

**TRACHYNILYSIN, A PROTEIN NEUROTOXIN ISOLATED
FROM STONEFISH (*SYNANCEIA TRACHYNIS*) VENOM,
INCREASES SPONTANEOUS QUANTAL ACETYLCHOLINE
RELEASE FROM *TORPEDO MARMORATA*
NEUROMUSCULAR JUNCTIONS**

by

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ABSTRACT. Trachynilysin, a neurotoxin isolated from the venom of the stonefish (*Synanceia trachynis*, Scorpaenidae), produced a marked increase in the frequency of spontaneous miniature endplate potentials (MEPPs) at *Torpedo* neuromuscular junctions. The periods of high frequency MEPP discharges were of variable duration and were followed by periods of rest. In addition, trachynilysin increased as a function of time the proportion of larger than normal MEPPs, the so-called "giant" MEPPs. Trachynilysin did not affect the junctions when applied in Ca²⁺-free medium supplemented with EGTA, but the subsequent addition of Ca²⁺ caused a rapid increase in MEPP frequency, even when the toxin was washed out of the Ca²⁺-free medium. Thus, trachynilysin binding to nerve terminals is not dependent on external Ca²⁺, but the cation is required for trachynilysin-elicited quantal transmitter release. The effect of trachynilysin on MEPP frequency was unaffected by the Ca²⁺ channel blockers ω -conotoxin GVIA, ω -agatoxin IVA and Gd³⁺, which indicates that the toxin's action involves Ca²⁺ entry via a pathway independent of voltage-sensitive Ca²⁺ channels. Pre-treatment of the junctions with concanavalin-A prevented the trachynilysin-induced enhancement of quantal transmitter release, which suggests that the toxin interacts with or binds to a glycoprotein on the surface of motor nerve terminals.

RÉSUMÉ. La trachynilysine, isolée du venin du poisson-pierre, *Synanceia trachynis*, augmente la libération quantique spontanée d'acétylcholine à la jonction neuromusculaire squelettique de *Torpedo marmorata*.

La trachynilysine, une neurotoxine isolée du venin du poisson-pierre, *Synanceia trachynis* (Scorpaenidae), provoque une augmentation très importante de la fréquence des potentiels de plaque motrice miniatures (PPMM) à la jonction neuromusculaire de la Torpille. Ces périodes de libération accrue d'acétylcholine ont une durée variable et alternent avec des périodes de repos. De plus, la trachynilysine augmente en fonction du temps la proportion de PPMM dits "géants" qui diffèrent des potentiels miniatures habituels par leur grande amplitude. La trachynilysine n'a pas d'action sur les terminaisons nerveuses motrices dans un milieu dépourvu de Ca²⁺ et contenant de l'EGTA. Cependant, l'addition ultérieure de Ca²⁺ au milieu expérimental augmente la fréquence de PPMM, même lorsque la trachynilysine a été préalablement retirée du milieu dépourvu de Ca²⁺. Ces résultats suggèrent que la fixation de la trachynilysine sur les terminaisons nerveuses ne requiert pas la présence de Ca²⁺. En revanche, son action sur la libération d'acétylcholine dépend strictement de la présence de Ca²⁺ dans le milieu extracellulaire. L'effet de la trachynilysine sur la fréquence des PPMM n'est pas affecté par les inhibiteurs des canaux Ca²⁺ (ω -conotoxine GVIA, ω -agatoxine IVA et Gd³⁺). Ceci suggère que la

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trachynilysine favorise l'entrée du Ca^{2+} dans les terminaisons par des voies différentes de celles des canaux Ca^{2+} . Le prétraitement des terminaisons nerveuses par la concanavaline-A bloque l'augmentation de la libération quantique d'acétylcholine provoquée par la trachynilysine, ce qui suggère que la toxine interagit avec ou se lie à une glycoprotéine présente à la surface des terminaisons nerveuses.

Key words. *Synanceia trachynis* - *Torpedo marmorata* - Quantal acetylcholine release - Miniature endplate potentials - Neuromuscular junction - Trachynilysin - Concanavalin A.

The venomous property of certain fish has long been recognised (Bottard, 1889a, 1889b), and more than 200 species of marine fish are known to possess some form of venom apparatus capable of inflicting serious and occasionally fatal wounds (Russell, 1965; Halstead, 1988). Numerous venomous fish belonging to the family Scorpaenidae ("scorpionfish") inhabit shallow waters over wide areas of the tropical Indo-Pacific and the Red Sea. Members of this family include three species of stonefish (*Synanceia*), which possess numerous pairs of well-developed venom glands and are among the most venomous fish known to man (Wiener, 1958; Sutherland, 1983; Halstead, 1988; Gwee *et al.*, 1994; Goudey-Perrière and Perrière, 1998). Scorpionfish envenomations occur chiefly in fishermen and swimmers as a result of contact with sharp dorsal, pelvic or anal spines which readily penetrate the skin of the victims (Bottard, 1889b), and stonefish envenomation is still an occupational hazard in many areas of the world (Chan *et al.*, 1996).

Our previous observation that low concentrations of *S. trachynis* venom markedly enhance spontaneous quantal acetylcholine release, detected as an increase in MEPPs or currents at mouse and frog skeletal neuromuscular junctions (Kreger *et al.*, 1993), prompted us to purify the venom constituent responsible for the observed stimulation of neurotransmitter release. Fractionation of *S. trachynis* venom by sequential anion-exchange fast protein liquid chromatography (FPLC) and size exclusion FPLC yielded a highly purified membrane-perturbing (haemolytic) protein toxin named trachynilysin (Colasante *et al.*, 1996). Trachynilysin dramatically increases spontaneous quantal acetylcholine release at the frog neuromuscular junction and depletes small clear synaptic vesicles containing acetylcholine, but does not affect the number or the immunoreactivity of large dense core vesicles (LDCV) containing neuropeptides (Colasante *et al.*, 1996). The insensitivity of LDCV to trachynilysin was further examined by determining whether trachynilysin could elicit catecholamine release from LDCV in bovine chromaffin cells. Unlike the case of LDCV in motor nerve terminals, trachynilysin evoked sustained exocytosis of LDCV containing catecholamines, but only in the presence of extracellular Ca^{2+} (Meunier *et al.*, 2000). Thus, the mechanisms for the exocytosis of LDCV from motor nerve terminals and neuroendocrine cells are distinct.

In the present study, we have examined: (i) the ability of trachynilysin to elicit neurotransmitter release from motor nerve terminals innervating skeletal muscle from *Torpedo marmorata*, (ii) the influence of extracellular Ca^{2+} on trachynilysin binding and action, and (iii) the Ca^{2+} pathway required for trachynilysin action. We found that trachynilysin's action on the *Torpedo* neuromuscular junction involves Ca^{2+} -independent, concanavalin-A-sensitive binding of toxin, and that the toxin dramatically enhances asynchronous acetylcholine release via a Ca^{2+} entry pathway which is distinct from one involving voltage-sensitive Ca^{2+} channels.

MATERIALS AND METHODS

Torpedo marmorata (obtained alive from the Marine Station of Arcachon, France) were maintained in a filtered and aerated aquarium at 12°C prior to sacrifice, and skeletal neuromuscular preparations were isolated from the pelvic fin (Brochier *et al.*, 1991). The isolated nerve-muscle preparations were stretched at their resting length, pinned in a Rhodorsil-lined (Rhône-Poulenc, St. Fons, France) Plexiglas chamber (200 ml capacity), and superfused with a physiological solution having the following composition (mM): NaCl, 280; KCl, 3; CaCl₂, 2; MgCl₂, 1.8; glucose, 5.5; urea, 300; NaHCO₃, 5; and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), 5 mM (pH 7.4). The Ca²⁺ concentration was varied in some experiments, as indicated in the Results section.

Electrophysiological recordings

Membrane potentials and spontaneous MEPPs were recorded from endplate regions at room temperature (20-22°C), with intracellular microelectrodes filled with 3 M KCl (8-12 MΩ resistance), using conventional techniques and an Axoclamp-2A system (Axon Instruments, Foster City, CA, U.S.A.). Recordings were made continuously from the same endplate before and after application of the agents tested. After amplification, electrical signals were displayed on a digital oscilloscope and were simultaneously recorded on video tape with the aid of a modified digital audio processor (Sony PCM 701 ES) and a video cassette recorder (Sony SLC9F). Data were collected and digitised with the aid of a computer equipped with an analogue and digital I/O interface board (DT2821, Data Translation Marlboro, U.S.A.) at a sampling rate of 25 kHz. Computerised data acquisition and analysis was performed with a program kindly provided by Dr. John Dempster (University of Strathclyde, Scotland).

Three to six individual experiments were performed for each condition studied and the results were calculated as the means ± standard error of the mean (S.E.M.). Statistical analysis was performed with the Student's t-test or the Mann-Whitney test, with $p < 0.05$ indicating significance.

Trachynilysin

Lyophilised stonefish (*S. trachynis*) venom was obtained as previously described (Kreger, 1991), and trachynilysin was isolated from a solution of reconstituted venom by sequential anion-exchange FPLC and size-exclusion FPLC (Colasante *et al.*, 1996). α -agatoxin IVA and α -conotoxin GVIA were purchased from Latoxan (Rosans, France), and tetrodotoxin and concanavalin-A were obtained from Sigma-Aldrich Chimie (Saint Quentin Fallavier, France).

RESULTS

Trachynilysin increases spontaneous quantal transmitter release

Exposure of fish neuromuscular preparations to 30-60 μM trachynilysin, in the presence of the standard physiological solution containing 2 mM Ca²⁺, induced asynchronous release of acetylcholine quanta, detected as an increase in MEPP frequency. The response elicited by 30 μM trachynilysin developed after a delay of about 10-20 min exposure to the toxin and was characterised by the appearance of high frequency MEPP discharges of variable duration, followed by periods of rest. The frequency of MEPPs oscillated from a control level of about 0.4-0.5 events/second (Hz) to 100-150 Hz during

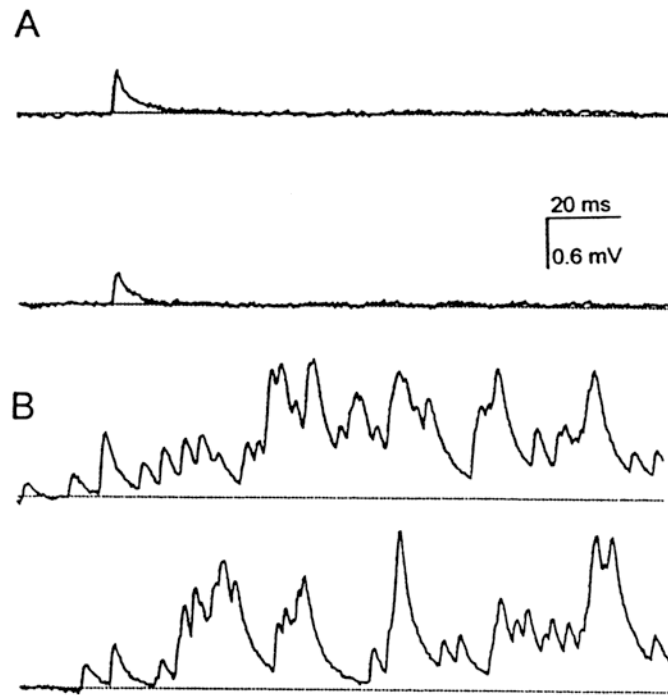


Fig. 1. Effect of trachynilysin on miniature endplate potentials recorded on the same junction before (A) and 10 min after (B) the addition of 60 μ M trachynilysin to the standard medium containing 1 μ M TTX bathing the isolated pelvic fin muscle from *Torpedo marmorata*. The resting membrane potential of the muscle fibre during the recordings was -86 mV. Notice the high frequency and the temporal summation of some of the spontaneous quantal events.

a given burst of MEPPs. Thus, the pattern of MEPP frequency changes elicited by 30 μ M trachynilysin was irregular and unpredictable. However, 60 μ M trachynilysin produced continuous bursts of high frequency MEPPs (Fig. 1) with only a few resting periods between bursts. Trachynilysin-induced MEPP bursts were not affected by blockade of voltage-dependent Na⁺ channels in motor axons and nerve terminals, by 1 μ M tetrodotoxin (Fig. 1). Therefore, it is unlikely that the trachynilysin-induced bursts of spontaneous MEPP discharges were caused by spontaneous firing of axons or nerve terminals.

As shown in the MEPP amplitude distribution histograms (Fig. 2), trachynilysin produced a marked increase in the occurrence of the so-called "giant" MEPPs (i.e., potentials larger than twice the modal amplitude of MEPPs). The proportion of such giant potentials (relative to the total number of MEPPs) increased with time, from 2.5 \pm 0.2% after 5 min to about 26 \pm 3.1% (n = 3) after 20 min of trachynilysin action, and no obvious difference in their frequency or time course was noticed when tetrodotoxin (1 μ M) was present in the physiological solution.

The increased MEPP frequency induced by trachynilysin was irreversible; i.e., washing out trachynilysin from the medium did not suppress the asynchronous quantal transmitter release. Trachynilysin (30-60 μ M) did not affect the resting membrane potential of the muscle fibres (n = 2).

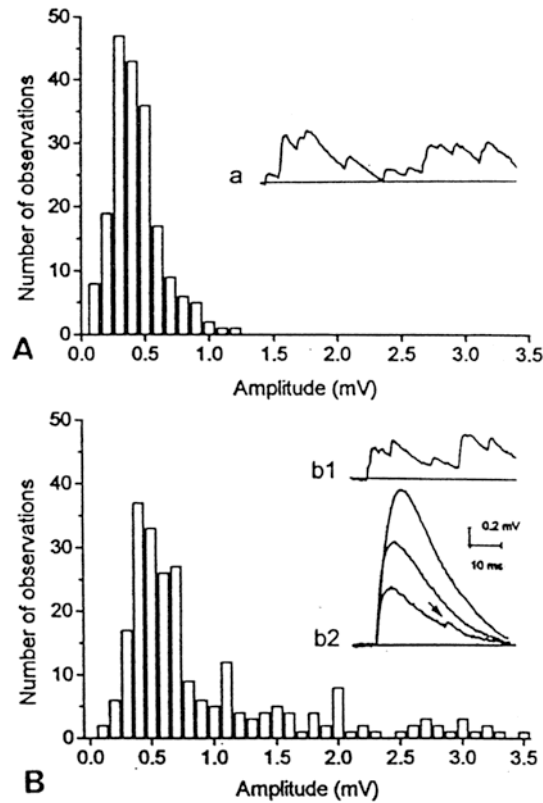


Fig. 2. Miniature endplate potential amplitude distribution recorded from the same neuromuscular junction after 10 min (A) and 20 min (B) exposure to trachynilysin (60 μ M). Insets in A (a) and B (b1) show typical high frequency MEPPs induced by trachynilysin. Notice in b2, superimposed traces of large amplitude events ("giant" MEPPs) responsible for the change in the amplitude distribution shown in B. The arrow in b2 indicates the presence of a MEPP which appears during the decay phase of a giant MEPP. The isolated pelvic fin muscle was bathed in standard physiological solution containing 1 μ M TTX. Resting membrane potential during measurements was -33 mV in (A) and -31 mV in (B). Calibration in b2 applies also to a and b1.

Extracellular Ca^{2+} is required for trachynilysin action

To ascertain whether trachynilysin needs Ca^{2+} as a cofactor to aid binding to an extracellular receptor in motor endings, neuromuscular preparations were pre-equilibrated in fish standard saline without Ca^{2+} and containing the Ca^{2+} -chelator EGTA (1 μ M). Under these conditions, the MEPP frequency was 0.21 ± 0.1 per second ($n=3$). Subsequent exposure to 60 μ M trachynilysin for 1 hr failed to increase MEPP frequency (0.15 ± 0.08 per second; $n=3$). However, extensive washing to remove unbound trachynilysin, and replacing the Ca^{2+} -free and EGTA-supplemented medium with standard fish saline containing Ca^{2+} (2 μ M), resulted in a significant stimulation of quantal transmitter release (the mean MEPP frequency was 40 ± 0.6 per second [$n=3$]). Thus, trachynilysin binding to nerve terminals is not dependent on external Ca^{2+} , but the cation is required for trachynilysin-elicited quantal transmitter release.

Ca²⁺ entry through voltage-gated Ca²⁺ channels is not required for trachynilysin action

Since extracellular Ca²⁺ is essential for trachynilysin-induced stimulation of quantal transmitter release, it was of interest to examine the possible involvement of voltage-sensitive Ca²⁺ channels in this effect. N and P/Q type Ca²⁺ channels are known to contribute to Ca²⁺-dependent acetylcholine release in pure cholinergic nerve endings from the *Torpedo* electric organ (Moulian and Morot-Gaudry-Talarmin, 1993); thus, we decided to determine whether selective Ca²⁺ channel blockers could modify the ability of trachynilysin to increase MEPP frequency at the fish neuromuscular junction. We found that application of ω -conotoxin GVIA (1.5 μ M) and ω -agatotoxin IVA (0.5 μ M), which block N- and P-type Ca²⁺ channels, respectively (Kerr and Yoshikami, 1984; Protti *et al.*, 1993), did not prevent the increase in MEPP frequency caused by 60 μ M trachynilysin. This observation suggests that these Ca²⁺ channels are not required for trachynilysin-mediated, spontaneous quantal transmitter release. However, in order to exclude the possibility that voltage-sensitive Ca²⁺ channels insensitive to ω -conotoxin GVIA and ω -agatotoxin IVA may be involved in trachynilysin's action, we applied Gd³⁺, a rather general Ca²⁺ channel blocker (Docherty, 1988; Molgó *et al.*, 1991) which also blocks stretch-activated ion channels (Yang and Sachs, 1989). Gd³⁺ (0.25 μ M) did not inhibit trachynilysin-elicited quantal acetylcholine release. Thus, the spontaneous quantal acetylcholine release induced by trachynilysin probably involves Ca²⁺ entry *via* a pathway independent of voltage-sensitive Ca²⁺ channels.

Concanavalin-A prevents trachynilysin's ability to increase quantal acetylcholine release

Our observation that trachynilysin binding is independent of external Ca²⁺ suggested that the toxin may act by binding to the nerve terminal membrane. Therefore, we decided to determine whether concanavalin-A (a lectin from *Canavalia ensiformis*), which binds firmly to *N*-acetyl- β -D-glucosaminyl residues (reviewed by Lin and Levitan, 1991), interferes with the action of trachynilysin at the fish neuromuscular junction. For this purpose, preparations were first incubated for 1 hr with the standard solution containing 500 μ g concanavalin-A/ml, in order to prebind the lectin binding sites, and unbound lectin was removed by washing the preparations with the standard solution. Preincubation with concanavalin-A caused a small reduction (30 \pm 3.5%) in MEPP frequency in all junctions investigated ($n=4$) and prevented trachynilysin's ability to increase MEPP frequency. The mean MEPP frequency in junctions incubated only with concanavalin-A for 60 min was 0.6 \pm 0.18 ($n=4$) compared to 0.7 \pm 0.2 ($n=3$) in time-matched, concanavalin-A-pretreated, trachynilysin-treated junctions. Interestingly, concanavalin-A was ineffective when applied after trachynilysin had increased the MEPP frequency. These results support the view that the action of concanavalin-A involves inhibition of trachynilysin binding to its receptor.

DISCUSSION

This is the first report to provide direct evidence that trachynilysin, a neurotoxic protein isolated from *S. trachynis* venom, increases spontaneous quantal acetylcholine release in *Torpedo* nerve-muscle preparations. Several characteristics of the action of trachynilysin in the present study are similar to observations previously made with *S. trachynis* venom in frog and murine neuromuscular preparations (Kreger *et al.*, 1993)

and with trachynilysin in the frog neuromuscular junction (Colasante *et al.*, 1996).

The present data confirm and extend our previous studies with purified trachynilysin. Thus, our results indicate that the trachynilysin-elicited increase in spontaneous quantal transmitter release involves a presynaptic mechanism requiring the influx of Ca^{2+} , since a significant change in MEPP frequency was not detected in Ca^{2+} -free, EGTA-containing medium. Furthermore, trachynilysin was found to increase the proportion of larger than normal MEPPs, the so-called "giant" MEPPs. Such large potentials may result from quantal acetylcholine release either from oversized synaptic vesicles or early endosomes (see Bauerfeind *et al.*, 1994). However, an unexpected finding was that the Ca^{2+} influx pathway evoked by trachynilysin was insensitive to N- and P-type Ca^{2+} channel blockers, as well as to the trivalent cation Gd^{3+} . The lack of involvement of voltage-gated Ca^{2+} channels raised the possibility that the stimulation of transmitter release by trachynilysin may result from Ca^{2+} influx mediated by the activation of another type of channel, or by the formation of channels by trachynilysin. Preliminary studies have revealed that trachynilysin forms pores in the cytoplasmic membrane of neuronal cells (Ouanounou *et al.*, 1999). Furthermore, evidence that trachynilysin increases intracellular Ca^{2+} levels in hippocampal neurones and chromaffin cells has been reported (Chameau *et al.*, 1997; Meunier *et al.*, 2000). Thus, it is likely that membrane pores formed by trachynilysin contribute to increase intracellular Ca^{2+} in motor nerve terminals and, hence, to quantal transmitter release.

The present results also indicate that extracellular Ca^{2+} is not required for trachynilysin binding to its membrane receptor(s) and that there probably is very little dissociation of trachynilysin from its nerve terminal receptor site(s). The idea that the effect of concanavalin-A involves inhibition of trachynilysin binding to its receptor is supported by our observation that the lectin was ineffective when applied after trachynilysin had increased MEPPs. This strongly suggests that trachynilysin interacts with or binds to a glycoprotein on the surface of motor nerve terminals. Clearly, further work is required to understand the action of trachynilysin at the molecular level. However, the striking similarities that exist between phyla in their responses to trachynilysin highlight the fundamental conservation of synaptic mechanisms during evolution.

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