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Biochemical Pharmacology 63 (2002) 1113–1118

Biochemical Pharmacology

Characterization of the mechanism underlying stonustoxin-mediated relaxant response in the rat aorta *in vitro*

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Received 26 July 2001; accepted 21 December 2001

Abstract

Stonustoxin (SNTX) is a lethal factor isolated from the venom of the stonefish *Synanceja horrida*. Although SNTX exhibits a multitude of biological activities, the primary cause of death upon administration of the toxin is attributed to marked hypotension. We investigated the possible mechanisms underlying the vascular hyporeactivity of this novel toxin. Cumulative doses of SNTX (5–320 ng/mL) induced concentration-dependent relaxation in phenylephrine (PE)—precontracted rat aortic rings with intact endothelium. Endothelium removal abolished the relaxation induced by SNTX. Tetraethylammonium (TEA), an inhibitor of K^+ channels, partially inhibited SNTX-induced relaxation. Similarly, SNTX-induced relaxation was partially attenuated by the SP receptor antagonist (NATB), whereas the inducible iNOS inhibitor, AMT–HCl, completely abolished the relaxation caused by SNTX. From the results obtained, it can be postulated that a component of SNTX-mediated vasorelaxation is *via* binding of either SNTX or SP to the SP receptors that are located on the endothelial cells. Occupation of these SP receptors causes subsequent production of NO and activation of K^+ channels, thus leading to vasorelaxation of the rat aortic rings.

Keywords: Stonustoxin; K^+ channel; Nitric oxide; Substance P receptor; Hypotension

1. Introduction

Venom isolated from the stonefish has been documented to cause marked hypotension and respiratory difficulties in envenomed animals; and in severe cases, have caused death and poisoning to humans [1]. The clinical manifestation of envenoming includes excruciating pain, edema induction, respiratory difficulties, paralysis and cardiovascular collapse [2].

SNTX is a lethal protein of molecular mass 149 kDa isolated from the venom of *Synanceja horrida* [3]. In previous studies, anesthetized rats in our laboratory have shown that the primary cause of death upon administration of SNTX may be attributed to marked hypotension [4]. The endothelium-dependent relaxation in response to SNTX is associated with the formation of cyclic guanosine monophosphate (cGMP), and these effects were abolished by L-NAME and methylene blue [4]. Administration of SNTX decreases blood pressure in experimental animals, and SNTX dose-dependently relaxes isolated rat aortic rings precontracted with PE.

The aim of this study was to characterize the mode of action of SNTX in isolated rat aortic rings, and the possible mechanisms underlying the vascular hyporeactivity of this novel toxin. The influence of several pharmacological agents—TEA; the iNOS inhibitor, AMT–HCl; and the SP receptor antagonist, NATB on the SNTX-induced vasorelaxation of isolated rat aorta were examined. To study the potential role of potassium (K^+) channel activity in SNTX-induced vasodilation, we tested the hemodynamic effects of the non-selective potassium channel inhibitor, TEA, on rat aortic rings. K^+ channels have been reported to be important in the regulation of arterial tone [5,6]. Both an intact endothelium and membrane potential of smooth muscle are important in the regulation of vascular tone [6–8], but a possible role of factors derived from the endothelium and K^+ channel activation in SNTX-induced relaxation in arteries has yet to be explored.

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Abbreviations: SNTX, stonustoxin; PE, phenylephrine; TEA, tetraethylammonium; SP, substance P; NATB, *N*-acetyl-L-tryptophan-3,5-bis(trifluoromethyl)-benzyl ester; L-NAME, L-*N*-*G*-nitro arginine methyl ester; AMT–HCl, 2-amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine; NOS, nitric oxide synthase.

0006-2952/02/$ – see front matter
PII: S0006-2952(02)00857-2

In the present work, we also investigated the effects of a SP receptor antagonist in the hypotensive response induced by the injection of SNTX in rats. Initial experiments were conducted to determine whether SNTX induces the relaxation of the rat aorta by a mechanism partially dependent on the local release of SP acting on the SP receptor.

2. Materials and methods

2.1. Preliminary studies in the rat aortic ring

The concentration–response of the endothelium-intact and -denuded aortic ring to SNTX in male Sprague–Dawley rats was determined. In PE (7.5×10^{-3} M)-precontracted rat aortic rings with intact endothelium, acetylcholine (ACh) (2 μM) produced nearly complete relaxation ($82 \pm 5\%$, $N = 5$). For endothelium-denuded aortic rings, ACh failed to exhibit any relaxant activity of PE-precontracted rings.

2.2. Tissue preparation

Male Sprague–Dawley rats weighing 200–250 g were killed by cervical dislocation. The thoracic aorta was dissected out, cleaned of surrounding tissue, and cut into rings of approximately 4 mm in length. For experiments that required the presence of intact endothelium, special care was taken, so as not to damage the endothelium. The preparation was then transferred to a 2.5 mL organ bath filled with Kreb's solution (in mM): 118 NaCl, 4.8 KCl, 1.2 $CaCl_2$, 2.4 $MgSO_4$, 25 $NaHCO_3$, 1.2 KH_2PO_4 and 11 D-(+)-glucose 11. The rings were then suspended in organ chambers, and a force–displacement transducer (Ugo Basile model 7006) was used to measure isometric force. The organ chamber was filled with 2.5 mL of Kreb's solution and continuously gassed with 95% O_2 and 5% CO_2. All experiments were performed at $37 \pm 1°$, and the basal tone was adjusted to a tension of 1.5–2.0 g.

2.3. Experimental protocol

Aortic rings were precontracted submaximally with 7.5×10^{-5} M PE. To test the integrity of the endothelium, ACh (2×10^{-6} M) was added to the bath after the PE-induced contraction reached a plateau. Tissues exhibiting less than 70% relaxation to ACh were not used. For the SNTX dose–response curves, PE (7.52×10^{-5} M) was added to the bath, and once the contractile response reached a stable plateau, concentration–response curves to SNTX were constructed in a cumulative manner. In a separate series of experiments, TEA (3×10^{-3} M), AMT–HCl (5×10^{-4} M) or NATB (5×10^{-4} M) were added 30 min before the initial addition of PE and cumulative dose–response relaxation curves of SNTX were recorded. Drug-induced relaxation was expressed as a percentage of the PE-induced increase in tension.

2.4. Data collection and statistical analysis

Data are collected and analyzed using a MacLab/400^{TM}. Values are expressed as percentages (mean ± SEM) of the contraction induced by PE, and N referred to the number of experiments. The statistical significance of the differences between the mean values is evaluated by unpaired Student's t-test. P-values that are less than 0.05 were considered statistically significant.

3. Results

3.1. Relaxant response to SNTX

Progressive addition of SNTX produced a sustained, dose-dependent relaxation of rat isolated aortic rings that were precontracted with PE, and a maximum relaxation of $72.7 \pm 6\%$ was obtained, with an IC_{50} value of 132.7 ng/mL for the toxin (Fig. 1). The response to SNTX was abolished by mechanical removal of the endothelium. Representative traces in Fig. 2 show that cumulative addition of SNTX reduced the PE-evoked contraction in a concentration-dependent manner.

3.2. Effect of K^+ channel blocker (TEA) and iNOS inhibitor, AMT–HCl on SNTX-mediated vasorelaxant action

Since SNTX caused endothelium-dependent relaxation in the rat aorta, we examined the possible involvement of endothelium-derived factors in the vascular response to

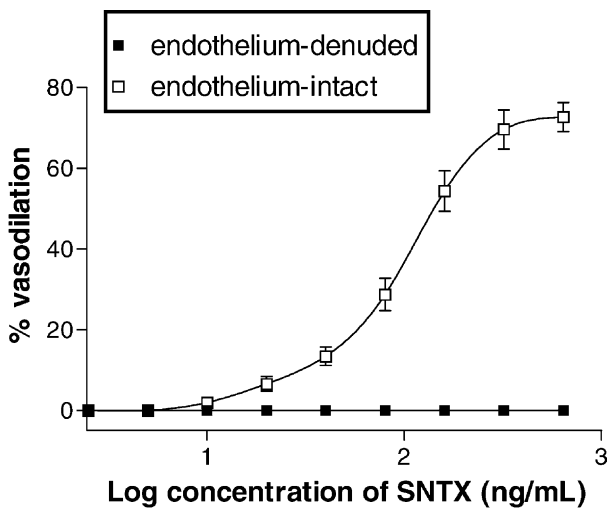

Fig. 1. Relaxation induced by SNTX in precontracted rat aortic rings with and without the presence of endothelium. Concentration–response curves to SNTX in endothelium-denuded ($N = 4$) (■), and endothelium-intact ($N = 6$) (□); rat aortic rings. Results are mean ± SEM of N experiments.

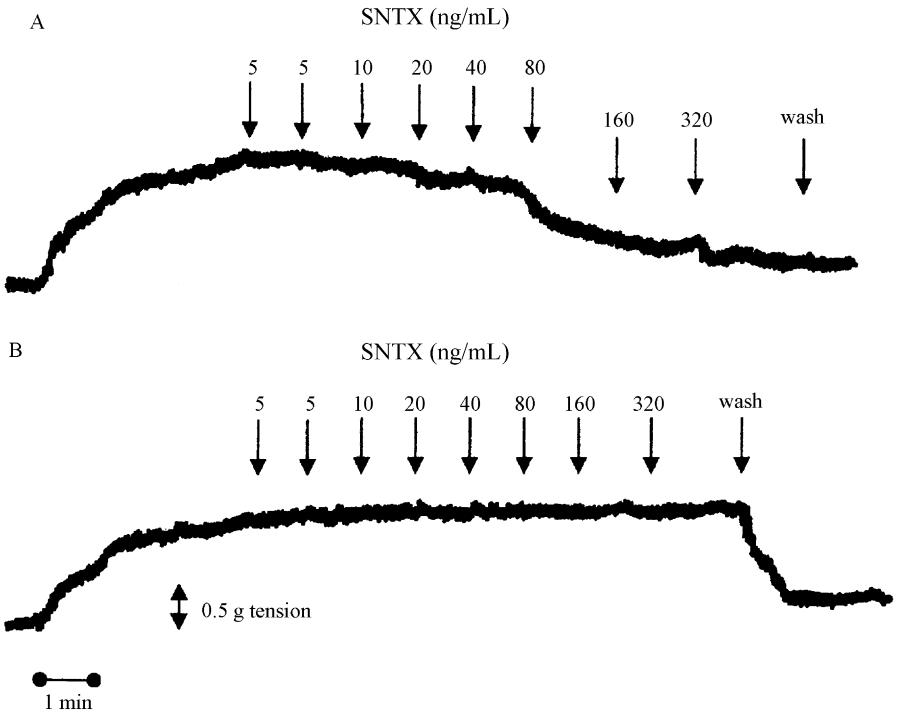

Fig. 2. The effect of SNTX on precontracted rat aortic rings. SNTX caused a dose-dependent relaxation in intact aortic rings (curve A). In aortic rings denuded of endothelium, the relaxation response was abolished (curve B).

SNTX. Cumulative applications of SNTX-induced endothelium-dependent relaxation of PE-precontracted rat aorta rings. SNTX-induced relaxation response was significantly ($P < 0.05$) reduced in the presence of TEA (from 72.7 ± 6 to $25.4 \pm 8\%$) as compared to control rat aortic rings (Fig. 3). Similarly, the vasodilatory effect of SNTX was blocked when iNOS was inhibited with AMT–HCl, a specific inhibitor of iNOS (Fig. 3).

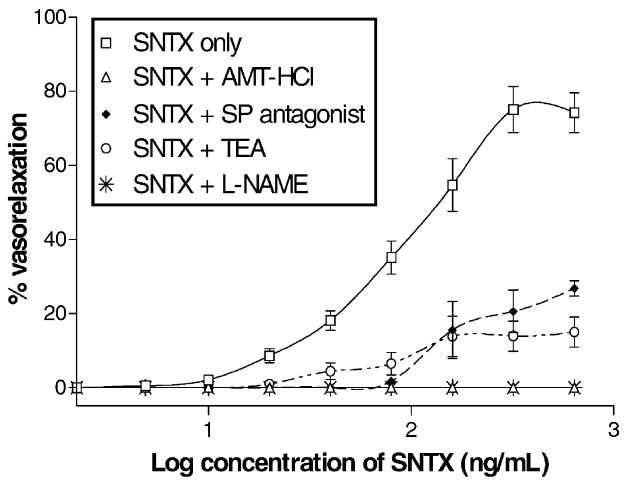

Fig. 3. Relaxation induced by SNTX in PE-precontracted rat aortic rings: in presence of the K^+ channel inhibitor, TEA ($N = 5$) (○); in presence of the specific iNOS inhibitor, AMT–HCl ($N = 4$) (△); in presence of the SP receptor antagonist, *N*-acetyl-L-tryptophan-3,5-bis(trifluromethyl)-benzyl ester (5×10^{-4} M) ($N = 5$) (◆); in presence of L-NAME ($N = 4$) (✳); and in absence of either TEA, AMT–HCl, L-NAME or the SP receptor antagonist (□). Results are mean ± SEM of *N* experiments.

3.3. Effect of SP receptor antagonist, NATB on SNTX-mediated vasorelaxant action

It is possible that SNTX induces vasorelaxation of rat aortic rings by a mechanism partially dependent on the release of neuropeptides, possibly SP that acts on the tachykinin NK_1 receptor. In order to ascertain the effects of SP receptors on the vasorelaxant effect of SNTX, we tested the ability of a SP receptor antagonist (NATB) to inhibit SNTX-induced relaxation of the rat aorta. Fig. 4 shows that the SP receptor antagonist was able to block the SP-induced vasorelaxation. The relaxant effect of SNTX was also partially blocked by NATB at a concentration

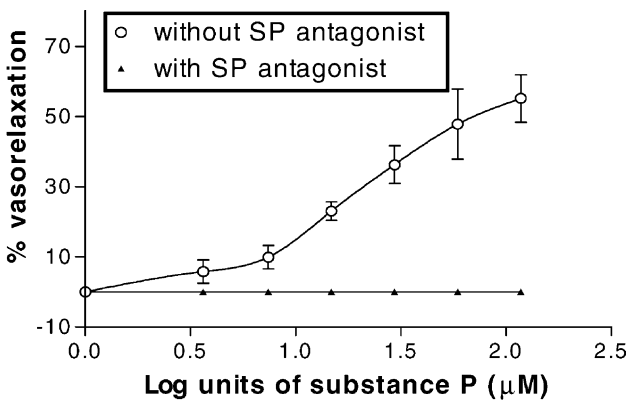

Fig. 4. Vasorelaxant response of SP in PE-precontracted aortic rings with ($N = 4$) (▲); and without ($N = 4$) (○) the SP receptor antagonist, *N*-acetyl-L-tryptophan-3,5-bis(trifluromethyl)-benzyl ester (5×10^{-4} M). Results are mean ± SEM of *N* experiments.

that abolished the responses to SP (5×10^{-4} M) ($N = 5$, Fig. 3).

4. Discussion

SNTX produces a cumulative dose-dependent relaxation in precontracted rat aortic rings. The mechanism by which SNTX produces this vasodilation is not completely understood, although our laboratory has previously reported that inhibition of NO production and preventing NO interaction with guanylate cyclase prevented vasodilation in rat aortic strips [4]. In an attempt to better elucidate the possible mechanisms underlying SNTX-induced vasorelaxation, we tested selective pharmacological agents on isolated aortic rings.

Relaxation caused by SNTX was inhibited by mechanical removal of the endothelium, thus supporting a role of the endothelium in the SNTX-mediated vasorelaxation as previously reported in rat aortic strips [4]. SNTX-induced vasorelaxation was reduced when rat aortic rings were treated with the non-selective K^+ channel inhibitor, TEA. Several physiological stimuli such as NO [9,10] or endothelium-derived hyperpolarization factor (EDHF) [11–13] can modulate K^+ channel activity. At the concentration used in this study (3×10^{-3} M), TEA is most likely blocking Ca^{2+}-dependent K^+ channels (K_{Ca}) [14]. By blocking K^+ channels, TEA inhibits voltage-dependent Ca^{2+} influx brought about by depolarization of vascular smooth muscle cells [14]. K^+ channel blockers have been shown to alleviate septic shock in animal models, which could be attributed to their ability to prevent the induction of iNOS either directly [15], or indirectly through inhibition of the release of tumor necrosis factor [16]. Since TEA reduces the relaxant effect of SNTX, it is likely that abnormal activation of K^+ channels underlies SNTX-induced relaxation of the rat aorta.

Involvement of iNOS in the vascular hyporeactivity in isolated rat aortic rings was assessed using specific iNOS inhibitor. iNOS is a Ca^{2+}-independent isoform, and its activation leads to NO overproduction, and inappropriate vasodilation by activation of the enzyme guanylate cyclase [17,18]. Although iNOS is not normally present in untreated cells, its expression can be induced in almost any cell-type by bacterial lipopolysaccharide (LPS), cytokines, and other agents. There are reports of constitutive iNOS expression in certain cells [19–23]. In this study, both L-NAME (3×10^{-3} M) and the specific iNOS inhibitor AMT–HCl (5×10^{-4} M) also inhibited the vasorelaxant effect of SP in rat aortic rings at the same concentrations that blocked the relaxant response to SNTX (results not shown).

In view of the abrogation of the relaxant effect of SNTX in the presence of the iNOS inhibitor, it is interesting to note that SNTX shares many similar biological and pharmacological properties with several cytokines, notably tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interferon-γ (IFN-γ). This suggests the possibility that SNTX might act by mimicking these immune modulators. Cytokines are typically associated with edema, erythema and hypotension, and are involved in the inflammatory response *via* activation and suppression of multiple genes [24]. One important gene that is induced by these cytokines is that of inducible NOS II found in vascular endothelial cells [25]. Attenuation of the relaxant response of SNTX by the specific iNOS inhibitor in PE-precontracted rat aortic rings suggests that SNTX may act as a 'functional cytokine' to protect stonefish from would-be predators. There have been previous reports of other animals utilizing their own nervous systems to generate substances that confer protective capability against predators, and many of these components have been shown to have mammalian homologues. For example, the enzyme phospholipase A_2 present in the venom of snakes is also found in mammals, and likewise, endothelins is the mammalian homologue of sarafatoxins [26]. Certain frogs also produce toxic peptides that are discharged from their skins for protection against predators [27]. It has been suggested that nitric oxide produced by vascular expression of iNOS activates calcium-dependent potassium channels [28]. As NO activates K_{Ca} channels in smooth muscle and TEA reduces the effect of SNTX, we suggest that SNTX stimulates iNOS which, in turn, activates K_{Ca} channels to cause muscle relaxation.

Agonists such as SP can increase NO production through interactions with receptors on the endothelial cells, following which, the endothelium generates nitric oxide to relax the underlying smooth muscle [29]. In epicardial coronary arteries, SP causes relaxation that is mediated largely by nitric oxide [29] and in LPS-activated macrophages, SP stimulates NO production in a time- and concentration-dependent manner by augmenting the induction of iNOS expression [30]. SNTX-induced relaxation was partially inhibited by SP receptor antagonist, indicating that SNTX either binds directly to SP receptors, or indirectly activates SP receptors through a component that is capable of stimulating the release of endogenous SP with subsequent activity at the SP receptors. In our study, when the isolated rat aortic rings were incubated with either L-NAME or the iNOS inhibitor, AMT–HCl, the relaxant effect of SP was totally inhibited (results not shown), suggesting that SP acts indeed by stimulating NO production.

The present study confirmed that SNTX induces endothelium-dependent vasorelaxation of the rat aorta. We have found that the vasodilatory effect of SNTX is individually reduced by TEA and SP, respectively, and blocked by AMT–HCl. The ability of the SP receptor antagonist to partially block the relaxant activity of SNTX suggests the involvement of the SP receptors in the vasodilatory effect of SNTX in the rat aortic rings. Following administration of SNTX, occupation of the SP receptors possibly leads to an increase in calcium concentration, thereby stimulating endothelial NO biosynthesis. Although

AMT–HCl is a specific iNOS inhibitor, it may be that at the concentration used in our study, AMT–HCl may similarly be active against the non-inducible NOS, such as endothelial NOS (eNOS) that is commonly found in endothelial cells. By inhibiting NOS, abrogation of NO production prevents the increase in intracellular concentration of cGMP. cGMP has been reported to cause vasodilation by several mechanisms, one of which involves the activation of calcium-sensitive potassium channels by cGMP-dependent protein kinase that results in subsequent membrane hyperpolarization [9]. In this respect, it is interesting to note that previous studies done in our laboratory showed that inhibition of cGMP blocks SNTX-induced vasorelaxation [4].

As both L-NAME and AMT–HCl inhibit the SP-induced relaxation, therefore, it is likely that in the rat aortic rings, SP acts by stimulating NO production. The SP antagonist was found to partially inhibit the SNTX vasorelaxing action, thus suggesting that a component of SNTX-mediated vasorelaxation is mediated either through direct binding of SNTX to the SP receptors, or *via* the release of SP from the SP-containing terminals in the walls of aorta.

From the results obtained, it can be postulated that in the rat aortic rings, SNTX increases NO production and mediates its vasorelaxant effect either through interaction with the SP receptors, or alternatively causes the release of SP that subsequently binds to the SP receptors. Following NO production, increase in the levels of cGMP in the smooth muscle cells subsequently activates K^+ channels, thereby, causing muscle membrane hyperpolarization and vasorelaxation, as summarized in Fig. 5.

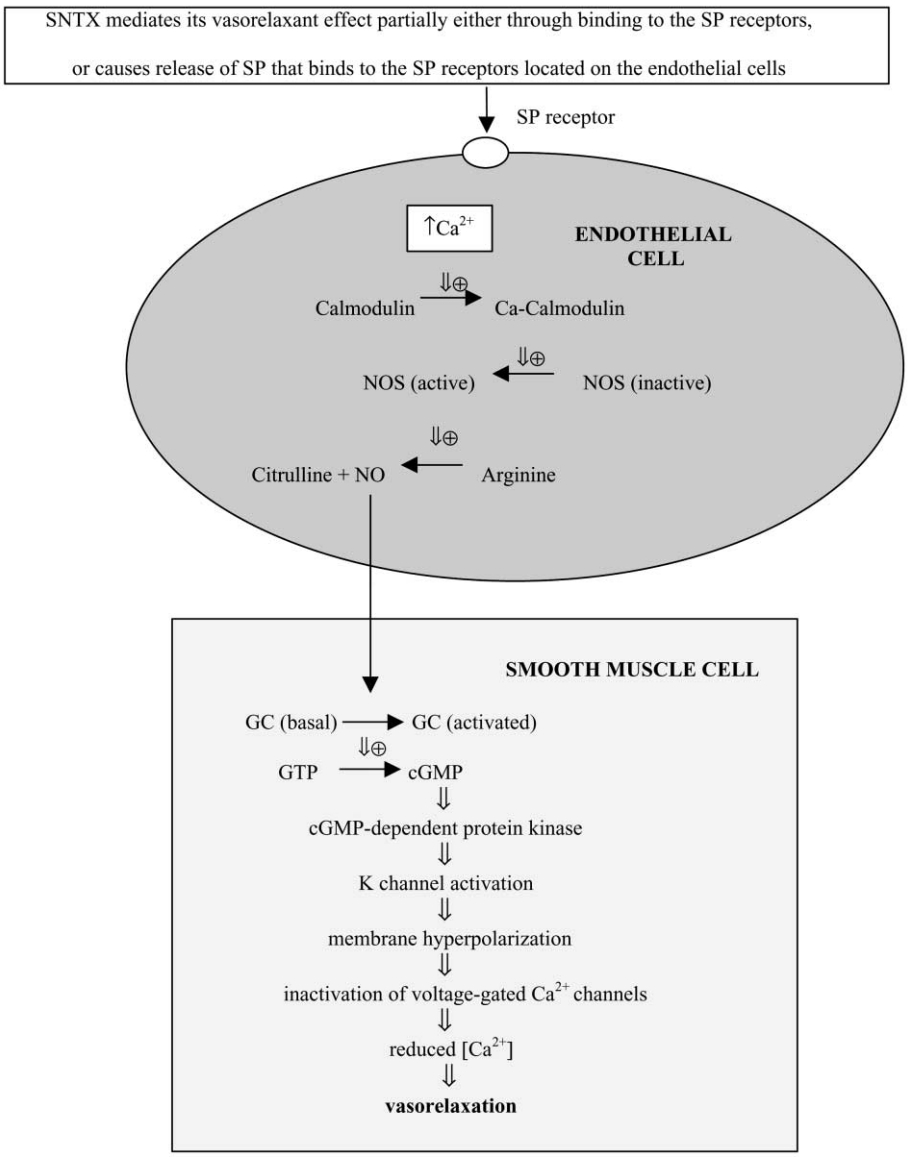

Fig. 5. Postulated components of SNTX-mediated vasorelaxant mechanism in the endothelium-intact rat aortic ring.

Acknowledgments

The authors thank the National University of Singapore for the research scholarship awarded to J.M.L. Sung. This project was supported by a National University of Singapore Grant RP3982340.

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