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Mechanism of nicotine-evoked release of [^{3}H]-noradrenaline in human cerebral cortex slices

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1 The mechanism of stimulation of noradrenaline (NA) release by nicotine (NIC) was investigated in human cerebral cortex slices preloaded with [^{3}H]-noradrenaline.

2 NIC (10–1000 μM) increased [^{3}H]-NA release in a concentration-dependent manner.

3 NIC (100 μM)-evoked [^{3}H]-NA release was largely dependent on external Ca^{2+}, and was attenuated by ω-conotoxin GVIA (0.1 μM) but not by nitrendipine (1 μM).

4 Tetrodotoxin (1 μM) and nisoxetine (0.1 μM) attenuated the NIC (100 μM)-evoked release of [^{3}H]-NA.

5 Mecamylamine (10 μM), dihydro-β-erythroidine (10 μM) and *d*-tubocurarine (30 μM), but not α-bungarotoxin (α-BTX, 0.1 μM), attenuated the NIC (100 μM)-evoked release of [^{3}H]-NA.

6 NIC (100 μM)-evoked release of [^{3}H]-NA was not affected by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 30 μM) and D(−)-2-amino-5-phosphonopentanoic acid (D-AP5, 100 μM), but attenuated by MK-801 (10 μM). MK-801 (0.1–1000 μM) displaced the specific binding of [^{3}H]-nisoxetine with K_i values of 91.2 μM. NIC (100, 300 and 1000 μM) did not induce [^{3}H]-D-aspartate release in human cerebral cortex slices.

7 NIC (100 μM)-evoked release of [^{3}H]-NA was attenuated by 7-nitroindazole (10 μM), N^G-nitro-L-arginine methyl ester HCl (L-NAME, 30 μM), N^G-monomethyl-L-arginine acetate (L-NMMA, 300 μM). [^{3}H]-NA release induced by NIC (100 μM) was attenuated by methylene blue (3 μM) and 1*H*-[1,2,4]oxadiazole[4,3-α]quinoxalin-1-one (ODQ, 10 μM), and enhanced by zaprinast (30 μM).

8 In conclusion, NIC stimulates the release of [^{3}H]-NA through activation of α-BTX-insensitive nicotinic acetylcholine receptors in the human cerebral cortex slices and this action of NIC is associated with modulation of the NO/cGMP pathway.

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Keywords: Nicotine; noradrenaline release; human cerebral cortex

Abbreviations: α-BTX, α-bungarotoxin; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; D-AP5, D(-)-2-amino-5 phosphonopentanoic acid; DHBE, dihydro-β-erythroidine; d-TC, d-tubocurarine; MCA, mechamylamine; NA, noradrenaline; NIC, nicotine; NO, nitric oxide; TTX, tetrodotoxin; VSCC, voltage-sensitive calcium channel

Introduction

Nicotine (NIC) is generally accepted as an active alkaloid in tobacco, it produces many CNS effects, some of which are considered to be beneficial, e.g. mood elevation, arousal, and learning and memory enhancement (Pomerleau, 1992). The behavioral effects of NIC are attributed to its action on the nicotinic acetylcholine receptors in the CNS. Nicotinic acetylcholine receptors are a family of cationic channels composed of five subunits. There are nine different α subunits (α2–α10) and three different β subunits (β2–β4) in mammalian CNS (Lindstrom, 1997; Elgoyhen *et al*., 2001). Nicotinic acetylcholine receptors are suggested to be involved in pathological events in human brain associated with Alzheimer's disease (Nordberg *et al*., 1992), epilepsy (Steinlein *et al*., 1995), schizophrenia (Leonard *et al*., 1996) and Parkinson's disease (Baron, 1996).

Nicotinic acetylcholine receptors are widely distributed in various brain regions of humans and animals (Naeff *et al*., 1992; Rubboli *et al*., 1994). Nicotinic acetylcholine receptors are located not only on cell body and dendrites but also on presynaptic terminals. Nicotinic agonists have been shown to evoke the release of several neurotransmitters from brain regions *via* acting on presynaptic nicotinic acetylcholine receptors (Wonnacott, 1997). It appears that distinct subtypes of presynaptic nicotinic acetylcholine receptors regulate the release of different neurotransmitters. For example, α-bungarotoxin (α-BTX) blocked NIC-evoked release of acetylcholine from rat superior cervical ganglion (Liang & Vizi, 1997) but not of dopamine from mouse striatum (Grady *et al*., 1992).

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The mechanisms underlying the stimulatory effects of NIC on neurotransmitters were proposed. NIC causes conformational changes of nicotinic acetylcholine receptor to increase the cation permeability (Karlin, 1993). Influx of cations to nerve terminals may cause exocytosis independently of voltage-sensitive Ca^{2+} channels (VSCC) (Mulle *et al*., 1992) or depolarization and activation of local VSCC resulting in Ca^{2+}-dependent transmitter release (McGehee *et al*., 1995). Involvement of glutamate in NIC-evoked release of catecholamines has been reported (Toth *et al*., 1992; Wonnacott *et al*., 2000).

Noradrenergic pathways in the cerebral cortex have been suggested to play roles in various animal behaviors, including memory consolidation (Zornetzer *et al*., 1978), anxiety (Redmond & Huang, 1979) and sleep (Aston-Jones & Bloom, 1981). Nicotinic acetylcholine receptors, present in high density in mammalian cerebral cortex (Clarke *et al*., 1985; Paterson & Nordberg, 2000) are also reported to be involved in above listed behaviors (Ague, 1973; Castellano, 1976; Domino & Yamamoto, 1965). In addition, NIC facilitated NA release from rat cerebral cortex in *in vivo* studies using microdialysis (Toth *et al*., 1992) and in *in vitro* superfusion studies using rat (Westfall, 1974) and human (Pittaluga *et al*., 1999) cerebral cortex slices. According to the rank order of potency of agonists, the β4-containing nicotinic acetylcholine receptor has been proposed to be responsible for the stimulatory effect of NIC on NA release in rat cerebral cortex (Anderson *et al*., 2000). Although NIC-induced NA release was reported in human cerebral cortex *via* nicotinic acetylcholine receptor (Pittaluga *et al*., 1999), its exact mechanism is not yet known.

Therefore, the present study aimed to investigate the underlying mechanisms of NIC-evoked release of NA in human cerebral cortex slices.

Methods

Subjects and materials

Written consent having been given by the patients, permission was given for this study by the Ethics Committee of Chorbak National University Hospital. Parts of the temporo-basal region of cerebral cortex were surgically removed from 15 male patients (aged 21.3 ± 4.2 years) who were undergoing neurosurgery for severe epilepsy that was not controlled by anti-epileptic drugs. All had declared a history of 'non-smoking'. After premedication with atropine, fentanyl and flunitrazepam, anaesthesia was induced by thiopental and maintained with nitrous oxide and isoflurane. Pancuronium was used for muscular relaxation. Resected cortical tissue (approximately 1.5 gm) contained in oxygenated ice-cold modified Krebs-HEPES buffer (KHB: 25 mM HEPES-sodium salt, 100 mM NaCL, 5 mM KCl, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM glucose, 0.1 mM ascorbic acid, pH 7.4) was transported to the laboratory. The cortical tissue was weighed, damaged parts removed, about 800 mg was used in [^{3}H]-NA or [^{3}H]-D-aspartate release experiments (400 mg/24-well culture plate), and the remaining tissue (about 300 mg) was stored for use in [^{3}H]-nisoxetine binding assay.

Measurement of [^{3}H]-NA and [^{3}H]-D-aspartate release

Within 5 min after resection, the cortical tissue was chopped into slices (0.25 mm thick, 0.25 mm diameter), and were dispersed in oxygenated KHB. [^{3}H]-NA and [^{3}H]-D-aspartate release were measured essentially as described previously (Werling *et al*., 1987). Briefly, slices were incubated with 15 nM [^{3}H]-NA (specific activity, 62.3 Ci/mmol) at 37°C for 15 min or 50 nM [^{3}H]-D-aspartate at 37°C for 20 min. After three rinses with drug-free KHB, equal aliquots (400 μl) of tissue slice suspensions were transferred to nylon mesh baskets and incubated in 2 ml of oxygenated KHB in 24-well tissue culture plates for 5 min for determination of base-line release. The tissues were then transferred to wells containing NIC or high potassium in KHB for a further 5 min incubation. Finally, the tissues were transferred to wells containing 0.2 N HCl for 45 min to extract the radioactivity present in the tissues. The tritium content in 400 μl of releasate was determined by liquid scintillation counter. Competitive antagonists, enzyme inhibitors and transporter blockers were added 10 min prior to the baseline release period, and were present during the 5 min baseline release and stimulation periods. NIC-stimulated release of [^{3}H]-NA or [^{3}H]-D-aspartate were defined as the amount of tritium release into the medium containing (−) NIC minus the amount of tritium in the control buffer (baseline release), expressed relative to the calculated total tissue content of tritium at the initiation of each release period; and it is presented as fractional release (per cent of tissue content). Drug treatments were tested in quadruplicate in each experiment, and each experiment was replicated at least four times on brain samples from different individuals.

Measurement of [^{3}H]-nisoxetine binding

Immediately after resection (about 1.5 g/patient), part of the temporal cortex was rinsed with ice-cold buffer A (50 mM Tris-HCl, pH 7.4) and kept at −70°C. Pooled cortex samples were homogenized in 10 vol of buffer A. The homogenate was centrifuged at $27{,}000 \times g$ for 15 min at 4°C. The pellet was resuspended in 20 vol of buffer and kept on ice for 60 min to remove any endogenous ligand. The suspension was washed three times and centrifuged at $27{,}000 \times g$ for 15 min at 4°C. Membranes were resuspended in ice-cold buffer and kept at −70°C at a concentration of 2 mg protein/ml until assay. Human cerebral cortex membranes were thawed at room temperature and homogenized by five strokes in a Teflon glass homogenizer. Homogenized membranes were washed twice with 50 mM Tris buffer (pH 7.4) containing 300 mM NaCl, 5 mM KCl and 0.1% BSA. The total volume in each tube was 0.5 ml and triplicate samples were used. The final membrane concentration was adjusted to 500 μg protein/tube. Non-specific binding was determined in the presence of 10 μM desipramine. After incubation at 4°C for 4 h, the incubation was terminated by an addition of 4 ml ice-cold buffer and rapid filtration through glass fibre filters (Type G-7; Inotech, Zurich, Switzerland) under pressure using a cell harvester (Inotech, Switzerland). The filters were washed three times with an additional 3 ml buffer, transferred to scintillation vials, soaked in 0.5 ml of absolute ethanol, and counted in

3 ml scintillation vials using a liquid scintillation counter (Tri-Carb TR-2300; Packard, Groningen, Netherlands) at an efficiency of about 40%. Displacement data were analysed by a computer program LIGAND (Munson & Rodbard, 1980).

Analysis of data

The data are expressed as mean with s.e.mean. EC_{50} value was estimated by fitting the data to a sigmoidal logistic equation using a biostatistical software PRISM (Graph Pad, San Diego, CA, U.S.A.). Results were analysed by one-way ANOVA. When significant treatment effects were found ($P<0.05$), differences between two means were determined by Student's *t*-test for unpaired observations.

Drugs used

1-[7,8-^{3}H]Noradrenaline (55.4 Ci/mmol), [N-methyl-^{3}H]-nisoxetine (67.9 Ci/mmol) and D-[2,3-^{3}H]aspartate (16.1 Ci/mmol) were purchased from Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). (−)-Nicotine tartrate, yohimbine HCl, tetrodotoxin, nitrendipine, ω-conotoxin GVIA, α-bungarotoxin, desipramine, nisoxetine, MK-801, D(−)-2-amino-5-phosphonopentanoic acid, 6-cyano-7-nitro-quinoxaline-2,3-dione, 7-nitroarginine, N^G-nitro-L-aginine methyl ester HCl, N^G-monomethyl-L-arginine acetate, methylene blue, zaprinast and 1*H*-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one were purchased from RBI (Natick, MA, U.S.A.).

Results

NIC caused a concentration-dependent release of [^{3}H]-NA from human cerebral cortex slices. The EC_{50} was 15.3 ± 1.2 μM and the maximal response (1.42 ± 0.04% of total [^{3}H]-NA in tissue slices) was attained at 300 μM of NIC (Figure 1).

Base-line release of preloaded [^{3}H]-NA was 3.19 ± 0.12% ($n=16$) of total [^{3}H]-NA content of human cerebral cortex slices. It was not changed by any treatment in this experiment. NIC caused a concentration-dependent release of [^{3}H]-NA. The EC_{50} was 15.3 ± 1.2 μM and the maximal response (1.42 ± 0.04% over base-line release of total [^{3}H]-NA in tissue slices) was attained at 300 μM of NIC (Figure 1).

In this study, a maximal dose of NIC (100 μM) was chosen to examine the mechanism of NIC-induced release of [^{3}H]-NA. One hundred μM of NIC stimulated 1.40 ± 0.05% of total [^{3}H]-NA in human cerebral cortex slices. We examined the effect of yohimbine, an antagonist for α_2 adrenoceptor, to evaluate the presynaptic regulation of [^{3}H]-NA release induced by NIC in human cerebral cortex slices. Yohimbine (1 μM) did not influence the base-line release, but enhanced the 100 μM of NIC by about 13% (Table 1).

Calcium dependence of the NIC-evoked release of [^{3}H]-NA was studied. In the absence of Ca^{2+} in the incubation media, the NIC-evoked [^{3}H]-NA release was significantly reduced by about 80%. NIC-evoked release of [^{3}H]-NA was attenuated by 0.1 μM of ω-conotoxin GVIA, a selective N-type Ca^{2+} channel blocker (Olivera *et al*., 1984). However, nitrendipine (1 μM), a L-type calcium channel blocker (Watanabe *et al*., 1995), did not influence the NIC-evoked [^{3}H]-NA release (Table 1).

To understand the localization of site of action of NIC, the effect of tetrodotoxin (TTX), a voltage-sensitive sodium-channel blocker, on NIC-induced release of [^{3}H]-NA was examined. TTX (1 μM) significantly attenuated the NIC-evoked release of [^{3}H]-NA (Table 1).

The influence of nisoxetine on the NIC-stimulated release of [^{3}H]-NA was examined to elucidate the involvement of NA transporter in human cerebral cortex. Nisoxetine (0.1 μM), a selective NA transporter blocker, significantly reduced the

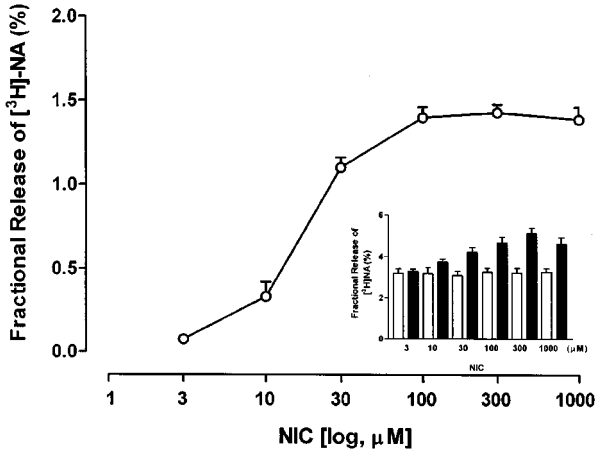

Figure 1 Concentration-response curve for the nicotine (NIC)-evoked [^{3}H]-NA release from human cerebral cortex slices. Each value represents the mean with standard error from four independent experiments from different individuals. NIC-stimulated release of [^{3}H]-NA was defined as the amount of tritium release into the medium containing (−) NIC (dark bar in inserted figure) minus the amount of tritium in the control buffer (baseline release, clear bar in inserted figure), expressed relative to the calculated total tissue content of tritium at the inflation of each release period; and it is presented as fractional release.

Table 1 Influence of yohimbine (Yoh), tetrodotoxin (TTX), removal of Ca^{2+} from incubation media and calcium channel blockers, nitrendipine (NTD) and ω-conotoxin GVIA (ω-Ctx), on the nicotine (NIC, 100 μM)-stimulated release of [^{3}H]-NA from human cerebral cortex slices

Treatment	*Fractional release of* [^{3}H]NA
Control (NIC 100 μM)	1.51 ± 0.05
NIC + Yoh 100 μM	1.74 ± 0.05[a]
NIC in the absence of Ca^{2+} with EGTA 1 mM	0.28 ± 0.02[a]
NIC + NTD 1 0.1 μM	1.47 ± 0.02
NIC + ω-Ctx 0.1 1 μM	0.50 ± 0.04[a]
NIC + TTX 1 μM	0.13 ± 0.04[a]

NIC-stimulated release of [^{3}H]-NA was defined as the amount of [^{3}H]-NA released into the medium containing 100 μM NIC, minus the amount released into the medium containing testing drug (base-line release), expressed relative to the calculated total tissue content of [^{3}H]-NA at the beginning of each release period. Testing drugs were added 10 min prior to the base-line release period, and were present during the 5 min base-line release and stimulation periods. Each value denotes the mean with s.e.mean from six independent experiments on brain samples from different individuals. [a]Significantly different from the value of NIC 100 μM alone according to the analysis of variance (one-way ANOVA) and unpaired Student's *t*-test ($P<0.05$).

NIC-induced [^{3}H]-NA release leaving base-line release unaffected (Table 1).

To find the subtype of nicotinic acetylcholine receptor responsible for the stimulatory effect of NIC on [^{3}H]-NA release in human cerebral cortex, the influence of nicotinic acetylcholine receptor antagonists on the NIC-evoked [^{3}H]-NA release was examined. The concentrations of nicotinic acetylcholine receptor antagonists used in this experiment are known to antagonize the action of NIC on neurotransmitter release in various brain regions (Sacaan *et al*., 1995; Clarke & Reuben, 1996; Kofalvi *et al*., 2000). NIC-evoked release of [^{3}H]-NA was attenuated by mechamylamine (MCA, 10 μM), dihydro-β-erythroidine (DHβE, 10 μM) and *d*-tubocurarine (*d*-TC, 30 μM). However, no effect was observed with α-bungarotoxin (α-BTX, 100 nM) (Figure 2).

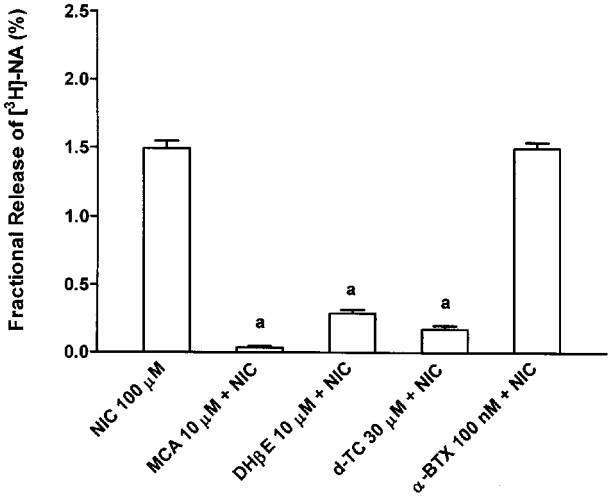

Figure 2 Effect of mechamylamine (MCA, 10 μM), dihydro-β-erythroidine (DHβE, 10 μM) and *d*-tubocurarine (*d*-TC, 30 μM) or α-bungarotoxin (α-BTX, 100 nM) on the nicotine (NIC, 100 μM)-evoked [^{3}H]-NA release from human cerebral cortex slices. Values are means$\pm$s.e.mean of six independent experiments. a: Significantly different from control according to the analysis of variance (one-way ANOVA) and unpaired Student's *t*-test ($P<0.05$).

The influence of antagonists for NMDA receptor on the NIC-evoked release of [^{3}H]-NA was examined in human cerebral cortex slices. The NIC-evoked release of [^{3}H]-NA was not influenced by CNQX (30 μM) and D-AP5 (100 μM), but significantly attenuated by MK-801 (10 μM) (Figure 3b).

MK-801 acts as a NA transporter blocker (Callado *et al*., 2000) and NIC-evoked [^{3}H]-NA release was inhibited by nisoxetine in this experiment. We, therefore, examined the influence of MK-801 on the specific binding of [^{3}H]-nisoxetine in human cerebral cortex membranes. MK-801 displaced the specific binding of [^{3}H]-nisoxetine with a K_i value of 91.2 ± 3.5 μM (Figure 3a). The effect of NIC on the [^{3}H]-D-aspartate release was also examined to check whether NIC induces glutamate release in preparations used in this study. NIC at doses of 30, 100 and 300 μM did not induce the [^{3}H]-D-aspartate release in this preparation.

NIC stimulates nitric oxide (NO) synthase in brain tissues of experimental animals (Fedele *et al*., 1998). The influences of chemicals which can alter NO and/or cGMP levels on the NIC-induced release of [^{3}H]-NA were tested to examine whether the NIC-evoked stimulation of [^{3}H]-NA is related with NO. Inhibitors of NO synthase, 7-nitroindazole (7-NI, 10 μM), N^G-nitro-L-arginine methyl ester (L-NAME, 30 μM) and N-monomethyl-L-arginine (L-NMMA, 300 μM), attenuated the NIC-induced release of [^{3}H]-NA. Inhibitors of guanylate cyclase, methylene blue (Met-Blue, 3 μM) and ODQ (10 μM) also attenuated the [^{3}H]-NA release. Zaprinast (30 μM), a cGMP-phosphodiesterase inhibitor, enhanced the NIC-induced [^{3}H]-NA release in human cerebral cortex slices (Figure 4).

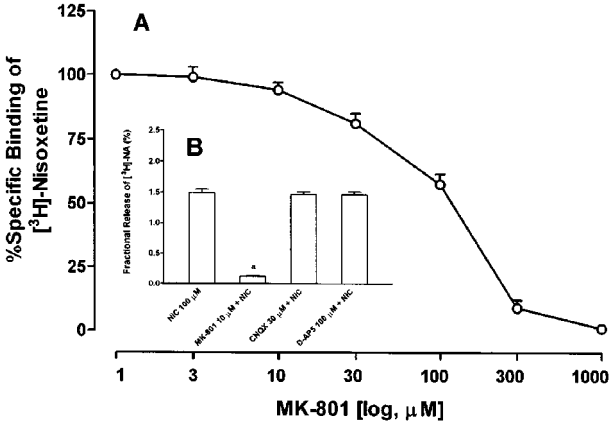

Figure 3 Displacement of specific binding of [^{3}H]-nisoxetine by MK-801 in human cerebral cortex membranes (A). Values are the mean with s.e.mean obtained from four independent experiments. Effect of MK-801 (10 μM), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 30 μM) or D(−)-2-amino-5-phosphonopentanoic acid (D-AP5, 100 μM) on the nicotine (NIC, 100 μM)-evoked [^{3}H]-NA release from human cerebral cortex slices. (B) Values are means$\pm$s.e.mean of six independent experiments. a: Significantly different from control according to the analysis of variance (one-way ANOVA) and unpaired Student's *t*-test ($P<0.05$).

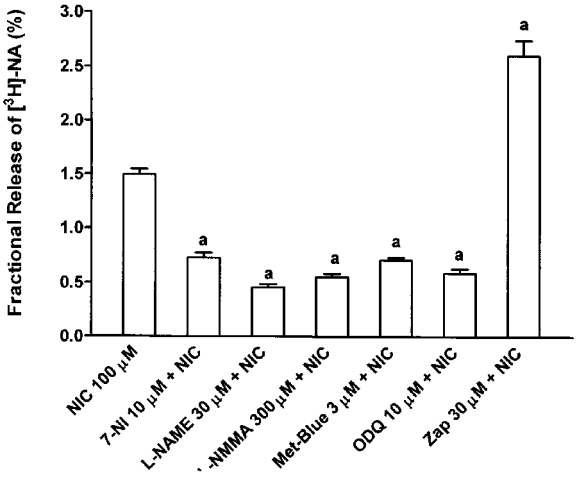

Figure 4 Effect of 7-nitroarginine (7-NI, 10 μM), N^G-nitro-L-arginine methyl ester (L-NAME, 30 μM), N^G-monomethyl-L-arginine (L-NMMA, 300 μM), methylene blue (Met-Blue, 3 μM), 1*H*-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ, 10 μM) or zaprinast (Zap, 30 μM) on the nicotine (NIC, 100 μM)-evoked [^{3}H]-NA release from human cerebral cortex slices. Values are means$\pm$s.e.mean of six independent experiments. a: Significantly different from control according to the analysis of variance (one-way ANOVA) and unpaired Student's *t*-test ($P<0.05$).

Discussion

In the present study, NIC stimulated [^{3}H]-NA release in a dose-dependent manner. The EC_{50} value for NIC obtained in this study (15 μM) is higher than those previously reported in frontal cortex of the rat (2 μM) (Anderson *et al*., 2000) and lower than that in hippocampal slices and locus ceruleus (LC) of the rat, 0.9 and 6.5 μM, respectively (Clarke & Reuben, 1996; Gallardo & Leslie, 1998). The reason for this discrepancy is not known. It could be related to differences in tissue preparations, species used, time of exposure to NIC or the involvement of different type(s) of nicotinic acetylcholine receptor. The NIC-evoked release of [^{3}H]-NA was potentiated in the presence of yohimbine, an α_2 adrenoceptor blocker. The presynaptic α_2 adrenoceptor-mediated feedback regulation of NA release in human cerebral cortex (Raiteri *et al*., 1992a) and the enhancement by α_2 adrenoceptor blockers of NIC-induced release of NA in bovine cerebral arteries have been reported (Sanchez-Merino *et al*., 1991). Our data suggests that the α_2 adrenoceptor operates on the process of NA release induced by NIC in human cerebral cortex.

Removal of Ca^{2+} from incubation media attenuated the NIC-evoked release of [^{3}H]-NA up to 80% in human cerebral cortex slices. Nicotinic acetylcholine receptors are capable of increasing intracellular Ca^{2+} concentration because of their high Ca^{2+} permeability (Mulle *et al*., 1992). Besides this action of NIC on their receptors, the contribution of VSCCs in stimulation of neurotransmitters release has been reported. Nicotine-evoked dopamine release was inhibited by ω-conotoxin GVIA, a N-type VSCC blocker, in rat striatum (Harsing *et al*., 1992). N-type VSCCs are also known to be involved in NA release evoked by NIC in bovine chromaffin cells (Fernandez *et al*., 1995). In the present study, NIC-evoked release of [^{3}H]-NA was not attenuated by nitrendipine, a L-type VSCC blocker, but by ω-conotoxin GVIA. L-type VSCC is located on neuronal cell bodies and not usually involved in neurotransmitter exocytosis in nerve terminals. Whereas N-type VSCC has been shown to be presented in nerve terminals, where they play a key role in neurotransmitter release. Our data shows the involvement of N-type, but not L-type, VSCC in NIC-evoked release of [^{3}H]-NA in human cerebral cortex slices. However, it has been reported that ω-conotoxin GVIA is reported to be a potent and reversible blocker of the nicotinic acetylcholine receptor-induced currents in the chromaffin cells (Fernandez *et al*., 1995). Thus, it is probable that entry of monovalent cations and Ca^{2+} through nicotinic acetylcholine receptors induces depolarization following activation of VSCCs as demonstrated in porcine adrenal chromaffine cells (Li & Forsberg, 1996). As a consequence of the elevated intracellular Ca^{2+} level due to Ca^{2+} influx through nicotinic acetylcholine receptors and activation of VSCCs, [^{3}H]-NA release from the vesicular pool may be triggered in human cerebral cortex slices in this study.

TTX has been used as a tool to exclude indirect drug effects due to its pharmacological property as a blocker of the voltage-dependent Na^+ channels. TTX-dependence of neurotransmitter release evoked by nicotinic agonists can vary according to the brain regions, tissue preparations, neurotransmitter systems and studies of the same system. Nicotine-induced dopamine release has been found TTX-sensitive in the striatum and nucleus accumbens but not in the cerebral cortex in *in vivo* microdialysis (Marshall *et al*., 1997). In striatal slices, dopamine release induced by nicotinic agonists is sensitive or insensitive to TTX (Sacaan *et al*., 1995). The NA release induced by nicotinic acetylcholine receptor stimulation was inhibited by TTX in hippocampal slices (Sacaan *et al*., 1995) but not in synaptosomal preparation (Clarke & Reuben, 1996). In the present study, NIC-evoked [^{3}H]-NA release from human cerebral cortex slices was substantially inhibited by TTX. This result suggesting that at least part of the NIC-evoked [^{3}H]-NA release is dependent on Na^+ channels modulating axonal conduction. In a view of the very varicous nature of noradrenergic neurons in CNS, TTX may inhibit NIC-evoked [^{3}H]-NA release by acting on the preterminal nicotinic acetylcholine receptors, the term introduced by Wessler (1992). However, it is apparent that TTX do not directly inhibit nicotinic acetylcholine receptor function (Marks *et al*., 1995; Marshall *et al*., 1996).

In this study, NIC-evoked release of [^{3}H]-NA was attenuated by nisoxetine, a selective NA transporter blocker in human cerebral cortex slices. Inhibition of nicotinic acetylcholine receptor-mediated release of NA by monoamine transporter blockers has been reported in rat hippocampal slices (Hennings *et al*., 1999). Nicotinic acetylcholine receptor-mediated currents are inhibited by tricyclic antidepressants in SH-SY5Y cells (Rana *et al*., 1993). In *in vivo* studies, behavioural effects of NIC were inhibited by cocaine and its analogues (Lerner-Marmarosh *et al*., 1995). Therefore, our results confirm the blockade of nicotinic acetylcholine receptor-mediated effects by monoamine transporter blocker. However, the exact mechanism of blockade of NIC-evoked release of [^{3}H]-NA by nisoxetine is not clear. It has been reported that monoamine transporter blockers have affinity to channel site of nicotinic acetylcholine receptors (Lerner-Marmarosh *et al*., 1995). There is no correlation between the inhibitory effect of monoamine transporter blockers on NA uptake and on NIC-evoked release of NA (Hennings *et al*., 1999). Thus the antagonism of NIC-evoked [^{3}H]-NA release by nisoxetine can be explained by the antagonistic action of this compound on nicotinic acetylcholine receptors. Contrary to our results, Anderson *et al*. (2000) reported the potentiation of NIC-evoked release of [^{3}H]-NA by nisoxetine in rat hippocampal slices. This discrepancy may be due to the difference in the concentration of nisoxetine. Therefore, the exact mechanism of this action of nisoxetine remains to be elucidated.

In this study, we examined the sensitivity of NIC-evoked release of [^{3}H]-NA to several nicotinic acetylcholine receptor antagonists to identify the responsible receptor subtype(s). In human cerebral cortex slices, MCA, a noncompetitive nicotinic acetylcholine receptor channel blocker, and *d*-TC, a nonselective competitive nicotinic acetylcholine receptor antagonist, inhibited the NIC-evoked [^{3}H]-NA release, indicating that the action of NIC was mediated by nicotinic acetylcholine receptor. The MCA-sensitive [^{3}H]-NA release, by NIC in human cerebral cortex slices has been reported previously (Pittaluga *et al*., 1999). It is also known that there are two main sub-populations, $\alpha4\beta2$ and $\alpha7$, of nicotinic acetylcholine receptors in human brain (Paterson & Nordberg, 2000). DHβE has been known to be an antagonist for α-BTX-insensitive neuronal types of nicotinic acetylcholine receptor, including $\alpha4\beta2$ nicotinic acetylcholine receptor (Decker *et al*., 1995). In our study, NIC-evoked release of

[^{3}H]-NA was inhibited by DHβE, indicating that the effect of NIC was mediated by α/β heteromeric receptors. It is unlikely that the α7 homomeric nicotinic acetylcholine receptor is involved in NIC-evoked release of [^{3}H]-NA because α-BTX had no effect. Our results indicate that the [^{3}H]-NA release by NIC may be attributed to the action on the α-BTX-insensitive neuronal type of nicotinic acetylcholine receptor in human cerebral cortex.

Nicotine stimulation of glutamate release has been reported in various regions of the rat brain (Perez de la Mora *et al*., 1991). Evidence has been provided for the involvement of glutamate in NIC-evoked release of catecholamines (Toth *et al*., 1992; Wonnacott *et al*., 2000). In human cerebral cortex slices, release of [^{3}H]-NA by stimulation of ionotropic glutamate receptors (Fink *et al*., 1992) and inhibition of NIC-evoked release of [^{3}H]-NA by antagonists for these sites (Pittaluga *et al*., 1999) in human cerebral cortex have been reported. Thus, we tried to verify the involvement of glutamate in NIC-evoked release of [^{3}H]-NA. In accordance with a previous report, MK-801 attenuated the NIC-evoked release of [^{3}H]-NA from human cerebral cortex. However, CNQX, an antagonist of the non-NMDA receptors, and D-AP5, a competitive NMDA receptor antagonist, at concentrations reported to antagonize the stimulatory effect of glutamate on NA release (Raiteri *et al*., 1992b; von Kugelgen & Starke, 1995) did not influence the NIC-evoked [^{3}H]-NA release. Our findings do not agree with the study showing that NIC-evoked [^{3}H]-NA release was inhibited by a competitive blocker of NMDA receptors, CGS-19755 (Pittaluga *et al*., 1999). To address this issue, we examined the effect of NIC on the [^{3}H]-D-aspartate release. [^{3}H]-D-aspartate, the unmetabolizable analogue of glutamate, has been widely used to study the activity of the glutamergic neurons. Similar characteristics of release of endogenous glutamate and [^{3}H]-D-aspartate in various preparations of the rat hippocampus have been reported (Muzzolini *et al*., 1997). In the present study, NIC failed to induce release of [^{3}H]-D-aspartate at concentrations that are used to stimulate [^{3}H]-NA release. These results, and the lack of antagonism of NIC-evoked release of [^{3}H]-NA by CNQX and D-AP5, suggest that the endogenous glutamate may not be involved in NIC-evoked release of [^{3}H]-NA in human cerebral cortex slices. It should be noted that our experimental system may not be sensitive enough to measure the NIC-evoked release of [^{3}H]-D-aspartate. Antagonism of NIC-evoked release of [^{3}H]-NA by MK-801 could be attributed to direct inhibition of nicotinic acetylcholine receptor function (Ramoa *et al*., 1990). The other possible mechanism could be inhibition of transporter-mediated release of [^{3}H]-NA by NIC. Findings that inhibition of specific binding of [^{3}H]-nisoxetine in our present study and [^{3}H]-desmethylimipramine (Rogers & Lemaire, 1992) by MK-801 indicate this compound has affinity to NA transporter. Inhibition of NA uptake by MK-801 further supports this hypothesis (Callado *et al*., 2000).

Nitric oxide is a gaseous messenger molecule, and is formed by NO synthase (NOS), an enzyme requiring Ca^{2+}-calmodulin, in response to an increase in intracellular Ca^{2+} ions (Moncada *et al*., 1991; Garthwaite, 1995). Localization of NOS and its activation by depolarization in human cerebral cortex has been reported (Egberongbe *et al*., 1994; Fontana *et al*., 1997). NO activates guanylyl cyclase and causes the elevation of cGMP that is involved in neurotransmission (Garthwaite, 1995). Neuronal nicotinic acetylcholine receptors activation elevates the intracellular Ca^{2+} concentration through receptors themselves owing to their Ca^{2+} permeability (Mulle *et al*., 1992) and VSCC (Li & Forsberg, 1996). Nicotine-induced NO release has been demonstrated in the hippocampal slices (Smith *et al*., 1998) and in various brain regions in *in vivo* experiments (Pogun *et al*., 2000). There has been accumulating evidence for a role of nitric oxide in the pharmacological actions of NIC. Enhancement of active avoidance learning (Yilmaz *et al*., 2000) and cGMP generation (Fedele *et al*., 1998) by systemically administered NIC are abolished by NOS inhibition in rats. It is well established that NO modulates various neurotransmitters (Prast & Philippu, 2001). NO donors increase the release of NA in the cerebral cortex (Montague *et al*., 1994). Reversal of NA transporter is involved in stimulation of NA release by both NO and nicotinic acetylcholine receptor agonists (Lonart & Johnson, 1995; Kiss *et al*., 1996). In the present study, we examined the involvement of NO in the stimulatory effect of NIC on [^{3}H]-NA release in human cerebral cortex slices. In human cerebral cortex slices, NIC-evoked release of [^{3}H]-NA was inhibited by NOS inhibitors (7-NI, L-NMMA and L-NAME), indicating that NO is involved. Attenuation of NIC-evoked release of [^{3}H]-NA by methylene blue and ODQ, inhibitors of guanylyl cyclase, and potentiation by zaprinast, a cGMP-phosphodiesterase inhibitor, indicate the involvement of cGMP. These results in the present study suggest that NO is involved in NIC-evoked release of [^{3}H]-NA release in human cerebral cortex slices.

In summary, the present study demonstrates that NIC stimulates the release of [^{3}H]-NA through activation of α-BTX-insensitive nicotinic acetylcholine receptors in the human cerebral cortex slices. Pharmacological characterization indicates that modulation of the NO/cGMP pathway is involved in the action of NIC. Nicotinic acetylcholine receptors in the cerebral cortex are of particular interest because of their possible roles in cognition, memory, arousal, attention and anxiety (Levin, 1992). A reduction of nicotinic acetylcholine receptors in the cerebral cortex has been reported both *in vitro*, with postmortem brain tissue (Flynn & Mash, 1986), and *in vivo*, with positron emission tomography (Nordberg *et al*., 1992). Lesions of the LC noradrenergic system result in deficits in sustained attention (Carli *et al*., 1983) and shifting attention (Devauges & Sara, 1990). Impaired learning associated with decreased cortical NA level was demonstrated in rats with lesions of the LC (Anlezark *et al*., 1973). Results from the present study provide a neurochemical background for the cognitive enhancing property of NIC in human (Sunderland *et al*., 1988). We should point out that long-term treatment with anti-epileptic drugs might affect nicotinic acetylcholine receptors (Diamond *et al*., 1983; Kamiya *et al*., 2001; Yoshimura *et al*., 1995). Cortical samples used in our experiment were obtained from patients who had been treated with various anti-epileptic drugs for various periods without complete suppression of seizures. In addition, mutated nicotinic acetylcholine receptors are related with epilepsy (Kuryatov *et al*., 1997).

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