venoms. The ability of sea snake antivenom, raised against a true sea snake venom, being effective in neutralising the taxonomically divergent sea krait venom is consistent with the remarkable parallel but independent streamlining of both marine lineages. In addition, tiger snake antivenom may not be as effective as previously thought in neutralising all sea snake venoms.

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