Journal of Venom Research ISSN: 2044-0324 J Venom Res, 2011, Vol 2, 1-5

RESEARCH REPORT

TTX, cations and spider venom modify avian muscle tone *in vitro*

Volker Herzig[α,]*, Wayne C Hodgson[β] and Edward G Rowan[γ]

[α]Division of Chemical and Structural Biology, Institute for Molecular Bioscience, The University of Queensland, Building 80, Service Road, St Lucia, QLD 4072, Australia, [β]Monash Venom Group, Department of Pharmacology, Monash University, Clayton, Victoria 3800, Australia, [γ]Strathclyde Institute of Pharmacy and Biomedical Sciences, University of Strathclyde, Glasgow, G4 0NR, United Kingdom

*Correspondence to: Volker Herzig, Email: schnakenstich@yahoo.com, Tel: +61 7 3346 2014, Fax: +61 7 3346 2101

Received 21 December 2010; Accepted 02 January 2011; Published online 02 January 2011

ABSTRACT

Agents that reduce skeletal muscle tone may have a number of useful clinical applications, *e.g.*, for muscle spasticity and other muscle disorders. Recently, we reported that the venoms of two species of Australian theraphosid (Araneae, Theraphosidae) spiders (*Coremiocnemis tropix* and *Selenotholus foelschei*) reduced the baseline tension of chick *biventer cervicis* nerve-muscle preparation. The purpose of this study was to determine the underlying physiology mediating the change in muscle tension, which was addressed by conducting isometric tension experiments. We found that $MgCl_2$ (20mM), $CaCl_2$ (20mM), tetrodotoxin (1μM) or *C. tropix* venom (2μl/ml) produced a similar decrease in baseline tension, whereas d-tubocurarine (100μM), gadolinium (1mM), verapamil (10mM), an increase in osmotic pressure by the addition of glucose (40mM), or the presence/absence of electrical stimulation did not produce a significant change in baseline tension. We suggest that mechanosensitive or muscle TTX-sensitive sodium channels are activated during muscle stretch. This may have implications for the treatment of stretch induced muscle damage.

KEYWORDS: Theraphosidae, *Coremiocnemis tropix*, venom, baseline muscle tension, chick *biventer cervicis* nerve-muscle preparation

INTRODUCTION

Animal venoms contain numerous pharmacologically active peptides and enzymes that can disrupt the normal physiology of cells. Snake venoms can affect the clotting cascade, bind to receptors at the neuromuscular junction and have the ability to target nerve terminals, alter the propagation of the nerve terminal action potential and disrupt neurotransmitter transmitter release (Kini, 2005; Hodgson et al, 2007; Servent and Fruchart-Gaillard, 2009). Scorpion venoms contain numerous peptides that interact with a large range of ion channels and receptors (Billen et al, 2008; Prestipino et al, 2009). A number of these peptides have been identified to selectively block insect ion channels (de Lima et al, 2007), thus having the potential to be developed as novel insecticides. Additionally scorpion venoms have been used therapeutically to treat, and when conjugated to an appropriate ligand, to visualise tumors of the CNS, *i.e.*, chlorotoxin isolated from the venom of *Leiurus quinquestriatus* (Sontheimer, 2008; Orndorff and Rosenthal, 2009). In common with scorpion venoms spider venoms contain a myriad of peptide and non-peptide ion channel toxins that can block or activate voltage dependent ion channels (Estrada et al, 2007) and are recognised as a source of bioactive molecules that can be used to identify therapeutic targets, elucidate mechanisms of action and design novel pharmaceutical drugs. In a recent study, we reported that bath application of venoms from the Australian theraphosid (Araneae, Theraphosidae) spiders *Coremiocnemis tropix* (Herzig and Hodgson, 2009) and *Selenotholus foelschei* (Herzig and Hodgson, 2008) on the isolated chick *biventer cervicis* nerve-muscle preparation cause a significant reduction in the resting (10mN) baseline muscle tension. This effect has not been reported before, and agents that relax skeletal muscle have the potential to be of medical value in the treatment of muscle spasticity and other muscle disorders. The present study therefore aims to elucidate the

mechanisms underlying the observed reduction in muscle tone induced by *C. tropix* venom by mimicking the venom-induced relaxation using other pharmacological or physiological agents. Besides *C. tropix* venom, we have tested gadolinium (antagonist of mechanosensitive channels), verapamil (antagonist of voltage-gated calcium channels), d-tubocurarine (d-TC, antagonist of nicotinic acetylcholine receptors), tetrodotoxin (TTX, antagonist of voltage-gated sodium channels), $MgCl_2$ and $CaCl_2$ (both to increase the extracellular ion concentrations), and glucose (to increase osmotic pressure). A separate control experiment was used to examine whether the presence/absence of electrical stimulation had any effect on the baseline muscle tension.

MATERIALS AND METHODS

Venom collection

The venom from female *C. tropix* spiders was collected by applying electrostimulation to the venom apparatus using a recently described method (Herzig and Hodgson, 2009).

Isolated chick biventer cervicis nerve-muscle preparation

We used the chick *biventer cervicis* nerve-muscle preparation according to our previous method (Herzig and Hodgson, 2008) with some modifications. Briefly, *biventer cervicis* muscles were removed from male chicks (8-15 days old), mounted in glass (10ml) organ baths and maintained at 34ºC under 1g resting tension in a physiological saline solution of the following composition: 118.4mM NaCl, 4.7mM KCl, 1.2mM $MgSO_4$, 1.2mM KH_2PO_4, 2.5mM $CaCl_2$, 25mM $NaHCO_3$, 11.1mM D-glucose and bubbled continuously with 95% O_2 + 5% CO_2 to maintain the pH between 7.2 - 7.4. Isometric contractions were measured via a Grass transducer (FTO3) connected via either one or two Quad Bridge modules (ADInstruments, Australia) to a Powerlab 4/20, MacLab/4s or MacLab/8e system (all ADInstruments, Australia). Twitches (where applicable) were evoked by stimulating the motor nerve (supramaximal voltage, duration 0.2ms, 0.1Hz) via silver electrodes connected to a Grass S88 stimulator. Nerve-mediated (indirectly evoked) twitches were confirmed by the addition of d-TC (10µM) a blocker of postsynaptic nicotinic acetylcholine receptors. Muscles that did not respond to acetylcholine, carbachol and KCl were excluded from this study.

In order to minimise variation, the same muscles were used for control and treatment (consisting of various pharmacological or physiological agents). Hence, each muscle first received 10µl/ml Milli-Q water as control and was monitored over 60min. Without receiving a washout, the baseline was then manually re-adjusted to the initial starting value and the respective treatment was applied immediately before the muscle was monitored for another 60min. This treatment schedule allowed each muscle to act as its own control. Each muscle was only used for a single treatment. The applied treatments consisted of *C. tropix* venom (2µg/ml, n = 5), gadolinium (1mM, n = 7), verapamil (10mM, n = 4), d-TC (100µM, n = 6), TTX (1µM, n = 6), $MgCl_2$ (20mM, n = 17), $CaCl_2$ (20mM, n = 6), and glucose (40mM, n = 4). A separate control experiment was carried out to test for effects on the baseline muscle tension when the electrical stimulation was turned on (n = 6) or off (n = 8). In case of the muscles that received the electrical stimulation of the motor nerves, only the baseline tension in the 10sec interval between twitches was analysed to allow for comparability with the non-stimulated muscle.

Drugs and chemicals

d-TC and TTX were obtained from Sigma Chemical Co (St Louis, MO, USA). Gadolinium, verapamil, $MgCl_2$ (20mM), $CaCl_2$ (20mM), and glucose were supplied by Sigma-Aldrich Company Ltd (Dorset, England). Stock solutions of drugs were made up in Milli-Q water unless otherwise stated.

Statistical analysis

Sigma-Plot 11.0 (Systat Software Inc., San Jose, California, USA) was used for all statistical analysis. The baseline muscle tension values for control and treatment for each group were compared by using either a t-test or (in cases where the normality test failed) a Mann-Whitney rank sum test. For all tests, significance levels were set to 0.05 and data are expressed as mean minus standard error of the mean (S.E.M.).

RESULTS AND DISCUSSION

Physical stretch of muscle

We have previously shown that the venom from female *C. tropix* spiders not only reduced the baseline muscle tension but also produced an irreversible block of nerve-evoked twitch tension on *biventer cervicis* nerve-muscle preparations (Herzig and Hodgson, 2008). A possibility to explain the reduced muscle tension therefore would be that the omission of the electrically induced twitches resulted in a relaxation of the muscle. Stretching of skeletal muscles results in a number of physiological effects (*e.g.*, to activate the contractile apparatus resulting in an increase of the force of contraction or to increase transmitter release) all whose end point is to elevate cytosolic calcium ions (see Rosenberg, 2009 for a recent review on calcium entry in skeletal muscle). Stretching of the muscle deforms both the cytoskeleton and cell membrane and these changes in shear forces are thought to cause ion channels and receptors to undergo conformational changes and open or activate. By blocking the muscle from being stretched during electrically induced twitches, *C. tropix* venom might have induced a decrease in the cytosolic calcium levels, leading to a decrease in muscle tension. This hypothesis, however, is not substantiated by our data (Figure 1A and B) that show that the absence of nerve-evoked twitches does not induce the muscle to relax significantly more than the time-matched control that received the nerve-evoked twitches. We also confirm our earlier observation on avian nerve-muscle preparations that *C. tropix* venom significantly reduces resting tension when the tissue was subjected to a resting tension of about 10mN (Figure 1C) (Herzig and Hodgson, 2009).

At frog neuromuscular junctions it was demonstrated that stretch of skeletal muscles induces an increase in spontaneous and evoked transmitter release mediated by mechanical tension on synaptically located membrane

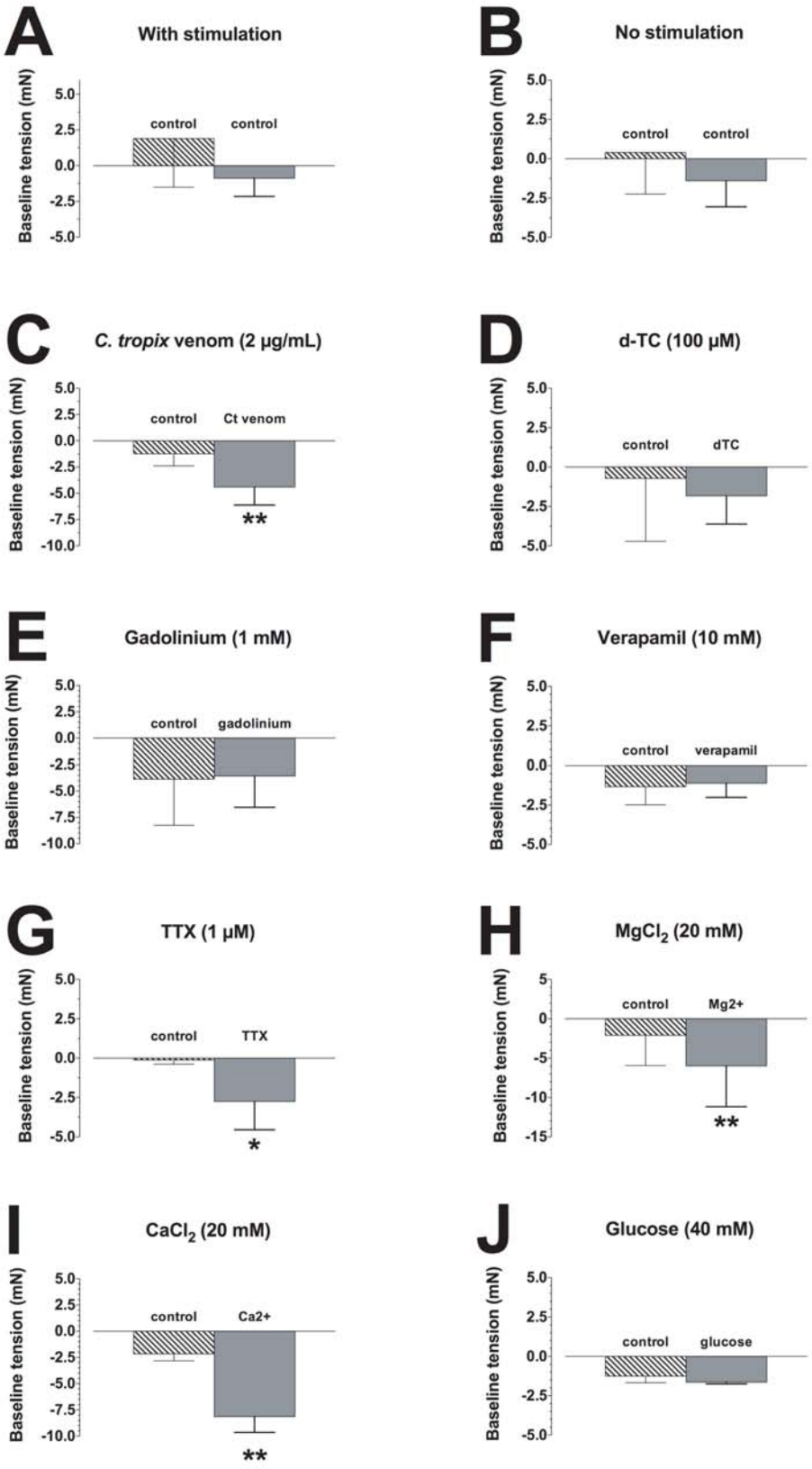

Figure 1. Baseline muscle tension values (inclding S.E.M.) after various treatments as recorded using a chick *biventer cervicis* nerve-muscle preparation. No effect of the stimulation of the motor nerve on the baseline muscle tension could be observed when tested under the presence (**A**) and absence (**B**) of electrical stimulation of the motor nerve. In the case of **A**, only the baseline tension in between twitches was analysed to allow for comparability with the non-stimulated muscle. All of the remaining treatments were carried out in the absence of electrical stimulation. These treatments include *C. tropix* venom (2µg/ml, **C**), d-TC (100µM, **D**), gadolinium (1mM, **E**), verapamil (10mM, **F**), TTX (1µM, **G**), $MgCl_2$ (20mM, **H**) and $CaCl_2$ (20mM, **I**), and glucose (40mM, **J**). Significant differences are indicated by *($P < 0.05$) and **($P < 0.01$).

bound integrins (Chen and Grinnell, 1995). It has been suggested that this effect is limited to the frog neuromuscular junction (Grinnell et al, 2003), as transmitter release is not significantly changed when adult rat diaphragm neuromuscular junctions were subjected to stretch. The effects of stretch on transmitter release on avian neuromuscular junctions has not been adequately studied, but if stretch increased transmitter release, it would be predicted to result in the activation of postsynaptic receptors and subsequent depolarisation of the multiply innervated muscle fibres to generate a sustained tonic contracture. As the nicotinic acetylcholine receptor antagonist d-TC (Figure 1D) did not significantly reduce baseline tension, we assume that despite the evidence for stretch-induced increased transmitter release in the frog, block of augmented acetylcholine release from stretched avian neuromuscular junctions cannot account for the reduction of baseline tension seen by *C. tropix* venom.

Stretch-activated ion channels (SACs)

The method used in the present study includes the application of a pre-tension of 10mN to the chick biventer muscles. Hence, there exists the possibility that this pre-tension (*i.e.*, stretch) caused the activation of SACs. Guharay and Sachs (1984) already reported that stretch causes the activation of SACs in chick skeletal muscle. It was found that the current evoked by the stretch appears neither to be due to activation of the nicotinic acetylcholine receptor ion channel complex nor due to the opening of Ca^{2+} activated K^+ channels. The current was further shown to have a reversal potential of around -30mV, to be cation (Ca^{2+}, K^+ and Na^+) selective, and to only poorly discriminate between Na^+ and K^+ ions. Based upon these biophysical properties, activation of this channel will result in depolarisation of skeletal muscle cells, and if the depolarisation is sufficiently large, the contractile apparatus would be activated through the subsequent influx of calcium ions through excitation-coupled calcium entry.

Further studies on chick embryonic myoblasts (Shin et al, 1996) demonstrated that stretch elevated intracellular Ca^{2+} concentration sufficiently, resulting in the activation of Ca^{2+}-activated K^+ channels, and these effects were completely blocked by 10μM gadolinium. The lack of effect of gadolinium (up to 50μM) in our study (Figure 1E) would either suggest that blocking calcium influx through SACs does not account for the effect of *C. tropix* venom on baseline tension or that the effect was caused by a toxin from the venom that acts on a sub-type of SACs, which are insensitive to gadolinium.

Voltage-gated ion channels

Calcium entry in skeletal muscle requires the functioning of L-type voltage-gated calcium channels (VGCCs). These ion channels mediate excitation-contraction coupling by acting as voltage sensors that trigger the opening of ryanodine receptors and induce calcium release from the sarcoplasmic reticulum. It was shown that the IVS6 segment of the L-type channel is critical for the binding of phenylalkylamines (verapamil) and accounts for the sensitivity of muscle contraction to these drugs (Schuster et al, 1996). As spider venoms are a rich source of ion channel toxins (Herzig et al, 2011) the venom of *C. tropix may* contain compounds that will block L-type calcium channels and account for the change in baseline tension. However, the lack of effect of verapamil (Figure 1F) would suggest that L-type calcium channels are not implicated in the effect of *C. tropix* venom.

Voltage-gated sodium channels (VGSCs) are sensitive to stretch, which can result in an increased permeability to sodium ions (Wallace et al, 1998; Shcherbatko et al, 1999; Tabarean et al, 1999). It has been suggested that stretch activates mechanosensitive sodium channels, which leads to an increase in intracellular sodium (Wolf et al, 2001). This increased sodium influx has two different effects: Firstly, it induces the sodium-calcium exchanger to pump in 'reverse-mode' (*i.e.*, calcium is pumped into the cell and sodium is pumped out of the cell) and secondly it induces membrane depolarization, which causes more sodium channels to open and activates VGCCs. The resulting increase in intracellular calcium activates the contractile apparatus, which results in shortening of muscle sarcomeres and a subsequent increase in baseline muscle tension. In all cases the effects of activating the sodium channel can be blocked by application of the selective sodium channel blocker TTX. The reduction in baseline tension induced by TTX (Figure 1G) and the lack of action of d-TC would suggest a postsynaptic mechanism of action for TTX, *i.e.*, acting through muscle sodium channels as opposed to neuronal sodium channels. The reduction of baseline muscle tension by excess Mg^{2+} (Figure 1H) and Ca^{2+} ions (Figure 1I) could further be explained by these cations blocking VGSCs, as suggested by another study (Yamamoto et al, 1984). The lack of effect of the osmotic equivalence of these ions through adding 40mM glucose (Figure 1J) to the physiological salt solution strongly suggests a pharmacological role for these ions. We have not measured the cation concentration from the venom of *C. tropix,* however, a related theraphosid spider *Aphonopelma steindachneri* (previously named *Eurypelma californicum* according to Platnick, 2010) is reported to contain approximately 100nM of cations (Savel-Niemann and Roth, 1989), well below the threshold that would affect the opening of sodium channels.

CONCLUSIONS

Our results show that excess Ca^{2+} and Mg^{2+} as well as TTX and *C. tropix* venom reduces the baseline muscle tension in the chick *biventer cervicis* nerve-muscle preparation. Based on the lack of effect of verapamil and d-TC, we can exclude that blocking voltage-gated calcium channels (VGCC) and nicotinic acetylcholine receptors are responsible for the reduced baseline tension. Our data strongly suggests that muscle TTX-sensitive sodium channels are activated tonically during muscle stretch and that blocking these channels causes a reduction in baseline resting tension through an as yet undefined mechanism The role of the sodium channel in muscle disease has seen a new resurgence as mutations of voltage-gated sodium channels have been recognised to be involved in a number of myotonias (see Platt and Griggs, 2009 for a review). Our data would suggest that skeletal muscle sodium channels ($Na_v1.4$) play an indirect role in activating the contractile apparatus. Further detailed electrophysiological, and calcium imaging experiments will be employed to explore this hypothesis.

The question that remains open is which of the possible mechanisms that we have shown to reduce the muscle

tension is employed by *C. tropix* venom. While Ca^{2+} and Mg^{2+} have been shown to be present in another theraphosid spider venom (Savel-Niemann and Roth, 1989), their quantity in the low nanomolar range would not have been sufficient to explain the present results induced by millimolar concentration of these ions. Although gadolinium did not show any effect on the muscle tension in the present experiments, there still exists the possibility that a toxin from *C. tropix* venom acted on a different sub-type of SACs, which is insensitive to gadolinium. Whereas theraphosid spider venoms are known to contain blockers of mechanosensitve channels such as M-TRTX-Gr1a (new toxin name according to ArachnoServer (Herzig et al, 2011), previously known as GsMTx4) from the venom of the theraphosid spider *Grammostola rosea* (Suchyna et al, 2000), more detailed experiments would be required to prove this assumption. Our most favourable explanation is that *C. tropix* venom reduced the tension by blocking muscle TTX-sensitive sodium channels. According to the spider toxin database ArachnoServer (Herzig et al, 2011), VGSC are among the main targets for spider toxins.

ACKNOWLEDGEMENTS

VH was funded by fellowships from the DAAD (Deutscher Akademischer Austauschdienst) and DFG (Deutsche Forschungsgemeinschaft). VH and WCH were supported by the ARC (Australian Research Council). We wish to thank Alan Harvey for helpful comments on the manuscript.

STATEMENT OF COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

d-TC; d-tubocurarine
SACs; stretch activated ion channels
TTX; tetrodotoxin
VGCCs; Voltage-gated calcium channels
VGSCs; Voltage-gated sodium channels

REFERENCES

Billen B, Bosmans F and Tytgat J. 2008. Animal peptides targeting voltage-activated sodium channels. Curr Pharm Des, 14, 2492-2502.

Chen BM and Grinnell AD. 1995. Integrins and modulation of transmitter release from motor nerve terminals by stretch. Science, 269, 1578-1580.

De Lima ME, Figueiredo SG, Pimenta AM et al. 2007. Peptides of arachnid venoms with insecticidal activity targeting sodium channels. Comp Biochem Physiol C Toxicol Pharmacol, 146, 264-279.

Estrada G, Villegas E and Corzo G. 2007. Spider venoms: a rich source of acylpolyamines and peptides as new leads for CNS drugs. Nat Prod Rep, 24, 145-161.

Grinnell AD, Chen BM, Kashani A, Lin J, Suzuki K and Kidokoro Y. 2003. The role of integrins in the modulation of neurotransmitter release from motor nerve terminals by stretch and hypertonicity. J Neurocytol, 32, 489-503.

Guharay F and Sachs F. 1984. Stretch-activated single ion channel currents in tissue-cultured embryonic chick skeletal muscle. J Physiol, 352, 685-701.

Herzig V and Hodgson WC. 2008. Neurotoxic and insecticidal properties of venom from the Australian theraphosid spider *Selenotholus foelschei*. NeuroToxicology, 29, 471-475.

Herzig V and Hodgson WC. 2009. Intersexual variations in the pharmacological properties of *Coremiocnemis tropix* (Araneae, Theraphosidae) spider venom. Toxicon, 53, 196-205.

Herzig V, Wood DLA, Newell F et al. 2011. ArachnoServer 2.0, an updated online resource for spider toxin sequences and structures. Nucleic Acids Research, in press, DOI 10.1093/nar/gkq1058.

Hodgson WC, Dal Belo CA and Rowan EG. 2007. The neuromuscular activity of paradoxin: a presynaptic neurotoxin from the venom of the inland taipan (*Oxyuranus microlepidotus*). Neuropharmacology, 52, 1229-1236.

Kini RM. 2005. Structure-function relationships and mechanism of anticoagulant phospholipase A_2 enzymes from snake venoms. Toxicon, 45, 1147-1161.

Orndorff RL and Rosenthal SJ. 2009. Neurotoxin quantum dot conjugates detect endogenous targets expressed in live cancer cells. Nano Letts, 9, 2589-2599.

Platnick NI. 2010. The world spider catalog, version 10.5. American Museum of Natural History, http://research.amnh.org/entomology/spiders/catalog/index.html

Platt D and Griggs R. 2009. Skeletal muscle channelopathies: new insights into the periodic paralyses and nondystrophic myotonias. Curr Opin Neurol, 22, 524-531.

Prestipino G, Corzo G, Romeo S et al. 2009. Scorpion toxins that block transient currents (I(A)) of rat cerebellum granular cells. Toxicol Letts, 187, 1-9.

Rosenberg PB. 2009. Calcium entry in skeletal muscle. J Physiol, 587, 3149-3151.

Savel-Niemann A and Roth D. 1989. Biochemical analysis of tarantula venom (*Eurypelma californicum*). Naturwissenschaften, 76, 212-213.

Schuster A, Lacinová L, Klugbauer N, Ito H, Birnbaumer L and Hofmann F. 1996. The IVS6 segment of the L-type calcium channel is critical for the action of dihydropyridines and phenylalkylamines. EMBO J, 15, 2365-2370.

Servent D and Fruchart-Gaillard C. 2009. Muscarinic toxins: tools for the study of the pharmacological and functional properties of muscarinic receptors. J Neurochem, 109, 1193-1202.

Shcherbatko A, Ono F, Mandel G and Brehm P. 1999. Voltage-dependent sodium channel function is regulated through membrane mechanics. Biophys J, 77, 1945-1959.

Shin KS, Park JY, Ha DB, Chung CH and Kang MS. 1996. Involvement of K(Ca) channels and stretch-activated channels in calcium influx, triggering membrane fusion of chick embryonic myoblasts. Dev Biol, 175, 14-23.

Sontheimer H. 2008. An unexpected role for ion channels in brain tumor metastasis. Exp Biol Med (Maywood), 233, 779-791.

Suchyna TM, Johnson JH, Hamer K et al. 2000. Identification of a peptide Toxin from *Grammostola spatulata* spider venom that blocks cation-selective stretch-activated channels. J Gen Physiol, 115, 583-598.

Tabarean IV, Juranka P and Morris CE. 1999. Membrane stretch affects gating modes of a skeletal muscle sodium channel. Biophys J, 77, 758-774.

Wallace RH, Wang DW, Singh R et al. 1998. Febrile seizures and generalized epilepsy associated with a mutation in the Na1-channel beta1 subunit gene SCN1B. Nat Genet, 19, 366-370.

Wolf JA, Stys PK, Lusardi T, Meaney D and Smith DH. 2001. Traumatic axonal injury induces calcium influx modulated by tetrodotoxin-sensitive sodium channels. J Neurosci, 21, 1923-1930.

Yamamoto D, Yeh JZ and Narahashi T. 1984. Voltage-dependent calcium block of normal and tetramethrin-modified single sodium channels. Biophys J, 45, 337-345.