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Nitric Oxide-Induced Vasorelaxation in Response to PnTx2-6 Toxin from *Phoneutria nigriventer* Spider in Rat Cavernosal Tissue

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Abstract

Introduction—Priapism is one of several symptoms observed in accidental bites by the spider *Phoneutria nigriventer*. The venom of this spider is comprised of many toxins, and the majority has been shown to affect excitable ion channels, mainly sodium (Na⁺) channels. It has been demonstrated that PnTx2-6, a peptide extracted from the venom of *P. nigriventer*, causes erection in anesthetized rats and mice.

Aim—We investigated the mechanism by which PnTx2-6 evokes relaxation in rat corpus cavernosum.

Main Outcome Measures—PnTx2-6 toxin potentiates nitric oxide (NO)-dependent cavernosal relaxation.

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Methods—Rat cavernosal strips were incubated with bretylium (3×10^{-5} M) and contracted with phenylephrine (PE; 10^{-5} M). Relaxation responses were evoked by electrical field stimulation (EFS) or sodium nitroprusside (SNP) before and after 4 minutes of incubation with PnTx2-6 (10^{-8} M). The effect of PnTx2-6 on relaxation induced by EFS was also tested in the presence of atropine (10^{-6} M), a muscarinic receptor antagonist, N-type Ca^{2+} channel blockers (ω -conotoxin GVIA, 10^{-6} M) and sildenafil (3×10^{-8} M). Technetium99m radiolabeled PnTx2-6 subcutaneous injection was administrated in the penis.

Results—Whereas relaxation induced by SNP was not affected by PnTx2-6, EFS-induced relaxation was significantly potentiated by this toxin as well as PnTx2-6 plus SNP. This potentiating effect was further increased by sildenafil, not altered by atropine, however was completely blocked by the N-type Ca^{2+} channels. High concentrated levels of radiolabeled PnTx2-6 was specifically found in the cavernosum tissue, suggesting PnTx2-6 is an important toxin responsible for *P. nigriventer* spider accident-induced priapism.

Conclusion—We show that PnTx2-6 slows Na^+ channels inactivation in nitrergic neurons, allowing Ca^{2+} influx to facilitate NO/cGMP signalling, which promotes increased NO production. In addition, this relaxation effect is independent of phosphodiesterase enzyme type 5 inhibition. Our data displays PnTx2-6 as possible pharmacological tool to study alternative treatments for erectile dysfunction.

Keywords

Corpus Cavernosum; Relaxation; PnTx2-6 Toxin; Nitric Oxide; Erectile Physiology

Introduction

A large number of pharmacological compounds and devices have been investigated for the treatment of erectile dysfunction (ED) [1], which is a multifactorial condition of increasing occurrence linked with endothelial integrity [2,3]. ED is estimated to affect more than 150 million men worldwide, and this number may double by the year 2025 [4]. Peptides isolated from the venom from arthropods, including spiders and scorpions, have shown to promote penile erection [5–7]. Both young and advanced aged individuals bit by the *Phoneutria nigriventer* spider, an aggressive South American arthropod, present many symptoms including priapism [8,9]. The effect of *P. nigriventer* venom in cavernosal tissue was first described by Lopes-Martins et al. [10] and it was quickly determined that the venom of the spider *P. nigriventer* is a rich source of bioactive peptides [11–14]. Most of these are neurotoxins [15] that interfere with ion channels function [16], particularly voltage-gated Na^+ channels [17–20]. One of the toxins from this spider, PnTx2-6, was recently named “eretina” because when this toxin was injected directly into the mouse penis, it induced an erection [6].

PnTx2-6, formally known as Tx2-6 [21] has been demonstrated to inhibit Na^+ channel fast inactivation [18] and delays the fast inactivation kinetics of neuronal-type Na^+ channels [22]. PnTx2-5 is a similar toxin extracted from the same fraction, and differs from PnTx2-6 by five amino acid residues [13]. Mice injected intraperitoneally (ip) with PnTx2-5 induced a toxic syndrome similar to PnTx2-6, including penile erection [23].

Our group previously demonstrated that intravenous or subcutaneous injection of PnTx2-6 increased intracavernosal pressure in anesthetized rats, resulting in improved erectile function and completely normalizing the severe ED observed in DOCA-Salt hypertensive animals. Furthermore, slices of rat corpus cavernosum incubated with PnTx2-6 exhibited an increase in nitric oxide (NO) production [24]. In addition, mRNA transcription of several murine genes from mouse erectile tissue was increased after PnTx2-6 treatment, resulting in

enhanced protein products important to relaxation mechanism in the penis [25]. Interestingly, one of these genes, *Sparc*, influences several biological activities, including vascular function as it modulates smooth muscle relaxation machinery [26]. Moreover, radiolabeled PnTx2-6 (^{125}I -PnTx2-6) was injected (s.c.) into mice, which was found to be localized in the testis [27].

NO mediates erection in human and animals [28,29] and NO released from nitrergic nerves, is the major relaxant factor involved in penile erection [30]. This relaxation response is mainly dependent upon cyclic GMP (cGMP) synthesized by soluble guanylyl cyclase (sGC) activation [30,31]. Deficiency in this erection pathway (NO/cyclic guanosine monophosphate [NO/cGMP]) is associated with many diseases [32,33]. In muscles, cGMP activates protein kinase G, which leads to decreases in cytosolic Ca^{2+} by various mechanisms [31,34]. The resultant fall in intracellular Ca^{2+} leads to relaxation of the vascular and cavernosal smooth muscle cells, and subsequently penile erection [35]. Relaxation is discontinued via phosphodiesterase enzyme type 5 (PDE5)-mediated cGMP hydrolysis [36,37].

Neuronal release or synthesis of NO depends on intracellular bioavailability of Ca^{2+} in the corpus cavernosum [38,39]. In cavernosal tissue, Ca^{2+} channels appear to have an indispensable role as increases in cytosolic Ca^{2+} , when coupled to calmodulin, activates nNOS [39,40]. Accordingly, relaxations elicited by nerve stimulation of human and canine cavernosal strips were sensitive to ω -conotoxin GVIA, an N-type Ca^{2+} channel blocker [41].

Aim

In this study we aimed to investigate whether PnTx2-6 was able to improve relaxation in rat-isolated cavernosum strips and determine the involvement of muscarinic receptors and N-type Ca^{2+} channels in the action of this toxin. Additionally, we explored a possible mechanism of PnTx2-6 on the cavernosal relaxation independent of PDE5 inhibition. Considering PnTx2-6 slows down Na^+ channels inactivation current [22], we hypothesized that this peptide indirectly induced Ca^{2+} channel conductance, via N-type Ca^{2+} channels, probably located in nitrergic neuronal endings, resulting in NO production in rat cavernosal strips. Furthermore, we speculated that there are specific sites of action for this toxin in the penis.

Methods

Animals

Male Sprague Dawley and Wistar rats (10–11 weeks old, 300–325 g) were used in these studies. All procedures were carried out in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Medical College of Georgia (Institutional Animal Care and Use Committee, number A3307-01, 10/23/2009) and by the Federal University of Minas Gerais on the use of animals in research and education. The animals were housed 4 per cage on a 12-h light/dark cycle and fed a standard rat chow diet and water ad libitum.

Drugs and Solutions

Physiological salt solution of the following composition was used: 130 mM NaCl, 14.9 mM NaHCO_3 , 5.5 mM dextrose, 4.7 mM KCl, 1.18 mM KH_2PO_4 , 1.17 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.6 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.026 mM EDTA. Atropine, phenylephrine (PE), sodium nitropruside (SNP), bretylium tosylate ([*o*-bromobenzyl] ethyldimethylammonium p-toluenesulfonate), ω -conotoxin GVIA were purchased from Sigma Chemical Co. (St. Louis, MO, USA) except sildenafil. All reagents used were of analytical grade. Stock solutions were prepared in deionized water and stored in aliquots at -20°C ; dilutions were prepared

immediately before use. Sildenafil citrate (Pfizer, Inc., New York, NY, USA) was reconstituted by suspending crushed Viagra (50 mg) tablets. The toxin PnTx2-6 from the venom of *P. nigriventer* spider was purified using HPLC as described previously [42] and kindly provided by the Ezequiel Dias Foundation (Brazil). Its sequence was confirmed by MALD-TOF mass spectrometry. Stannous chloride (SnCl_2) (Sigma), sodium borohydride (NaBH_4) (Merck, Whitehouse Station, NJ, USA); bovine serum albumin [BSA] (Sigma), and $^{99\text{m}}$ technetium (from the Radioisotopes Laboratory, School of Pharmacy—Federal University of Minas Gerais, Brazil) were used to radiolabel PnTx2-6 toxin.

Functional Studies in Cavernosal Strips

After euthanasia, penises were excised, transferred to ice-cold buffer, and dissected to remove the tunica albuginea. One crural strip preparation ($1 \times 1 \times 10$ mm) was obtained from each corpus cavernosum (two crural strips from each penis). Cavernosal strips were mounted in 4 mL myograph chambers (Danish Myo Technology, Aarhus, Denmark) containing buffer at 37°C continuously aerated with a mixture of 95% O_2 and 5% CO_2 . The tissue was stretched to a resting force of 3.0 mN and allowed to equilibrate for 60 minutes; during which time the solutions were replaced every 10 to 15 minutes. Changes in isometric force were recorded using a PowerLab/8SP data acquisition system (Chart software, version 5.0; ADInstruments, Colorado Springs, CO, USA). To verify the contractile ability of the preparations, a high potassium chloride (KCl) solution (120 mM) was added to the organ baths at the end of the equilibration period. All preparations were incubated for 35 minutes with bretylium tosylate (3×10^{-5} M) to block sympathetic nerve discharge. Cavernosal strips were contracted with PE (10^{-5} M) and relaxation was evoked by electrical field stimulation (EFS). Electrical stimuli were applied to strips placed between platinum pin electrodes, which were attached to a stimulus splitter unit (Stimu-Splitter, Med Lab, Loveland, CO, USA) connected to a Grass S88 stimulator (Astro-Medical, West Warwick, RI, USA). EFS was conducted at 50 V, 1-ms pulse width, and trains of stimuli lasting 10 seconds at varying frequencies (1 to 32 Hz) before and after incubation with PnTx2-6 (10^{-8} M) for 4 minutes. To evaluate cholinergic nerve-mediated response caused by the toxin, strips were incubated with atropine (10^{-6} M). The Ca^{2+} channel blocker ω -conotoxin GVIA (10^{-6} M) was used to evaluate the participation of N-type Ca^{2+} channels in EFS-induced relaxation in the presence of PnTx2-6. This specific Ca^{2+} channel inhibitor was added 4 minutes before the stimulation with PE. Cumulative concentration–response curves to SNP (10^{-8} M to 10^{-2} M; NO donor) were performed in the absence and presence of PnTx2-6. The participation of PDE5 in the relaxation evoked by EFS in presence of PnTx2-6 was evaluated using sildenafil (3×10^{-8} M), that was added 5 minutes before the EFS. Cumulative concentration–response curve to sildenafil were first performed to determine a dose used in the relaxation by EFS.

Radiolabeling of Toxin with $^{99\text{m}}$ Technetium

Radiolabeling was made using two reducing agents, the stannous chloride (SnCl_2) and sodium borohydride (NaBH_4) as described elsewhere [43,44]. Stannous chloride (2.0 mg/mL in HCl 0.25 M) and sodium borohydride (10.0 mg/mL in NaOH 0.1 M) were added to 50 μg of toxin. The pH was adjusted to 7.0 with HCl 0.25 M, using a pH indicator strip. Then, 111 MBq of sodium pertechnetate previously eluted $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ generator ($\text{Na}^{99\text{m}}\text{TcO}_4$) (IPEN/CNEN-SP, Brazil) were added. The mixture was kept at room temperature for 20 minutes. After that, the presence of impurities such as $^{99\text{m}}\text{TcO}_4^-$ (nonreduced technetium) and $^{99\text{m}}\text{TcO}_2$ (reduced technetium) were determined by a method adapted from the United States Pharmacopoeia by Nunan and collaborators [43]. Two chromatographic systems: ascending chromatography in silica gel 60 (Merck) and descending chromatography in Whatman paper N°1 (Whatman, Piscataway, NJ, USA) were utilized. In the first technique, 5.0 μL of labeling mixture were applied to silica gel 60 ($1.5 \times$

10 cm) and eluted with acetone (Merck). After that, the strip was divided in 10 segments of 1.0 cm and counted in Automatic Scintilador (ANSR-Abbot, USA). The $^{99m}\text{TcO}_2$ and the ^{99m}Tc -Toxin stayed at the application site (Rf: 0–0.1) and the $^{99m}\text{TcO}_4^-$ migrated to the top of the strip. The percentage of $^{99m}\text{TcO}_4^-$ was calculated. In the descending chromatography procedure, 5.0 μL of labeling mixture were applied to a Whatman N°1 paper strip (15 $\times$ 36 cm), previously saturated with 1.0% BSA (Sigma) solution. Saline was used to elute the mixture up to 15.0 cm from the origin. The strip was cut in 15 fragments of 1.0 cm each and counted using scintillation. $^{99m}\text{TcO}_4^-$ and ^{99m}Tc -Toxin migrated to the end of the strip (Rf: 0.7–1.0) and the reduced technetium ($^{99m}\text{TcO}_2$) stayed at the origin. The percentage of $^{99m}\text{TcO}_2$ that was not incorporated into toxin was calculated. Using the results (radioactivity values) obtained in the five chromatograms; the labeling yield was calculated according to following formula.

$$\% \text{ labeling yield} = \frac{\text{cpm } (^{99m}\text{Tc} - \text{toxin})}{\text{Total cpm } (^{99m}\text{Tc} - \text{toxin} + ^{99m}\text{TcO}_2 + ^{99m}\text{TcO}_4^-)} \times 100$$

The purification was performed by filtration using a cellulose ester membrane (Millipore, Billerica, MA, USA). This procedure was used to separate $^{99m}\text{TcO}_2$ from ^{99m}Tc -Toxin, as the percentage of $^{99m}\text{TcO}_4^-$ was not significant. After filtration, the labeling yield was determined by the formula showed above.

Biodistribution Studies

Anesthetized male Wistar rats were injected with 0.37 MBq of ^{99m}Tc -PnTx2-6 via subcutaneous (N = 5). After 20 minutes of administration, the animals were euthanized and testicle, brain and penis were removed and weighed. The radioactivity of the organs was measured by an automatic scintillation apparatus covering an energy window of 70–210KeV (ANSR-Abott, Chicago, IL, USA). The results were expressed as counts per minutes per gram of tissue (cpm/g).

Statistical Analysis

The results shown are expressed as mean values $\pm$ standard error of the mean. Relaxation is presented as percentage change from the PE-induced contraction. Relaxation response curves were fitted using a nonlinear interactive fitting program (Graph Pad Prism 4.0; GraphPAD Software Inc., San Diego, CA, USA). Differences were estimated by two-way analyses of variance and Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.

Results

PnTx2-6 Potentiated EFS-Induced Relaxation of Rat-Isolated Cavernosal Strips

Rat-isolated cavernosal strips incubated with bretylium tosylate were contracted by PE (10^{-5} M) and submitted to EFS before and after a 4-minute incubation with PnTx2-6 (10^{-8} M). PnTx2-6 does not change the basal tone in PE-induced contraction (data not shown). Pilot studies indicated that the best concentration and incubation time for PnTx2-6 was 10^{-8} M for 4 minutes (data not shown). The relaxation obtained by EFS was significantly potentiated in the presence of the toxin (Figure 1B). Time control curves were generated to ensure that the improved relaxation observed was indeed caused by the toxin (Figure 1A). This data suggest that the toxin facilitates the cholinergic and/or noncholinergic, nonadrenergic (NANC) transmission.

PnTx2-6 Potentiation Effect on the EFS-Induced Relaxation in Rat-Isolated Cavernosal Strips Is not Blocked by Atropine

To determine whether the potentiating effect induced by PnTx2-6, as demonstrated in Figure 1B was solely caused by acetylcholine (ACh) released from the nerve endings, we tested the effect of PnTx2-6 on EFS-mediated relaxation in cavernosal tissue incubated with bretylium tosylate and atropine (10^{-6} M). This dose of Atropine (10^{-6} M) blocked muscarinic receptors, as observed in relaxation dose–concentration curve to acetylcholine (data not shown), but does not abolish significantly the cavernosal relaxation evoked by EFS. Muscarinic receptor blockade by atropine did not alter the ability of PnTx2-6 (10^{-8} M) to potentiate the relaxation evoked by EFS (Figure 2), suggesting little or no involvement of ACh in the relaxation induced by toxin.

Effect of PnTx2-6 on EFS-Induced Relaxation of Rat-Isolated Cavernosal Strips is Upstream from sGC

To verify whether the PnTx2-6 induced potentiation effect that was demonstrated in Figure 1B was caused by the changes in the GC/cGMP pathway, we evaluated SNP (an NO donor) mediated cavernosal relaxation in the presence of the toxin. Treatment with PnTx2-6 (10^{-8} M) did not affect the cumulative SNP-induced relaxation–response curves performed in cavernosal strips treated with bretylium tosylate (3×10^{-5} M) and contracted with PE (10^{-5} M, Figure 3A). However, the PnTx2-6 potentiation effect on EFS-induced relaxation was further increased by SNP (3×10^{-6} M). These data indicated that PnTx2-6 does not act downstream from GC, instead suggesting an improvement in NO release.

The Effect of PnTx2-6 on EFS-Induced Relaxation of Rat-Isolated Cavernosal Strips Is Blocked by Ca^{2+} Channel Blocker

Using a specific N-type Ca^{2+} channel blocker to test whether the PnTx2-6 potentiation effect was caused by the changes in Ca^{2+} mobilization, we found that cavernosal strips incubated with bretylium tosylate and ω -conotoxin GVIA (10^{-6} M) exhibited a significantly impaired potentiation effect (Figure 4). This suggests a vital role of N-type Ca^{2+} channels in NANC nerve endings involved in the cavernosal relaxation effect evoked by PnTx2-6.

Biodistribution of $^{99\text{m}}\text{Tc}$ -PnTx2-6 in Rats Indicates Presence of Toxin in the Penis

PnTx2-6 radiolabeled $^{99\text{m}}\text{Tc}$ (0.37 MBq) was injected (s.c.) in five rats. The radioactivity in the brain, testicles and penis was assessed after 20 minutes to verify the presence of the PnTx2-6 in these organs (Figure 5). The labeling yield of the $^{99\text{m}}\text{Tc}$ -Toxin was $92.0 \pm 1.4\%$, indicating a good stability. The data obtained from 5 experiments showed an increased presence of radiolabeled PnTx2-6 in the penile tissue. These data suggest that the penis expresses receptors for PnTx2-6, which after injection, is localized to the penis where it mediates its effect.

Effect of PnTx2-6 Toxin on EFS-Induced Relaxation of Rat-Isolated Cavernosal Strips is Independent of PDE5

To determine if the relaxation effect of PnTx2-6 was dependent on PDE5 inhibition, we used PE contracted rat cavernosum strips in the presence of toxin (10^{-8} M) plus sildenafil (3×10^{-8} M, 5 minutes incubation) and performed EFS relaxation–response curves. Previously, a cumulative sildenafil relaxation–response curve was performed to confirm the sildenafil dose able to evoke approximately 25% of relaxation (Figure 6A). We observed a further increase in the relaxation of strips incubated with both, as compared with only PnTx2-6 (Figure 6B). These data reveal a role of PnTx2-6 that does not involve PDE5 inhibition.

Discussion

Considering that PnTx2-6 potentiates erection [6,24], we investigated this toxin's ability to facilitate relaxation in isolated strips from rat cavernosum tissue. Because of the significant increase in the rat corpus cavernosum relaxation evoked by EFS observed in the presence of PnTx2-6 (Figure 1B), we believe that the improvement in cavernosal relaxation involves the action of PnTx2-6 on Na^+ channels, inducing prolonged depolarization, leading to an increased Ca^{2+} influx and neuronal NO production.

Muscarinic receptor activation on cholinergic nerve terminals, leading to release of endothelium derived relaxation factors, has been suggested to contribute to the erectile response [45,46]. We observed that atropine (10^{-6} M), a muscarinic receptor antagonist, did not alter the PnTx2-6-mediated relaxation of the cavernosal tissue (Figure 2). This indicates that the PnTx2-6-induced potentiation of the EFS-induced relaxation does not involve muscarinic receptors in the penis. On the other hand, it has been demonstrated that the whole fraction PnTx2 of *P. nigriventer* spider induces the release of various neurotransmitters, including Ach in rat cerebrocortical synaptosomes [47]. We found no evidence for muscarinic activation via PnTx2-6. However, this may be explained because other PhTx2 fraction toxins may mediate Ach release.

Given that the NO/cGMP pathway has been considered a primary target to improve erectile function [36,48–50], we tested whether PnTx2-6 acts via facilitating NO production in the absence of EFS. Cumulative concentration–response curves to SNP (10^{-8} M to 10^{-2} M; NO donor) performed in the absence and in presence of PnTx2-6 were identical (Figure 3A). As PnTx2-6 was mechanistically shown to slow Na^+ channel inactivation [18], it is conceivable that this toxin does not influence SNP-induced cavernous tissue relaxation at low doses or in the absence of EFS. However, when relaxation was evoked by EFS plus SNP, the potentiating effect caused by this toxin was further improved (Figure 3B). These data suggest that PnTx2-6 toxin improves NO release upstream of sGC activation, thus facilitating the NO/cGMP pathway. Any effect of PnTx2-6 was observed when the NOS inhibitor (L-NAME) was used (data not shown). These results corroborated with data showing that in mice treated with L-NAME [6], as well as treated with a specific nNOS inhibitor (7-NI), there was an absence of toxic syndrome evoked by PnTx2-6 toxin [23].

It has been demonstrated that nerve action potentials open tetrodotoxin-sensitive Na^+ channels in nitrergic nerve terminals and promote Ca^{2+} influx, possibly through N-type Ca^{2+} channels [51]. Also, increases in cytosolic Ca^{2+} participates in activation of nNOS in the presence of calmodulin [40]. In our experiments, treatment with ω -conotoxin GVIA abolished the improvement on the EFS-induced relaxation produced by PnTx2-6 (Figure 4). These results are in agreement with experiments showing the effects of Ca^{2+} antagonists in nitrergic nerve function in isolated canine corpus cavernosum suggesting that N-type Ca^{2+} channels are necessary to increase cytosolic Ca^{2+} in the penis [30,40].

Two genes were recently proposed to be involved in the relaxation of the smooth muscle, and may have important function in the penile erection induced by PnTx2-6 [25]. One of them, *ednrb*, was found to be over-expressed in mice injected with PnTx2-6 [25], and has been described as activated by endothelins [21], thus increasing NOS activity promoting NO production leading to an erection [30]. As PnTx2-6 has been involved in erection, we hypothesized that PnTx2-6 targets Na^+ channels slowing inactivation of the Na^+ current, leading to a prolonged depolarization and consequent increase in Ca^{2+} influx via N-type Ca^{2+} channels present in nitrergic nerve membranes. The increase in Ca^{2+} influx activates nNOS inducing NO production in the nerve endings, improving relaxation of vascular and cavernosal smooth muscle.

The s.c administration of radiolabeled (99m technetium) PnTx2-6 in rats demonstrated that this toxin is recruited to the penis. To our knowledge, no other toxin from the venom of *P. nigriventer* has been assayed by similar method, however, PnTx2-6 was labeled with iodine-125 and *in vivo* experiments demonstrated that uptake occurs primarily in the kidneys, suggesting renal excretion as a means for elimination. These data are in agreement with our results where we observed (99m technetium)-PnTx2-6 time-dependent increases in the kidney (data not show). Our results indicated a strong presence of labeled PnTx2-6 in the penis (Figure 5), suggesting the existence of binding sites for this toxin in the rat corpus cavernosum. Our study did not detect differences in the brain, as observed by Yonamine [27]. In addition, when compared with the penis (Figure 5), the amount of labeled toxin in this organ, was increased by a factor of 4. Based on these data, the role of PnTx2-6 seems to act locally, not centrally.

Improved cavernosal relaxation evoked by PnTx2-6 was found to be further increased in the presence of the PDE5 inhibitor, sildenafil (Figure 6B), a common therapeutic strategy to treat ED [52,53]. Considering that PnTx2-6 increases NO bioavailability in penile tissue thus enhancing erectile function, and increasing cGMP levels augments this response, we speculate that PnTx2-6 toxin regulates penile function independent of PDE5. PnTx2-6 a concentration equivalent to 25% of the relaxation response was used in a cumulative relaxation–response curve to sildenafil (Figure 6A). In the short incubation with sildenafil, no differences in PE contracted tonus were observed.

In summary, we speculate that PnTx2-6 facilitates NO released from nitrergic nerves activating sGS, thus increasing cGMP levels facilitating penile erection. We believe that binding sites for PnTx2-6 exist in the penis demonstrating local action. We also determined that the toxin's action is independent of muscarinic activation, or PDE5, however involves N-type Ca^{2+} channels. These data suggest a role for this toxin in current pharmacological therapies, in addition to using it for the development of alternative treatments for ED.

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Figure 1. PnTx2-6 (10^{-8} M) improves electrical field stimulation-induced relaxation in rat-isolated cavernosum strip. (A) Relaxation–response curve induced by a second set of stimulation 4 minutes after the initial set (in the absence of toxin to serve as time control) is not different from the control relaxation–response curve ($N = 6$). (B) The relaxation–response curve induced by electrical field stimulation (1–32 Hz) was significantly improved in the presence of PnTx2-6 ($N = 8$). * $P < 0.05$ (two-way analysis of variance followed by Bonferroni test).

Figure 2.

Atropine, a muscarinic receptor antagonist, did not change the PnTx2-6 potentiation effect on the electrical field stimulation (EFS)-induced relaxation in rat-isolated cavernosum strips. The relaxation–response curves induced by EFS (1–32 Hz) and potentiated by PnTx2-6 (10^{-8} M) was not altered in the presence of atropine (10^{-6} M, $N = 7$). $*P < 0.05$ (two-way analysis of variance followed by Bonferroni test).

Figure 3.

Relaxation induced by sodium nitroprusside in rat-isolated cavernosum strip was not affected by PnTx2-6 (A). Concentration-response curves to sodium nitroprusside (from 10^{-8} to 10^{-2} M) were performed in (10^{-5} M) phenylephrine-contracted cavernosal strips in the absence and presence of PnTx2-6 (10^{-8} M, N = 6). PnTx2-6 potentiation effect on the electrical field stimulation-induced relaxation is further increased by sodium nitroprusside (3×10^{-6} M, N = 6) (B). * $P < 0.05$ (two-way analysis of variance followed by Bonferroni test).

Figure 4.

Calcium channel blockers inhibit PnTx2-6 potentiation effect on the electrical field stimulation (EFS)-induced relaxation in rat-isolated cavernosum strips. The relaxation-response curves induced by EFS (1–32 Hz) were performed in (10^{-5} M) phenylephrine-contracted cavernosal strips in the presence and absence of PnTx2-6 (10^{-8} M), after previous treatment with ω -conotoxin GVIA (10^{-6} M, $N = 8$) a selective N-type calcium channel blocker.

Figure 5.

Radiolabeled PnTx2-6 was found to be significantly higher in the penis, as compared with other tissues. Brain, testicle, and penile tissue harvested from rats injected with labeled toxin (0.37 MBq) were tested. Increased presence of radiolabeled PnTx2-6 in the penile tissue suggested the presence of receptors sites for this toxin in the penis ($N = 5$). $*P < 0.05$ (two-way analysis of variance followed by Bonferroni test).

Figure 6.

Cumulative relaxation–response curve to sildenafil (A). PnTx2-6 potentiation effect on the electrical field stimulation (EFS)-induced relaxation in rat-isolated cavernosum strips is further increased by sildenafil (B). The relaxation–response curves induced by EFS (1–32 Hz) were performed in (10^{-5} M) phenylephrine-contracted cavernosal strips in the presence or absence of PnTx2-6 (10^{-8} M), 5 minutes after previous treatment with sildenafil (3×10^{-8} M), an inhibitor of phosphodiesterase type 5 ($N = 05$). $*P < 0.05$ vs. EFS + PnTx2-6 (two-way analysis of variance followed by Bonferroni test).