The effect of neosurugatoxin on the release of neurohypophysial hormones by nicotine, hypotension and an osmotic stimulus in the rat

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1 Experiments were carried out to test whether neosurugatoxin (NSTX) which blocks autonomic ganglia also acts centrally, like hexamethonium, on nicotinic cholinceptors involved in the neural control of release of vasopressin and oxytocin from the neurohypophysis.

2 In the water-loaded rat under ethanol anaesthesia, nicotine 100 µg i.v. produced a pressor and an antidiuretic response accompanied by an increase in the urinary excretion of vasopressin and of oxytocin-like radioimmunoactivity (OLRI). This indicates release of both vasopressin and oxytocin.

3 Under conditions in which tachyphylaxis was avoided, NSTX, 80 ng i.c.v., caused a prolonged inhibition of the release of both hormones by nicotine.

4 NSTX i.c.v. caused some reduction in the pressor response to nicotine. It is suggested that this response involves both central and peripheral stimulation of the sympathetic nervous system and that the central component is blocked by neosurugatoxin.

5 Muscarine, 40 ng i.c.v., produced a pressor and an antidiuretic response with increased urinary excretion of vasopressin and OLRI. All these effects were blocked by atropine but were not inhibited by NSTX.

6 Sodium nitroprusside (SN), 200 µg i.v., and hypertonic saline (HS; 1.54 M NaCl solution) 4 µl i.c.v., both produced antidiuretic responses accompanied by increased urinary excretion of vasopressin and OLRI. The ratio of the excretion of vasopressin to that of OLRI was 5.1 ± 1.3 (mean $\pm$ s.e.; $n = 8$) for SN and 1.2 ± 0.24 (mean $\pm$ s.e.; $n = 6$) for HS. NSTX 80 ng i.c.v., caused a significant reduction in the antidiuretic response to the hypotension induced with SN: the increased urinary excretion of vasopressin was also significantly reduced but not that of OLRI. NSTX had no effect on the response to HS.

7 We conclude that NSTX acts centrally on nicotinic cholinceptors to block the release of vasopressin and oxytocin by nicotine and the release of vasopressin, but not that of oxytocin, by hypotension. It does not inhibit the release of either hormone by a central osmotic stimulus.

Keywords: Neosurugatoxin; vasopressin; oxytocin; nicotinic receptors; muscarinic receptors; hypertonic saline

Introduction

Two outbreaks of food poisoning which occurred in Japan in 1957 and 1965 after ingestion of the Japanese Ivory mollusc, *Babylonia japonica*, produced a pattern of symptoms, including hypotension, mydriasis and constipation, reminiscent of the side-effects of ganglion blocking agents in man (Hirayama *et al.*, 1974). A toxin, IS-toxin, contained in a crude extract of the mollusc was found to block the ciliary ganglia in mice and the superior cervical ganglia in cats (Hirayama *et al.*, 1970). Kosuge *et al.* (1972) isolated a pure crystalline substance which they named surugatoxin. It was reported that this toxin caused selective block of nicotinic cholinceptors in sympathetic and parasympathetic ganglia and on adrenal chromaffin cells, without any action on muscarinic receptors in smooth muscle or on nicotinic cholinceptors at the neuromuscular junction (Hirayama *et al.*, 1974; Hayashi & Yamada, 1975; Brown *et al.*, 1976; Ascher *et al.*, 1979). It now appears that the mydriatic activity which was used in the isolation of surugatoxin is due to contamination with two other toxins (Kosuge *et al.*, 1982). A new toxin, neosurugatoxin, NSTX, has been isolated (Kosuge *et al.*, 1981; 1982) which possesses the same functional groups, myo-inositol, bromo-oxindole and dioxypyrimidine, as surugatoxin but differs from it in having a 6–7 rather than 6–6 heterocyclic ring system. NSTX evokes mydriasis in mice and

has an anti-nicotinic action on the guinea-pig ileum about 100 times as potent as that reported for surugatoxin with a pA_2 of 9.07 compared with 5.46 for hexamethonium (Kosuge *et al.*, 1982; Hayashi *et al.*, 1983; 1984). It also inhibits the secretion of catecholamines from cultured bovine adrenal medullary cells in response to nicotine (Bourke *et al.*, 1988) and carbachol (Wada *et al.*, 1989).

There has been considerable interest in the possibility that NSTX might block nicotinic cholinceptors in the CNS. This would be useful for identifying sub-types of receptors and for investigating cholinergic transmission in physiological systems. Specific binding of the toxin to brain membranes and inhibition of nicotine binding has been demonstrated (Hayashi *et al.*, 1983; 1984; Rapier *et al.*, 1985; Yamada *et al.*, 1985; 1988a; Billiar *et al.*, 1988). NSTX blocked the presynaptic nicotinic facilitation of [³H]-dopamine release from striatal nerve terminals (Rapier *et al.*, 1985). *In vivo* studies have demonstrated an inhibition of nicotine-induced antinociception in mice (Yamada *et al.*, 1988b) and a decrease in the frequency, although not the amplitude, of pulses of luteinizing hormone (LH) secretion in rats (Billiar *et al.*, 1988).

A well-established central action of nicotine discovered by Burn *et al.* (1945) is the release of vasopressin from the neurohypophysis. Hexamethonium i.c.v. blocks the release of vasopressin by nicotine and by hypotension (Bisset & Feldberg, 1977; Bisset & Chowdrey, 1984). It also blocks the release of vasopressin by application of hypertonic saline

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(HS) to the rat hypothalamo-neurohypophyseal explant (Sladek & Joynt, 1979) but not by i.c.v. injection in the rat (Bisset *et al.*, 1988; 1989). To test whether NSTX has a central as well as a peripheral action like that of hexamethonium, we have investigated the effect of i.c.v. NSTX on the release of vasopressin by nicotine, hypotension and i.c.v. HS. Release of vasopressin was measured by antidiuretic responses and increased urinary excretion of vasopressin in water-loaded rats under ethanol anaesthesia. In the same experiments, the excretion of oxytocin-like radioimmunoactivity (OLRI) was also measured as an indicator of oxytocin release (Bisset *et al.*, 1989; 1990b).

Methods

The experiments were carried out on Wistar rats under ethanol anaesthesia in which a constant fluid load equivalent to 8% body weight (220–230 g) was maintained. This produced a sustained diuresis. Drugs were injected i.v. (0.1–0.4 ml) into an external jugular vein or i.c.v. (1–4 μ l) into a lateral cerebral ventricle. Blood pressure was recorded from a femoral artery. The hypotensive response to sodium nitroprusside was integrated to provide a count comprising both amplitude and duration. The trachea was cannulated to maintain a free airway but artificial ventilation was not applied; amyl nitrite was administered by inhalation through the tracheal cannula. Urine flow was recorded from the cannulated bladder with a drop counter set to a 1 min time base. Samples of urine (5 ml) were collected during a control period at the start of each experiment, as soon as a steady diuresis had been achieved, and immediately after each drug injection. The antidiuretic response to nicotine, muscarine and HS, all of which produced a pressor response, was calculated as the percentage reduction in urine flow from the expression $100(A - B) \div A$, where A is the control rate of flow immediately before the injection and B the mean rate of flow during the period in which the 5 ml urine sample was collected after the injection. In calculating the antidiuretic response to sodium nitroprusside, the initial antidiuresis resulting from the renal vascular response to hypotension (Bisset & Chowdrey, 1984) was discounted. At the end of the collection, each sample of urine was acidified by the addition of 0.5 ml 1 M formic acid and stored at 4°C until extracted. Extracts were prepared with octadecasilyl silica (La Rochelle *et al.*, 1980) for estimation by radioimmunoassay of the content of vasopressin (AVP) and oxytocin-like radioimmunoactivity (OLRI). Urinary excretion of vasopressin and OLRI is expressed as the total amount (pg) contained in a 5 ml sample of urine. Full experimental details have been published (Bisset & Chowdrey, 1984; Bisset *et al.*, 1990b). In all experiments with nicotine, except those illustrated in Figure 9, atropine 200 μ g was given i.v. at the start of the experiment to prevent hypotension resulting from stimulation of parasympathetic ganglia which might have caused reflex release of vasopressin.

Drugs

Drugs used were nicotine hydrogen tartrate and sodium nitroprusside (BDH), atropine sulphate, hexamethonium bromide, muscarine chloride and noradrenaline hydrochloride (Sigma), doses of which are expressed in terms of the salts; amyl nitrite vitellae BPC (Evans); arginine vasopressin (1st International Standard for Arginine Vasopressin for Bioassay); the synthetic vasopressin antagonist, (+)-[CH₂]₅ D-Tyr(Et) valyl arginine vasopressin (VAVP) (Manning *et al.*, 1982) and neosurugatoxin (NSTX). The vasopressin antagonist was a generous gift from Dr Manning of the Department of Biochemistry & Molecular Biology, Medical College of Ohio, U.S.A. NSTX was isolated in the School of Pharmaceutical Science, University of Shizuoka by the method of Kosuge *et al.* (1982). A 100 μ g sample of toxin was dissolved

in 1 ml of phosphate buffered saline at pH 6.5 and dispensed into 10 aliquots of 100 μ l each containing 10 μ g which were stored at -20°C until use. All further dilutions were made with phosphate buffered saline.

Statistics

Results are expressed as mean $\pm$ s.e.mean. Significance of differences between means was determined by Student's *t* test, using a paired (Figures 3, 8 and 9) or unpaired (Figure 6) test as appropriate. A level of $P < 0.05$ was taken to be significant.

Results

Nicotine i.v. produced an antidiuretic and a pressor response with increased urinary excretion of both vasopressin and OLRI. The threshold dose for an antidiuretic response (ADR) was 50 μ g: a dose of 200 μ g frequently caused apnoea. A standard dose of 100 μ g was adopted which did not cause apnoea in any experiment. Figure 1 compares the antidiuretic and pressor responses to 100 μ g nicotine i.v. and 0.5 and 1.0 ng vasopressin i.v. before (a) and after (b) the injection i.v. of a synthetic vasopressin antagonist, (+)-[CH₂]₅ D-Tyr(Et) VAVP, which blocks both the V₁ (vascular) and V₂ (renal) receptors. In (a) the ADR to nicotine was similar to that produced by 1 ng vasopressin but the pressor response to nicotine was much larger and more prolonged. In (b) the antagonist blocked the antidiuretic and pressor responses to vasopressin but the pressor response to nicotine was unchanged and accompanied by a pronounced diuresis.

Figure 1 Blood pressure (upper traces) and urine flow (lower traces) in a water-loaded rat under ethanol anaesthesia. Each vertical line in the lower traces indicates the urine flow (μ l) in 1 min. AVP = arginine vasopressin, 0.5 and 1.0 ng; N = nicotine, 100 μ g; NA = noradrenaline, 1 μ g. Between (a) and (b), 4 μ g (+)-[CH₂]₅ D-Tyr (Et) VAVP was injected, and, between (b) and (c) 4 mg hexamethonium. All injections i.v.

Between (b) and (c), hexamethonium 4 mg was injected i.v.; this caused a large and sustained fall of blood pressure and antidiuresis. The pressor response to nicotine was abolished; the i.v. injection of 1 μ g noradrenaline then produced a rise of blood pressure and diuresis. In similar experiments the threshold dose of NSTX i.v. for producing a fall of blood pressure was 200 ng; a dose of 8 μ g i.v. blocked the pressor response to nicotine. The vasopressin antagonist was tested in five experiments and there was no significant difference between the pressor responses to nicotine before ($41 \text{ mmHg} \pm 7.05$) and after ($38 \text{ mmHg} \pm 7.05$) the antagonist.

Three series of experiments were carried out to test for an inhibitory effect of NSTX i.c.v. on the responses to nicotine. In the first series, three successive injections of nicotine i.v. were given to each of five rats. Four responded to 100 μ g; in the fifth the dose was increased to 200 μ g without causing apnoea. The first injection served as a control; 5 min before the second injection a single dose of 40 ng NSTX was injected i.c.v. One of these experiments is illustrated in Figure 2. The second injection of nicotine, N2, produced a smaller pressor response than the first injection, N1, a greatly reduced urinary excretion of vasopressin and only a small ADR. The pressor response to the third injection, N3, was considerably less than that to N2 and accompanied by a diuresis with a urinary excretion of vasopressin less than in a control sample of urine, C, collected at the beginning of the experiment before the first injection of nicotine. The urinary excretion of OLRI, in contrast with that of vasopressin showed little change. The mean values for the five experiments are shown in Figure 3. There was a significant difference in both the antidiuretic and pressor responses between N1 and N2 and between N2 and N3; N2 produced a significantly smaller urinary excretion of vasopressin than N1 and N3 did not differ significantly from the control. Although the urinary excretion of OLRI showed some decline from N1 to N2 and from N2 to N3, the differences were not significant.

Control experiments were carried out to test whether the diminished response to successive injections of nicotine could have been due to tachyphylaxis rather than an inhibitory effect of NSTX. Figure 4 illustrates an experiment in which two injections of nicotine at an interval of 60 min produced almost identical responses. However, tachyphylaxis was observed in three experiments in which the first injection of

Figure 3 Summary of results of 5 experiments including that illustrated in Figure 2 and with the same protocol and abbreviations. The mean interval between injections of nicotine N1 and N2 was 101 min and between N2 and N3, 82 min. The columns show mean responses with s.e. indicated by vertical bars.

nicotine produced an exceptionally prolonged ADR and subsequent injections were given after only a partial recovery. One of these experiments is illustrated in Figure 5. The first injection of 100 μ g of nicotine produced an extremely large rise of blood pressure and a persistent ADR. Although an interval of 65–75 min was allowed between injections there was a progressive decline in the pressor and antidiuretic responses and in the urinary excretion of vasopressin. The fourth injection produced a diuresis although the urinary excretion of vasopressin was still increased compared with the control. In contrast with vasopressin, the increased urinary excretion of OLRI did not decline. In the experiments of Figure 3, nicotine was injected at intervals of 65–140 min and the urine flow recovered completely between injections. However, in three of these experiments, the first injection of nicotine resulted in a relatively prolonged ADR which might have induced tachyphylaxis. Therefore, in order to exclude any possibility of tachyphylaxis, a second series of experiments was carried out (Figure 6) in which a single injection of 100 μ g nicotine was examined in two groups of rats. The first group served as a control. Each rat in the test

Figure 2 Blood pressure and urine flow in a water-loaded rat under ethanol anaesthesia, recorded as in legend to Figure 1. Three successive injections of nicotine, 100 μ g i.v., (N1, N2 and N3) were given; the interval between N1 and N2 was 75 min; and between N2 and N3, 65 min. Neosurugatoxin (NSTX) 40 ng i.c.v. was injected 5 min before N2. Arginine vasopressin (AVP) and oxytocin-like radioimmunoactivity (OLRI); total amount (pg) excreted in a 5 ml control sample of urine (C) collected at the start of the experiment, before N1, or in 5 ml samples collected after the injections of nicotine.

Figure 4 Water-loaded rat under ethanol anaesthesia. The effect of 2 injections of nicotine, 100 μ g i.v. (N1 and N2), at an interval of 60 min, on urine flow and blood pressure recorded as in legend to Figure 1, and on urinary excretion of arginine vasopressin (AVP) and oxytocin-like immunoreactivity (OLRI) in 5 ml samples of urine: C = control. Note the absence of tachyphylaxis.

Figure 5 Similar experiment to that in Figure 4 with four successive injections of nicotine, 100 μg i.v. (N1, N2, N3 and N4) at intervals of 65–75 min. The antidiuretic response to N1 had not fully recovered when N2 was given. The pressor and antidiuretic responses and the urinary excretion of arginine vasopressin (AVP) exhibited tachyphylaxis but not the excretion of oxytocin-like immunoreactivity (OLRI): C = control.

Figure 6 Results of experiments on 10 control (open columns) and 6 test (solid columns) rats water-loaded under ethanol anaesthesia. All received nicotine 100 μg i.v. The test rats were given 2 injections of neosurugatoxin (NSTX) 40 ng i.c.v., 20 min and 10 min before nicotine. Control samples of urine (C) were collected in each group. The columns show mean responses with s.e. indicated by vertical bars. Excretion of arginine vasopressin (AVP) and oxytocin-like immunoreactivity (OLRI) measured as total amount in 5 ml samples of urine.

group was given an injection of NSTX i.c.v. before nicotine. Since the response to nicotine was not completely inhibited 5 min after the injection of 40 ng NSTX in the first series of experiments, two injections of 40 ng NSTX were given in the present series, the first 20, and the second 10 min, before nicotine. Only one of the ten rats in the control group failed to respond to 100 μg nicotine. There was no significant difference between the pressor responses in the two groups, but the ADR was almost abolished in the test group. Nicotine increased the excretion of both vasopressin and OLRI significantly in the control group: after NSTX, there was no significant change in either vasopressin or OLRI in response to nicotine.

A third series of experiments was carried out in six rats to test whether NSTX acts selectively on nicotinic receptors. One of these experiments is illustrated in Figure 7, and the mean results of the six experiments are shown in Figure 8. Three injections of 20 ng muscarine i.c.v. (M1, M2 and M3) were given: M1 served as a control; two injections of 40 ng NSTX were given i.c.v. 20 and 10 min before M2 to conform with the procedure used in the second series of experiments and atropine, 200 μg i.v., was given between M2 and M3. Finally the response to nicotine, 100 μg i.v., was tested. Muscarine produced pressor and antidiuretic responses and a

Figure 7 Blood pressure and urine flow in a water-loaded rat under ethanol anaesthesia, recorded as in legend to Figure 1. Three successive injections of muscarine, 20 ng i.c.v. (M1, M2 and M3) were given followed by nicotine (N), 100 μg i.v. Two injections of neosurugatoxin (NSTX) 40 ng i.c.v., were given 20 min and 10 min before M2 and 200 μg atropine i.v. was injected between M2 and M3. Control (C) samples of urine were collected at the start of the experiment. Excretion of arginine vasopressin (AVP) and oxytocin-like immunoreactivity (OLRI) measured as total amount (pg) in 5 ml samples of urine.

significant increase in the urinary excretion of both vasopressin and OLRI: none of these effects was significantly reduced by NSTX but all were abolished by atropine. Nicotine produced a pressor and diuretic response but no increase in the urinary excretion of vasopressin or OLRI; this confirmed that central nicotinic cholinergic receptors had been effectively blocked by NSTX. At the end of each experiment an inhalation of amyl nitrite was given to produce hypotension; no antidiuretic response was observed. In contrast, the injection of hypertonic saline (HS; 1.54 M NaCl solution) 4 μl i.c.v. after nicotine in 2 experiments produced pressor and antidiuretic responses with an increase in the urinary excretion of vasopressin to 262 and 279 pg and that of OLRI to 813 and 781 pg. These preliminary results suggested that NSTX might have a differential effect on the release of vasopressin by hypotension and an osmotic stimulus. To investigate this, two further series of experiments were carried out, but using sodium nitroprusside (SN) in preference to amyl nitrite since the hypotensive responses to SN were more reproducible.

Figure 8 Summary of results of six experiments including that illustrated in Figure 7 and with the same protocol. The columns show mean responses with s.e. indicated by vertical bars.

Doses of 50, 100 and 200 μg SN in the same rat produced graded hypotensive and antidiuretic responses and increases in the urinary excretion of vasopressin. A dose of 200 μg was adopted as standard. Each rat was given two injections of either SN (8 experiments) or hypertonic saline (HS) (6 experiments). NSTX was given before the second injection, the first serving as a control. Two doses of toxin, 40 ng i.c.v. were given at an interval of 10 min, as in the experiments illustrated in Figures 6–8 but, in order to allow for the time to the maximum onset of action, the first NSTX injection was given 60 min before SN or HS. The results are shown in Figure 9. Both SN and HS increased the urinary excretion of OLRI as well as that of vasopressin but the mean vasopressin: OLRI ratio was 5.1 ± 1.3 for SN compared with

1.2 ± 0.24 for HS. After NSTX, SN produced a similar hypotensive response but there was a significant reduction in both the antidiuretic response and the increased urinary excretion of vasopressin. The reduction in the hypotension-induced vasopressin release was not due to tachyphylaxis since, in a control series of experiments in which two injections of SN were given at a similar time interval but without the intervention of NSTX, there was no significant difference in the responses. Interestingly, the increased urinary excretion of OLRI after SN was not reduced by NSTX. In contrast with the effects of a hypotensive stimulus, the responses to HS before and after NSTX were almost identical. Nicotine injected after SN or HS produced pressor and diuretic responses with no significant increase in the urinary excretion of vasopressin or OLRI compared with that in the controls. This demonstrated, as in the experiments illustrated in Figures 6 and 8, an effective block of central nicotinic cholinergic receptors.

Discussion

The fact that the antidiuretic response to i.v. nicotine in the rat was accompanied by an increase in the urinary excretion of OLRI as well as vasopressin indicates that both vasopressin and oxytocin are released. This contrasts with the cat in which i.v. nicotine has been shown to release vasopressin alone (Bisset *et al.*, 1975; Bisset & Feldberg, 1977). Many of the actions of nicotine, including the release of vasopressin, exhibit tachyphylaxis (Paton & Savini, 1968; Bisset & Feld-

Figure 9 Results of two series of experiments in water-loaded rats under ethanol anaesthesia in which two i.v. injections (SN1 and SN2; $n = 8$) of 200 μg sodium nitroprusside, or two i.c.v. injections (HS1 and HS2; $n = 6$) of 4 μl hypertonic saline (1.54 M NaCl solution) were given in each rat. Two injections of neosurugatoxin (NSTX) 40 ng i.c.v., were given 60 and 50 min before SN2 and HS2. Nicotine, 100 μg i.v., was injected after SN2 and HS2 to test for the effectiveness of NSTX in blocking nicotinic cholinergic receptors. A control sample of urine (C) was collected at the start of each experiment. Columns show mean responses with s.e. indicated by vertical bars. Excretion of arginine vasopressin (AVP) and oxytocin-like immunoreactivity (OLRI) measured as total amount in 5 ml samples of urine. The hypotensive response to SN is measured as an integrated count (see Methods).

berg, 1977; Wonnacott, 1990). In the present investigation, tachyphylaxis was observed in those control experiments in which the first injection of nicotine produced a relatively prolonged antidiuretic response with only partial recovery of the urine flow before the next injection. It is interesting to note however that, although the urinary excretion of vasopressin declined progressively with successive injections of nicotine in these experiments, the excretion of OLRI was unchanged. Also, in the first series of experiments in which three successive injections of nicotine were given in one rat, the second after 40 ng NSTX, the decline in the excretion of vasopressin with the second and third injections was not accompanied by any significant decrease in the excretion of OLRI. This dissociation emphasises the independence of the neural mechanisms which control the release of vasopressin and oxytocin. The possibility cannot be excluded that the results obtained in the first series of experiments were due at least in part to tachyphylaxis. However, the results obtained in the second and third series of experiments in which each rat received only one injection of nicotine and a larger dose of 80 ng NSTX was used, show unequivocally that this dose of NSTX blocks the release of both vasopressin and oxytocin by nicotine and that this inhibitory effect persists for at least 3 h. This prolonged block is consistent with a report that NSTX may irreversibly inactivate nicotine binding sites *in vitro* (Yamada *et al.*, 1985) and that inhibition of nicotine binding *in vivo* persists up to 7 days after i.c.v. injection of NSTX (Yamada *et al.*, 1988a).

Agonists acting on muscarinic cholinergic receptors have been shown to release vasopressin and oxytocin in the rat and this release is blocked by atropine (Kühn & McCann, 1971; Kühn, 1974; Clarke & Merrick, 1978; Bisset & Chowdrey, 1984; Iitake *et al.*, 1986; Shoji *et al.*, 1989). In our experiments, NSTX acted selectively on nicotinic, and not on muscarinic, receptors. The antidiuretic response to muscarine and the increased urinary excretion of vasopressin and OLRI which were blocked by atropine were not inhibited by doses of NSTX which blocked the response to nicotine in the same experiments.

Since the pressor response which accompanied the antidiuretic response to i.v. nicotine was blocked by i.v. hexamethonium but not by a vasopressin antagonist, it is due not to release of vasopressin but to stimulation of the sympathetic nervous system. The partial inhibition of this response by only 80 ng NSTX i.c.v. could not have been caused by a peripheral sympathetic block since the minimum dose of NSTX i.v. to block the pressor response to i.v. nicotine was 8 µg. This suggests that the pressor response is mediated in part by central sympathetic stimulation which is blocked by NSTX. In many experiments in which NSTX blocked the release of vasopressin, the residual pressor response was accompanied by diuresis, probably caused by an effect on renal haemodynamics of catecholamines released by the peripheral component of sympathetic stimulation.

Hypotension induced with i.v. sodium nitroprusside (SN) and an osmotic stimulus consisting of i.c.v. hypertonic saline (HS) caused an increase in the urinary excretion of vasopressin and OLRI, although not in the same ratio. This implies release of both vasopressin and oxytocin. A species difference is again involved since, in the cat, hypotension, haemorrhage, carotid occlusion and i.c.v. HS have all been shown to release vasopressin without oxytocin (Bisset *et al.*, 1989). Changes in blood pressure are monitored by peripheral receptors in the cardiovascular system (for review see Bisset & Chowdrey, 1988) which have an afferent input to the nucleus of the tractus solitarius (NTS) on the dorsal surface of the medulla. The NTS projects to the A1 group of noradrenergic neurones on the ventral surface which, in turn, selectively innervate the vasopressin-secreting cells in the supraoptic and paraventricular nuclei (SON and PVN) either directly or indirectly through the lateral preoptic nucleus (LPN) (Banks & Harris, 1984). It is now thought that this group of neurones is coincident with the nicotine sensitive

area which has been defined on the ventral surface of the medulla in the cat (Bisset *et al.*, 1975). Topical application of nicotine to this area causes a release of vasopressin without oxytocin which is blocked by hexamethonium (Bisset & Feldberg, 1977). The LPN, which is a part of the magnocellular basal nucleus lying dorsolateral to the SON provides the only known cholinergic input to this nucleus (Mason *et al.*, 1983; Hatton & Mason, 1985). Electrical stimulation in the region of the LPN causes an excitation of the vasopressin-secreting neurones in the SON which is blocked by hexamethonium (Hatton *et al.*, 1983). Intracellular recording from the neurones innervated by the LPN demonstrated depolarization after microperfusion with acetylcholine and this effect also was blocked by hexamethonium (Cobbett *et al.*, 1986). The SON was stained with a monoclonal antibody to the α sub-unit of the nicotinic receptor (Mason, 1985). However, a recent immunocytochemical study involving both light and electron microscopy failed to demonstrate a direct innervation of the cell bodies and dendrites in the SON and PVN by cholinergic synapses (Theodosis & Mason, 1988). This suggests the possibility of intervening neurones (Tribollet *et al.*, 1985; Raby & Renaud, 1989a; Pow & Morris, 1989; Jhamandas *et al.*, 1989; Renaud & Bourque, 1991) and is consistent with the finding that binding sites for nicotine and acetylcholine lie predominantly outside the SON and PVN (Clarke *et al.*, 1985; Sharp *et al.*, 1987; Härfstrand *et al.*, 1988). In previous work (Bisset & Chowdrey, 1984) we showed that i.c.v. hexamethonium blocked the antidiuretic response to amyl nitrite in the water-loaded rat preparation, although it has been reported not to inhibit the release of vasopressin by non-hypotensive or hypotensive haemorrhage (Iitake *et al.*, 1989). In the present experiments, i.c.v. NSTX, in the same dose which blocked the release of vasopressin and oxytocin by nicotine, blocked the antidiuretic response to amyl nitrite and caused a significant reduction in the antidiuretic response to SN and in the increased urinary excretion of vasopressin. This suggests that NSTX acts on the same or a similar type of receptor to that on which hexamethonium acts and confirms the existence of one or more cholinergic synapses in the central neural pathways controlling the release of vasopressin by hypotension. These synapses are probably the site of action of nicotine in releasing vasopressin and of NSTX in blocking this release. In contrast with vasopressin, the release of oxytocin by hypotension was not inhibited by NSTX. This is consistent with the absence of any direct cholinergic input to the oxytocin-secreting cells in the SON and PVN and with the recent suggestion that the release of oxytocin may be controlled by a direct projection from the NTS to the oxytocin-secreting cells in which the transmitter at the final synapse is not acetylcholine or noradrenaline but the peptide inhibin β (Sawchenko *et al.*, 1988; Raby & Renaud, 1989a,b; Bourque & Renaud, 1990; Renaud & Bourque, 1991). It is conceivable that nicotine may cause release of oxytocin by stimulating this efferent projection from the NTS.

The osmotic control of vasopressin release is still controversial. The main issue is whether the SON itself functions as an osmoreceptor or receives an afferent neural input from extraneous osmoreceptors in the circumventricular organs (Bisset & Chowdrey, 1988; Bisset *et al.*, 1990a). The entire system may be integrated in an 'osmoreceptor complex' (Honda *et al.*, 1990). Sladek & Joynt (1979) produced evidence from *in vitro* work with hypothalamic explants for a cholinergic input to the vasopressin-secreting cells in the SON from the organum vasculosum laminae terminalis (OVLT). Excitation of neurosecretory cells in the PVN by intracarotid HS was inhibited by microionophoretic application of hexamethonium and atropine (Akaishi & Negoro, 1983). In the water-loaded rat preparation, both hexamethonium and atropine failed to block the release of vasopressin by i.c.v. HS (Bisset *et al.*, 1988) and, in the present experiments, NSTX, in a dose which effectively inhibited release by nicotine or hypotension, caused no reduction in the excretion of either vasopressin or OLRI after i.c.v. HS.

Similarly, Iitake *et al.* (1989) reported that doses of i.c.v. hexamethonium and atropine which blocked the release of vasopressin in response to i.c.v. cholinergic agonists, did not block release by i.v. HS in conscious rats. There is, therefore, no evidence for a cholinergic link in osmotic control *in vivo*.

In conclusion, we have shown that NSTX, which is known to block peripheral nicotinic cholinergic receptors in autonomic ganglia and the adrenal medulla, also acts centrally to block the release of vasopressin and oxytocin by nicotine. It inhi-

bits the release of vasopressin but not that of oxytocin by hypotension and has no effect on the release of either hormone by an osmotic stimulus. Our experiments provide further evidence for a central action of NSTX on nicotinic cholinergic receptors and illustrate its potential for elucidating cholinergic pathways in the CNS.

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References

- AKAISHI, T. & NEGORO, H. (1983). Effects of microelectrophoretically applied acetylcholine- and angiotensin-antagonists on the paraventricular neurosecretory cells excited by osmotic stimuli. *Neurosci. Lett.*, **36**, 157–161.
- ASCHER, P., LARGE, W.A. & RANG, H.P. (1979). Studies on the mechanism of acetylcholine antagonists on rat parasympathetic ganglion cells. *J. Physiol.*, **295**, 139–170.
- BANKS, D. & HARRIS, M.C. (1984). Lesions of the locus coeruleus abolish baroreceptor-induced depression of supraoptic neurones in the rat. *J. Physiol.*, **355**, 383–398.
- BILLIAR, R.B., KALASH, J., ROMITA, V., TSUJI, K. & KOSUGE, T. (1988). Neosurugatoxin: CNS acetylcholine receptors and luteinizing hormone secretion in ovariectomized rats. *Brain Res. Bull.*, **20**, 315–322.
- BISSET, G.W. & CHOWDREY, H.S. (1984). A cholinergic link in the reflex release of vasopressin by hypotension in the rat. *J. Physiol.*, **354**, 523–545.
- BISSET, G.W. & CHOWDREY, H.S. (1988). Control of release of vasopressin by neuroendocrine reflexes. *Q. J. Exp. Physiol.*, **73**, 811–872.
- BISSET, G.W. & FELDBERG, W. (1977). Effect of hexamethonium on the release of vasopressin by nicotine and carotid occlusion. *J. Physiol.*, **267**, 30–31P.
- BISSET, G.W., CHOWDREY, H.S. & FELDBERG, W. (1990a). The effect of an osmotic stimulus on the release of neurohypophyseal hormones in the cat: preferential release of vasopressin with a possible involvement of the area postrema. *J. Neuroendocr.*, **2**, 201–207.
- BISSET, G.W., CHOWDREY, H.S., FAIRHALL, K.M. & GUNN, L.K. (1988). Is there a cholinergic link *in vivo* mediating the osmotic release of vasopressin in the rat? *J. Physiol.*, **398**, 82P.
- BISSET, G.W., CHOWDREY, H.S., FAIRHALL, K.M., FELDBERG, W. & GUNN, L.K. (1989). Osmotic control of release of vasopressin and oxytocin in the cat and rat. *Proceedings of the Fourth International Conference on the Neurohypophysis*. pp. 83–86. Oxford: Oxford Community Press.
- BISSET, G.W., CHOWDREY, H.S., FAIRHALL, K.M. & GUNN, L.K. (1990b). Central inhibition of γ -aminobutyric acid and muscimol of the release of vasopressin and oxytocin by an osmotic stimulus in the rat. *Br. J. Pharmacol.*, **99**, 529–535.
- BISSET, G.W., FELDBERG, W., GUERTZENSTEIN, P.G. & ROCHA E SILVA, JR (1975). Vasopressin release by nicotine: the site of action. *Br. J. Pharmacol.*, **54**, 463–474.
- BOURKE, J.A., BUNN, S.J., MARLEY, P.D. & LIVETT, B.G. (1988). The effects of neosurugatoxin on evoked catecholamine secretion from bovine adrenal chromaffin cells. *Br. J. Pharmacol.*, **93**, 275–280.
- BOURQUE, C.W. & RENAUD, L.P. (1990). Electrophysiology of mammalian magnocellular vasopressin and oxytocin neurosecretory neurones. *Frontiers in Neuroendocrinology*, **11**, 183–212.
- BROWN, D.A., GARTHWAITE, J., HAYASHI, E. & YAMADA, S. (1976). Action of surugatoxin on nicotine receptors in the superior cervical ganglion of the rat. *Br. J. Pharmacol.*, **58**, 157–159.
- BURN, J.H., TRUELOVE, L.H. & BURN, I. (1945). Antidiuretic action of nicotine and of smoking. *Br. Med. J.*, **i**, 403–406.
- CLARKE, G. & MERRICK, L.P. (1978). Tentative identification of the synaptic transmitters involved in the neural regulation of oxytocin release. *J. Physiol.*, **277**, 19–20P.
- CLARKE, P., SCHWARTZ, R., PAUL, S., PERT, C. & PERT, A. (1985). Nicotinic binding in rat brain: autoradiographic comparison of [3 H] acetylcholine, [3 H] nicotine and [125 I] alpha-bungarotoxin. *J. Neurosci.*, **5**, 1307–1315.
- COBBETT, P.J.R., MASON, W.T. & POULAIN, D.A. (1986). Intracellular analysis of control of rat supraoptic neurone (SON) activity *in vitro* by acetylcholine (ACh). *J. Physiol.*, **371**, 216P.
- HÄRFSTRAND, A., ADEM, A., FUXE, K., AGNATI, L. ANDERSSON, K. & NORDBERG, A. (1988). Distribution of nicotinic cholinergic receptors in the rat tel- and diencephalon: a quantitative receptor autoradiographical study using [3 H]-acetylcholine, [α - 125] bungarotoxin and [3 H]nicotine. *Acta Physiol. Scand.*, **132**, 1–14.
- HATTON, G.I. & MASON, W.T. (1985). Acetylcholine input to vasopressin neurones in the hypothalamus. In *Vasopressin*. ed. Schrier, R.W. pp. 361–366. New York: Raven Press.
- HATTON, G.I., HO, Y.W. & MASON, W.T. (1983). Synaptic activation of phasic bursting in rat supraoptic nucleus neurones recorded in hypothalamic slices. *J. Physiol.*, **345**, 297–317.
- HAYASHI, E., ISOGAI, M., KAGAWA, Y. & YAMADA, S. (1983). Characterization of central nicotinic receptors by [3 H]-nicotine: effect of neosurugatoxin. *Jpn. J. Pharmacol.*, (Suppl.) **33**, 89P.
- HAYASHI, E., ISOGAI, M., KAGAWA, Y., TAKAYANAGI, N. & YAMADA, S. (1984). Neosurugatoxin, a specific antagonist of nicotinic acetylcholine receptors. *J. Neurochem.*, **42**, 1491–1494.
- HAYASHI, E. & YAMADA, S. (1975). Pharmacological studies on surugatoxin, the toxic principle from Japanese ivory mollusc (*Babylonia japonica*). *Br. J. Pharmacol.*, **53**, 207–215.
- HIRAYAMA, H., GOHGI, K., URAKAWA, N. & IKEDA, M. (1970). A ganglion-blocking action of the toxin isolated from Japanese ivory shell (*Babylonia japonica*). *Jpn. J. Pharmacol.*, **20**, 311–312.
- HIRAYAMA, H., SUGIHARA, K., TSUYAMA, S., WAKIGAWA, K., OHKUMA, H. & GOHGI, K. (1974). A ganglion blocking action of toxic substances, IS-toxin and surugatoxin, from the Japanese ivory shell (*Babylonia japonica*). *Jpn. J. Pharmacol.*, **24**, 599–574.
- HONDA, K., NEGORO, H., DYBALL, R.E.J., HIGUCHI, T. & TAKANO, S. (1990). The osmoreceptor complex in the rat: evidence for intersections between the supraoptic and other diencephalic nuclei. *J. Physiol.*, **431**, 225–241.
- IITAKE, K., SHARE, L., OUCHI, Y., CROFTON, J.T. & BROOKS, D.P. (1986). Central cholinergic control of vasopressin release in conscious rats. *Am. Physiol. Soc.*, **251**, E146–E150.
- IITAKE, K., SHARE, L., BROOKS, D.P., CROFTON, J.T. & OUCHI, Y. (1989). Role of brain acetylcholine in vasopressin release during osmotic stimulation and haemorrhage. *Exp. Brain. Res.*, **75**, 47–52.
- JHAMANDAS, J.H., RABY, W., ROGERS, J., BUIJS, R.M. & RENAUD, L.P. (1989). Diagonal band projection towards the hypothalamic supraoptic nucleus: light and electron microscopic observations in the rat. *J. Comp. Neurol.*, **282**, 15–23.
- KOSUGE, T., ZENDA, H., OCHIAI, A., MASAKI, N., NOGUCHI, M., KIMURA, S. & NARITA, H. (1972). Isolation and structure determination of a new marine toxin, surugatoxin from the Japanese ivory shell, *Babylonia japonica*. *Tetrahedron Letts.*, **25**, 2545–2548.
- KOSUGE, T., TSUJI, K., HIRAI, K., YAMAGUCHI, K., OKAMOTO, T. & IITAKE, Y. (1981). Isolation and structure determination of a new marine toxin, neosurugatoxin, from the Japanese ivory shell, *Babylonia japonica*. *Tetrahedron Letts.*, **35**, 3417–3420.
- KOSUGE, T., TSUJI, K. & HIRAI, K. (1982). Isolation of neosurugatoxin from the Japanese ivory shell, *Babylonia japonica*. *Chem. Pharmacol. Bull.*, **30**, 3255–3259.
- KÜHN, E.R. (1974). Cholinergic and adrenergic release mechanism for vasopressin in the male rat: a study with injections of neurotransmitters and blocking agents into the third ventricle. *Neuroendocrinol.*, **16**, 255–264.
- KÜHN, E.R. & MCCANN, S.M. (1971). Release of oxytocin and vasopressin in laboratory rats after injection of carbachol into the third ventricle. *Neuroendocrinol.*, **8**, 48–58.
- LA ROCHELLE, F.T., NORTH, W.G. JR & STERN, P. (1980). A new extraction of arginine vasopressin from blood. The use of octadecasil silica. *Pflügers Arch.*, **387**, 79–81.

- MANNING, M., OLMA, A., KLIS, W.A., KOŁODZIEJCZYK, M., SETO, J. & SAWYER, W.H. (1982). Design of more potent antagonists of the antidiuretic responses to arginine vasopressin. *J. Med. Chem.*, **25**, 45–50.
- MASON, W.T. (1985). Staining of the magnocellular nuclei of the rat hypothalamus by a monoclonal antibody directed against the α -subunit of the nicotinic cholinergic receptor. *Neurosci. Letts.*, **59**, 89–95.
- MASON, W.T., HO, Y.W., ECKENSTEIN, F. & HATTON, G.I. (1983). Mapping of cholinergic neurones associated with rat supraoptic nucleus: combined immunocytochemical and histochemical identification. *Brain Res. Bull.*, **11**, 617–626.
- PATON, W.D.M. & SAVINI, E.C. (1968). The action of nicotine on the motor endplate in the cat. *Br. J. Pharmacol. Chemother.*, **32**, 360–380.
- POW, D.V. & MORRIS, J.F. (1989). Differential distribution of acetyl cholinesterase activity among vasopressin- and oxytocin-containing supraoptic magnocellular neurones. *Neuroscience*, **28**, 109–119.
- RABY, W.N. & RENAUD, L.P. (1989a). Dorsomedial medulla stimulation activates rat supraoptic oxytocin and vasopressin neurones through different pathways. *J. Physiol.*, **417**, 279–294.
- RABY, W.N. & RENAUD, L.P. (1989b). Nucleus tractus solitarius innervation of supraoptic nucleus: anatomical and electrophysiological studies in the cat suggest differential innervation of oxytocin and vasopressin neurones. *Prog. Brain Res.*, **81**, 319–332.
- RAPIER, C., HARRISON, R., LUNT, G.G. & WONNACOTT, S. (1985). Neosurugatoxin blocks nicotinic acetylcholine receptors in the brain. *Neurochem. Int.*, **7**, 389–396.
- RENAUD, L.P. & BOURQUE, C.W. (1991). Neurophysiology and neuropharmacology of hypothalamic magnocellular neurones secreting vasopressin and oxytocin. *Prog. Neurobiol.*, **36**, 131–169.
- SAWCHENKO, E., PLOTSKY, P.M., PFEIFFER, S.W., CUNNINGHAM, T. Jr., VAUGHAN, J., RIVIER, J. & VALE, W. (1988). Inhibit β in central neural pathways involved in control of oxytocin secretion. *Nature*, **334**, 615–617.
- SHARP, B.M., NICOL, S., CUMMINGS, S. & SEYBOLD, V. (1987). Distribution of nicotinic binding sites with respect to CRF and neurophysin immunoreactive perikarya within the rat hypothalamus. *Brain Res.*, **422**, 361–366.
- SHOJI, M., SHARE, L., CROFTON, J.T. & BROOKS, D.P. (1989). The effect on vasopressin release of microinjection of cholinergic agonists into the paraventricular nucleus of conscious rats. *J. Neuroendocrinol.*, **1**, 401–406.
- SLADEK, C.D. & JOYNT, R.J. (1979). Characterization of cholinergic control of vasopressin release by the organ-cultured rat hypothalamo-neurohypophyseal system. *Endocrinol.*, **104**, 659–663.
- THEODOSIS, D.T. & MASON, W.T. (1988). Choline acetyltransferase immunocytochemical staining of the rat supraoptic nucleus and its surroundings: a light- and electron microscope study. *Cell Tissue Res.*, **254**, 119–124.
- TRIBOLLET, E., ARMSTRONG, W.E., DUBOIS-DAUPHIN, M. & DREIFUSS, J.J. (1985). Extra-hypothalamic afferent inputs to the supraoptic nucleus area of the rat as determined by retrograde and anterograde tracing techniques. *Neuroscience*, **15**, 135–148.
- WADA, A., UEZONOA, Y., ARITA, M., TSUJI, K., YANAGIHARA, N., KOBAYASHI, H. & IZUMI, F. (1989). High-affinity and selectivity of neosurugatoxin for the inhibition of ^{22}Na influx via nicotinic receptor-ion channel in cultured bovine adrenal medullary cells: comparative study with histrionicotoxin. *Neurosci.*, **2**, 333–339.
- WONNACOTT, S. (1990). The paradox of nicotinic acetylcholine receptor upregulation by nicotine. *Trends Pharmacol. Sci.*, **11**, 216–219.
- YAMADA, S., ISOGAI, M., KAGAWA, T., TAKAYANAGI, N., HAYASHI, E., TSUJI, K. & KOSUGE, T. (1985). Brain nicotinic acetylcholine receptors. Biochemical characterization by neosurugatoxin. *Mol. Pharmacol.*, **28**, 120–127.
- YAMADA, S., USHIJIMA, H., NAKAYAMA, K., HAYASHI, E., TSUJI, K. & KOSUGE, T. (1988a). Intracellular injection of neosurugatoxin induces a prolonged blockade of brain nicotinic acetylcholine receptors. *Eur. J. Pharmacol.*, **156**, 279–282.
- YAMADA, S., KAGAWA, Y., TAKAYAMA, K., HAYASHI, E., TSUJI, K. & KOSUGE, T. (1988b). Inhibition by neosurugatoxin of nicotine-induced anti-nociception. *Brain Res.*, **375**, 360–362.

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