Br. J. Pharmacol. (1988), **93**, 275–280

The effects of neosurugatoxin on evoked catecholamine secretion from bovine adrenal chromaffin cells

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1 The effect of neosurugatoxin (NSTX), a toxin from the Japanese ivory mollusc (*Babylonia japonica*), on the nicotinic response of bovine adrenal chromaffin cells was examined.

2 NSTX inhibited acetylcholine- and nicotine-induced catecholamine secretion from the cultured cells with an IC_{50} against 5 μM nicotine of 30 nM.

3 This inhibitory effect was reversible and independent of the presence of agonist.

4 NSTX had no effect on the catecholamine release from cultured cells evoked by 50 mM K^+, or 1 μM histamine.

5 NSTX had no effect on the stimulation of phosphatidylinositol metabolism evoked by 100 μM muscarine.

6 These results suggest NSTX may be useful as a nicotinic receptor probe in tissues such as the adrenal and sympathetic ganglia where α-bungarotoxin is ineffective.

Introduction

In studies of receptor-ionophore function in adrenal chromaffin cells, there is currently a need for a better probe for the nicotinic receptor. Hexamethonium is a weak, non-competitive inhibitor of the nicotinic response (Kilpatrick *et al.*, 1981), while α-bungarotoxin binds with high affinity to the chromaffin cells but does not inhibit the nicotinic response (Kumakura *et al.*, 1980; Quik & Trifaro, 1982).

Neosurugatoxin (NSTX) is a novel marine toxin recently isolated from the Japanese ivory mollusc, *Babylonia japonica* (Kosuge *et al.*, 1981). The structure of NSTX is shown in Figure 1. The earliest studies with this toxin demonstrated the selectivity of NSTX for ganglionic nicotinic receptors over muscarinic receptors on smooth muscle. NSTX inhibited the contractile response of rat and guinea-pig ileum and cat nictitating membrane by acting at the ganglionic nicotinic receptors that facilitate postganglionic transmitter release. The target tissue responses to exogenous ACh (which occur via muscarinic receptors on the muscle surface) were unaffected by NSTX (Hirayama *et al.*, 1974; Hayashi & Yamada 1975; Hayashi *et al.*, 1984).

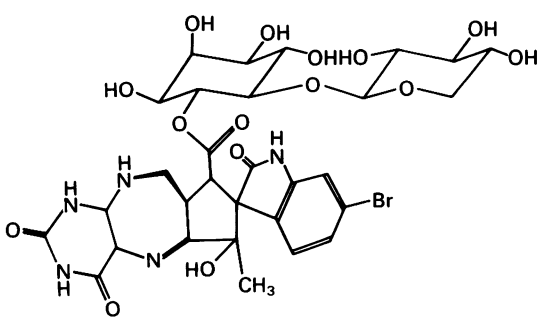

Figure 1 The molecular structure of neosurugatoxin (NSTX).

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The high affinity of the toxin for ganglionic nicotinic receptors over ganglionic muscarinic receptors has been demonstrated in the isolated superior cervical ganglion of the rat. Extracts of the Japanese ivory mollusc were shown to depress reversibly orthodromic transmission and antagonize the depolarizing action of carbachol on nicotinic receptors, while failing to reduce the depolarizing effects of muscarine (Brown *et al.*, 1976). The selectivity of NSTX for central nicotinic receptors has also been demonstrated. NSTX is a potent antagonist of [^{3}H]-nicotine binding to rat forebrain membranes but does not affect the binding of [^{3}H]-quinuclidinyl benzilate (QNB) to muscarinic receptors (Hayashi *et al.*, 1984; Yamada *et al.*, 1985; Rapier *et al.*, 1985).

In the present study, the action of NSTX on nicotine-induced catecholamine secretion and muscarine-induced phosphatidyl inositol (PI) metabolism

in bovine adrenal chromaffin cells has been investigated. Chromaffin cells are derived embryologically from the neural crest and are thus homologous with postganglionic neurones of the autonomic nervous system. The use of bovine chromaffin cells is convenient to demonstrate the specificity of NSTX for nicotinic receptors since secretion of catecholamines from these cells has been shown to be mediated by nicotinic but not muscarinic acetylcholine receptors (Wilson & Kirshner, 1977; Livett *et al.*, 1979; Mizobe *et al.*, 1979; Derome *et al.*, 1981; Livett & Boksa, 1984).

Methods

Adrenal chromaffin cells were isolated from adult bovine adrenal glands by collagenase digestion. Primary monolayer cultures of these cells were prepared following purification by Percoll density gradient centrifugation. The culture medium used comprised a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F-12 supplemented with 10% foetal calf serum, antibiotics and antimitotics. The cells were plated at a density of $2.5-4 \times 10^5$ cells per well into 24-well plastic culture dishes precoated with rat tail collagen. The cells were cultured in a humid atmosphere of 5% CO_2 in air at 37°C for three days to allow attachment of the cells (for full details, see Livett *et al.*, 1987a).

In the standard protocol to test the ability of NSTX to modify nicotine- or acetylcholine (ACh)-induced catecholamine secretion, culture dishes were removed from the incubator and equilibrated to room temperature (20–22°C) for 5 min. Each well then received 4 successive 5 min incubations with buffer of the following composition (mM): NaCl 154, KCl 2.6, K_2HPO_4 2.15, KH_2PO_4 0.85, $MgSO_4$ 1.18, D-glucose 10, $CaCl_2$ 2.2, BSA 0.5%, pH 7.4. The first two incubations were washes, the third a preincubation with the desired concentration of NSTX (or buffer for controls), and finally stimulation with 5 μM nicotine or 50 μM ACh in the presence (or absence) of NSTX.

The ability of NSTX to modify K^+- and histamine-evoked catecholamine release was tested to determine the specificity of NSTX for the nicotinic response. In these experiments, buffer with an additional 50 mM KCl (substituting for NaCl) or 1 μM histamine was used to stimulate the cells in place of nicotine. The stimulation period used for histamine-evoked secretion was 20 min.

For the study on the reversibility of inhibition of nicotine-mediated secretion by NSTX, the preincubation with NSTX was followed by washes with buffer for different lengths of time, with regular changes of the buffer. The cells were then stimulated with 5 μM nicotine for 5 min in the absence of NSTX.

At the end of the stimulation period, the incubation buffer was collected and mixed with perchloric acid (PCA, final concentration 0.4 M). The cells were extracted with 0.01 M PCA, and acidified to the same final PCA concentration. The protein precipitates were removed by centrifugation, and the incubation buffer and cell extracts assayed for adrenaline (Ad) and noradrenaline (NA) by separation with h.p.l.c. and quantitation with electrochemical oxidation, using dihydroxybenzylamine as an internal standard (Livett *et al.*, 1987b).

The effects of NSTX on phosphatidyl inositol (PI) metabolism were studied on cultured cells plated at a density of $1.2-2 \times 10^6$ cells per well in 6-well plastic culture dishes precoated with collagen. Culture medium was removed after 24 h and replaced with medium containing [^{3}H]-inositol (5–10 μCi ml^{-1}). The medium used for [^{3}H]-inositol loading was M199 supplemented with 2% foetal calf serum, antibiotics and antimitotics. The cells were returned to the incubator for 48–72 h to allow incorporation of the label.

In the standard protocol to test the ability of NSTX to modify muscarine-induced PI metabolism, the dishes were removed from the incubator and the loading medium removed. Each well then received two consecutive 5 min washes at 37°C with incubation buffer of the following composition (mM): NaCl 149, KCl 2.6, K_2HPO_4 2.15, KH_2PO_4 0.85, $MgSO_4$ 1.18, LiCl 5, glucose 10, pH 7.4. PI metabolism was then stimulated for 30 min at 37°C by 100 μM muscarine in the same incubation buffer. The effects of NSTX and atropine were determined by including the drug in both the second wash and the stimulation period. The incubation was terminated by replacing the buffer with 1 ml ice cold 15% trichloroacetic acid (TCA) and extracting the cells for 1 h. This TCA was collected into glass tubes, and the cells were rinsed with a further 0.5 ml of TCA which was collected after 5 min.

The extraction and analysis of inositol phosphates was performed using a modified procedure of Berridge *et al.* (1983). TCA was removed from the cell extracts with 3 ether washes, the aqueous phase neutralized and applied to a Bio-Rad AG1-X8 anion exchange column. [^{3}H]-inositol and [^{3}H]-glycerophosphoinositol were removed from the columns by eluting with 10 ml H_2O and 10 ml 60 mM sodium formate respectively. Total [^{3}H]-inositol phosphates were eluted with 2×3 ml 1.0 M ammonium formate in 0.1 M formic acid. These were collected and the radioactive content determined by gel-phase liquid scintillation counting (18–25% efficiency with aqueous counting scintillant (ACS)).

Drugs

Neosurugatoxin (NSTX) was a gift from Professor T. Kosuge, Shizuoka College of Pharmacy, Oshika,

Shizuoka 422, Japan. Percoll was obtained from Pharmacia PL Laboratories. Tissue culture media and antibiotics were obtained from Gibco Laboratories. Myo-[2-^{3}H]-inositol (1 mCi ml^{-1}) and ACS scintillation fluid were obtained from Amersham. The anion exchange resin used was Bio-Rad AG1-X8, 100–200 mesh, formate form. All other drugs and chemicals including collagenase, DNase and antimitotics were from Sigma Chemical Company.

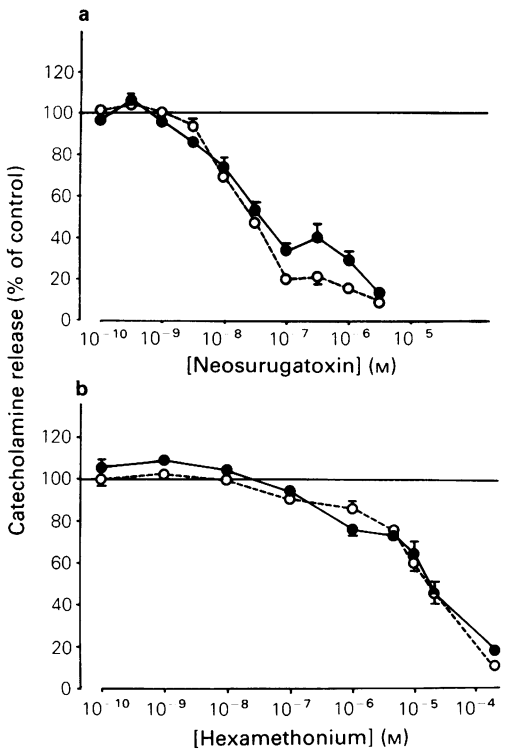

Figure 2 The effects of neosurugatoxin (0.1 nM–3 μM, a) and hexamethonium (0.1 nM–100 μM, b) on 5 μM nicotine-induced release of endogenous noradrenaline (○) and adrenaline (●) from cultured bovine adrenal chromaffin cells. Catecholamine release induced by 5 μM nicotine in the absence of inhibitor amounted typically to 12–20% cell content per 5 min for noradrenaline and 4–10% cell content per 5 min for adrenaline with the 10 cell preparations used for these data. Nicotine-induced catecholamine release in the presence of inhibitor is plotted as a % of the relevant control values. Results are mean for $n = 11–20$ for neosurugatoxin and $n = 6–12$ for hexamethonium; vertical lines show s.e.mean.

Results

At concentrations above 3 nM, NSTX produced a dose-dependent inhibition of 5 μM nicotine-evoked release of endogenous adrenaline (Ad) and noradrenaline (NA) (Figure 2a). The IC_{50} for inhibition of release of catecholamines from the cultured chromaffin cells was approximately 30 nM. Below this concentration, NSTX inhibited Ad and NA release equally. However, at concentrations higher than the IC_{50}, NSTX was a more potent inhibitor of NA release than Ad release. NSTX was 3 orders of magnitude more potent as a nicotinic antagonist on chromaffin cells than hexamethonium, which had an IC_{50} of approximately 10 μM against nicotine-evoked catecholamine release from chromaffin cells (Figure 2b).

The effect of NSTX on catecholamine release evoked by stimuli other than nicotine was also studied (Figure 3). NSTX (30 nM) inhibited 50 μM ACh-evoked NA and Ad release by 60% and 70% respectively. However, NSTX had no effect on NA or Ad release induced by 50 mM K^+ over a 5 min stimulation period, or the catecholamine release induced by 1 μM histamine over a 20 min incubation period.

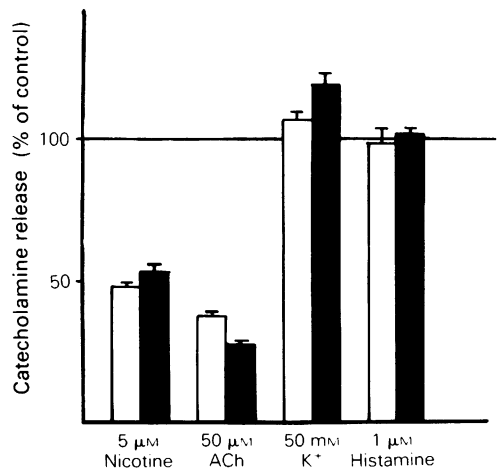

Figure 3 Effect of 30 nM neosurugatoxin on evoked release of endogenous noradrenaline (NA) (open columns) and adrenaline (Ad) (solid columns) from cultured bovine adrenal chromaffin cells. Test wells were preincubated for 5 min with toxin before stimulation with agonist. Catecholamine release induced by agonist in the absence of toxin amounted typically to (% cell content released per 5 min): 12–20% NA and 4–10% Ad for 5 μM nicotine, 13–15% NA and 4–6% Ad for 50 μM ACh, 12–15% NA and 3–5% Ad for 50 mM K^+ and (% cell content released per 20 min) 4–5% NA and 3–4% Ad for 1 μM histamine.

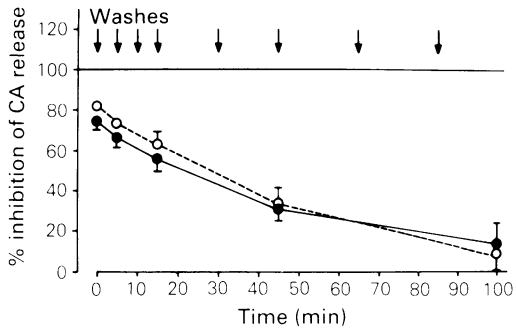

Figure 4 Assessment of the reversibility of inhibition of the release of endogenous noradrenaline (○) and adrenaline (●) release by 0.1 μM neosurugatoxin from cultured bovine adrenal chromaffin cells. The cells were preincubated with 0.1 μM neosurugatoxin for 5 min prior to different length wash regimes; the changes in incubation medium are indicated by arrows. The cells were then stimulated for 5 min with 5 μM nicotine in the absence of toxin. Nicotine-induced catecholamine release following preincubation with toxin is expressed as % inhibition of catecholamine release evoked by 5 μM nicotine over a 5 min stimulation period. Results are mean for $n = 6$ determinations; s.e.mean shown by vertical lines.

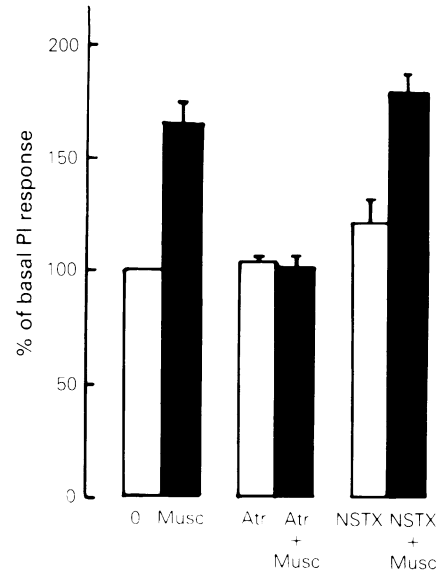

Figure 5 The effect of neosurugatoxin (300 nM) and atropine (1 μM) on metabolism of phosphatidyl inositol in cultured bovine adrenal chromaffin cells. The cells were loaded with [^{3}H]-inositol-containing medium (5–10 μCi ml^{-1}) for 48–72 h to allow incorporation of the label. Test wells were preincubated for 5 min with drug prior to stimulation for 30 min with 100 μM muscarine in the presence of drug. Results are expressed as a % of the PI response induced by a 30 min incubation with buffer alone. Results are mean for $n = 6$–10 determinations: s.e.mean shown by vertical lines. Three cell preparations were used to obtain these data.

Inhibition of the nicotinic response by NSTX was shown to be reversible, since the degree of inhibition was inversely related to the duration of the wash between the preincubation with the toxin and the final nicotinic stimulation in the absence of NSTX (Figure 4). The inhibition of catecholamine release by 0.1 μM NSTX fell from 75% inhibition when preincubation with NSTX was followed immediately by nicotinic stimulation, to 15% inhibition when the duration of the wash between pre-incubation with NSTX and the final stimulation was 100 min.

The effect of NSTX on stimulation of PI metabolism by 100 μM muscarine was studied (Figure 5). PI turnover was increased by 65% over basal when the cells were stimulated with 100 μM muscarine. NSTX (300 nM) had no effect on this muscarinic stimulation of PI metabolism, and only slightly enhanced basal PI turnover. By comparison, 1 μM atropine completely abolished the muscarinic activation of PI turnover but had no effect on basal PI metabolism.

Discussion

The present study demonstrates the high potency of NSTX for inhibiting the nicotinic response in chromaffin cells. Previous studies have shown that extracts of the Japanese ivory mollusc (*Babylonia japonica*) containing NSTX are potent antagonists of the nicotinic receptor response in sympathetic and parasympathetic ganglia but not at the neuromuscular junction (Hayashi & Yamada, 1975; Brown *et al.*, 1976; Ascher *et al.*, 1979). More recently, NSTX has been shown to antagonize nicotinic receptor function in the brain (Hayashi *et al.*, 1984; Yamada *et al.*, 1985; Rapier *et al.*, 1985), supporting the view that many central nicotinic receptors may be ganglionic in character (Schmidt *et al.*, 1980, Clarke *et al.*, 1985). It has been shown also that [^{3}H]-nicotine selectively labels nicotinic cholinoceptors in rat brain, and that neosurugatoxin is a potent noncompetitive antagonist of these receptors (Yamada *et al.*, 1985).

In our experience, NSTX is the most potent antagonist of nicotine-evoked release of catecholamines (IC_{50} 30 nM) in bovine chromaffin cells. The specificity of NSTX for the nicotinic receptor-ionophore complex was tested by studying its effects on catecholamine release evoked by K^+, which activates voltage-dependent Ca^{2+} channels directly by depolarizing the chromaffin cell membrane, to bring

about exocytosis (Douglas, 1975) and histamine, which evokes release via H_1-receptors (Livett & Marley, 1986). NSTX had no effect on either K^+-induced or histamine-induced catecholamine release, demonstrating the high specificity of the toxin for the nicotinic response.

Furthermore, the failure of NSTX to inhibit the enhancement of PI metabolism induced by muscarine demonstrates its selectivity for chromaffin cell nicotinic acetylcholine receptors over chromaffin cell muscarinic receptors.

In studies to determine whether inhibition of the nicotinic response by NSTX was reversible, it was shown that NSTX could exert its effects in the absence of nicotinic agonist, since inhibition of the nicotinic response occurred when preincubation with the toxin was followed by stimulation in the absence of NSTX. This suggests that NSTX is not a metaphilic antagonist of the nicotinic response. In addition, following preincubation with NSTX the inhibition of catecholamine release was almost fully reversed over a 100 min incubation in the absence of NSTX prior to nicotinic stimulation.

The nicotinic and muscarinic cholinoceptors on bovine adrenal chromaffin cells are known to be pharmacologically distinct from their muscle and ganglionic counterparts. Chromaffin cell nicotinic receptors are insensitive to blockade by α-bungarotoxin (Kumakura *et al.*, 1980; Quik & Trifaro, 1982), but are blocked by P-286, a drug that does not block sympathetic ganglia (Gardier *et al.*, 1960). Canine chromaffin cell muscarinic receptors have much lower affinities for muscarinic antagonists than smooth muscle muscarinic receptors (Henderson & Ungar, 1977). Bovine chromaffin cells are not stimulated by McN-A-343 or pilocarpine (Derome *et al.*, 1981) which are drugs active in autonomic ganglia (Bowman & Rand, 1984). The present study shows that NSTX is a selective antagonist of adrenal chromaffin cell nicotinic receptors, having no action on adrenal chromaffin cell muscarinic receptors. Due to its high potency, reversibility and selectivity, NSTX should be particularly useful for studies of nicotinic receptor function in the adrenal medulla, sympathetic ganglia and brain.

We thank Professor Kosuge for his generous gift of neosurugatoxin, and Mr Ken Mitchelhill for his excellent technical assistance. P.D.M. is a Queen Elizabeth II Fellow. The work was supported by a project grant to B.G.L.

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(Received May 12, 1987.
Accepted June 19, 1987.)