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Nitric oxide modulates the release of acetylcholine in the ventral striatum of the freely moving rat

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Abstract The influence of nitric oxide on acetylcholine release in the ventral striatum was investigated by the push-pull superfusion technique in the conscious, freely moving rat. Superfusion with the nitric oxide donors S-nitroso-N-acetylpenicillamine or with 3-morpholino-sydnonimine caused a pronounced increase in striatal acetylcholine release. This effect was prevented by superfusion with tetrodotoxin. Pre-superfusion with the guanylyl cyclase inhibitor methylene blue abolished the effect of 3-morpholino-sydnonimine. Superfusion of the ventral striatum with the guanylyl cyclase inhibitor LY83583 decreased acetylcholine release by 60% of basal release, whereas the less specific guanylyl cyclase inhibitor methylene blue was ineffective in this respect. Superfusion of the ventral striatum with inhibitors of nitric oxide synthase also led to different effects on basal acetylcholine release. Superfusion with L-N^G-methylarginine did not influence basal acetylcholine release, whereas superfusion with L-N^G-nitroarginine or with L-N^G-nitroarginine methyl ester led to a substantial decrease in acetylcholine output, the latter compound being more effective. The effect of L-N^G-nitroarginine was abolished by simultaneous superfusion with L-arginine.

The effects of NO donors and of LY83583 suggest that NO increases acetylcholine release, probably by a cGMP-dependent mechanism. The effectiveness of nitric oxide synthase inhibitors shows that the activity of striatal neurons is under the permanent influence of nitric oxide, that leads, via a direct or indirect mechanism, to continuous enhancement of acetylcholine release.

In conclusion, our findings suggest that NO synthesized in the ventral striatum acts as an intercellular messenger which modulates acetylcholine release.

Key words Acetylcholine release · Striatum · Nitric oxide · Push-pull technique

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Introduction

Nitric oxide (NO) is now considered to be a genuine second messenger in the central nervous system. NO is released post-synaptically upon stimulation of glutamate release sites. It diffuses rapidly and exerts its actions in surrounding target cells by increasing cGMP synthesis (Garthwaite 1991). In cerebellum of rats and mouse brain, cGMP has been shown to increase upon activation of ionotropic glutamate receptors (Wood et al. 1990). NO synthesis in the brain has gained special interest because NO seems to be involved in learning and memory (Böhme et al. 1993; Estall 1993). Long-term potentiation of synapses in the hippocampal CA1 region and long-term depression in the cerebellum have been shown to depend on the synthesis on NO (Haley et al. 1992; Shibuki and Okada 1991). It seems likely that NO acts, via cGMP dependent mechanisms, by modulating the release of certain neurotransmitters. Indeed, we have shown that superfusion of the basal forebrain with the inhibitor of NO-synthase L-N^G-nitroarginine (LNA) decreases the striatal acetylcholine output and that this effect is abolished by superfusion with the NO-donor 3-morpholino-sydnonimine (SIN-1) (Prast and Philippu 1992a). The striatum contains the highest activities of guanylyl cyclase and cGMP phoshodiesterase in the brain (Greenberg et al. 1978). Recently, it has been reported that NO may stimulate vesicular release independent of intracellular calcium and cGMP elevation (Meffert et al. 1994).

Aim of the present study was to further investigate the role of NO with respect to striatal cholinergic

neurons. For this purpose, the ventral striatum of conscious, freely moving rats was superfused by the push-pull technique with artificial cerebrospinal fluid (CSF) and the released acetylcholine detected in the superfusate. The experimental strategy included superfusion of the ventral striatum with NO donors, with inhibitors of NO synthase and with inhibitors of guanylyl cyclase.

Materials and methods

Push-pull technique. Male Wistar rats (230–260 g) were anaesthetized with sodium pentobarbital (40 mg/kg, i.p.) and ketamine (50 mg/kg, i.p.). The head was fixed in a stereotaxic frame. A guide cannula (outer diameter 1.25 mm, inner diameter 0.90 mm) with its stylet was stereotaxically inserted according to the atlas of Paxinos and Watson (1986) until the tip of the stylet was 2 mm above the left ventral striatum (coordinates of the stylet tip in mm: AP 1.0, L 2.4, V 5.5). The guide cannula was fixed with screws and dental cement. Two days after anaesthesia and operation, the stylet was removed and replaced by a push-pull cannula (outer needle: outer diameter 0.85 mm, inner diameter 0.5 mm; inner needle: outer diameter 0.2 mm, inner diameter 0.1 mm). The push-pull cannula was 2 mm longer than the guide cannula so as to reach the ventral striatum. The ventral striatum of the conscious, freely moving rat was superfused with CSF pH 7.2 which contained 1 µmol/l neostigmine. The superfusion rate was 20 µl/min. The superfusate was continuously collected in time periods of 10 min at − 25° C. Drugs were dissolved in CSF. Solutions of SIN-1 and of S-nitroso-N-acetylpenicillamine (SNAP) were prepared immediately before use and protected from light.

At the end of the experiment, the animal was sacrificed by an overdose of sodium pentobarbital, the brain removed and immersed in formaldehyde solution (4%). The position of the cannula was verified in histological slices stained with cresyl violet and luxol fast blue (Klüver and Barrera 1953). Experiments with unsatisfactory cannula localizations were discarded.

Determination of acetylcholine. Acetylcholine was determined in the superfusate by HPLC with electrochemical detection as described by Damsma et al. (1987) and modified by Prast and Philippu (1992b). Briefly, the HPLC equipment consisted of a pump (Kontron, 422), a pulse damper, a presaturation column (chromspher 5 C18, 100 × 3 mm), an injector (Rheodyne 7125) equipped with a 100 µl loop, a precolumn (chromspher 5 C18, 10 × 2.1 mm), an analytical column (chromspher 5 C18, 70 × 2 mm) which was converted to a cation exchange column by loading with sodium lauryl sulphate (100 mg/20 ml), a postcolumn enzyme reactor (10 × 2.1 mm) filled with lichrosorb-NH_2 and activated with glutaraldehyde (5 v/v% in 0.1 mol/l sodium hydrogen carbonate, pH 8.5) to which the enzymes acetylcholinesterase and choline oxidase were covalently bound, and an electrochemical detector (Bioanalytical Systems, West Lafayette, In., USA). The mobile phase, consisted of 100 mmol/l phosphate buffer, 5 mmol/l KCl, 1 mmol/l tetramethylammoniumhydroxid, 0.1 mmol/l EDTA, 1.0 ml/l, Kathon^R CG, pH 7.9. Acetylcholine was hydrolyzed to acetate and choline at the postcolumn reactor. Choline was subsequently oxidized by the choline oxidase to betaine and hydrogen peroxide which was electrochemically detected by a platinum electrode at + 500 mV. Acetylcholine was quantified using calibration curves from external standards that were injected at the beginning and at the end of the analysis. The detection limit for acetylcholine (signal/noise ratio = 3/1) was 30 fmol/sample.

Determination of nitrite. The formation of nitrite from the NO-donors was determined by the method of Green et al. (1982) as modified by Werner-Felmayer et al. (1993). The NO-donors were dissolved in CSF at 22° C and in equilibrium with air.

Drugs and reagents. SIN-1 (Cassella-AG, Frankfurt, Germany); SNAP (Dr. G. Kojda, Dept. of Pharmacology, Heinrich-Heine-Universität, Düsseldorf, Germany); 6-anilino-5, 8-quinolinedione (LY83583) (Calbiochem-Novabiochem. Corp., La Jolla, Calif., USA); L-arginine, L-N^G-methylarginine (LMA), L-N^G-nitroarginine (LNA), L-N^G-nitroarginine methyl ester (LNAME), choline oxidase (E.C.1.1.3.17), tetrodotoxin, methylene blue (Sigma, Deisenhofen, Germany); acetylcholinesterase (E.C.3.1.1.7.), (Boehringer, Mannheim, Germany); Chromspher 5 C18, (Chrompack, Middleburg, The Netherlands); Lichrosorb NH_2, (Merck, Darmstadt, Germany); Kathon^R CG, (Rohm & Haas, Frankfurt/Main, Germany).

Statistical analysis. Data were analyzed by Friedman's analysis of variance followed by Wilcoxon's signed-rank test for paired data. The two samples before superfusion with a drug were taken as control values.

Results

The mean basal release rate of acetylcholine in the ventral striatum was 342 ± 11 fmol/min (mean value ± SEM, $n = 81$). The release rate was fairly constant for at least 6 hours. Spontaneous changes in the release rate were negligible (Fig. 1), provided that acoustic or other exogenous, disturbing factors were avoided (not shown). Superfusion with 1 µmol/l tetrodotoxin decreased the acetylcholine output by 75%. The inhibitory effect of tetrodotoxin continued for at least 30 min after termination of superfusion with the drug (Fig. 1).

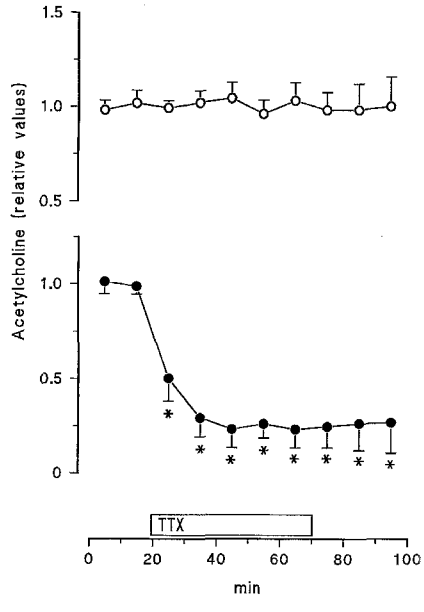

Fig. 1 Effect of superfusion with tetrodotoxin (1 µmol/l) on the release of acetylcholine in the ventral striatum. *Open circles*: basal release ($n = 8$) ; *Solid circles*: Superfusion with tetrodotoxin (TTX, 1 µmol/l, $n = 7$). *Horizontal bar* indicates the superfusion with the drug. The mean release rates in the first two samples of each group (basal release 295 ± 13 fmol/min; TTX 323 ± 28 fmol/min mean values ± SEM) were taken as 1.0. Mean values and SEM as *vertical bars*. $^*P < 0.05$

Effects of NO donors

The kinetics of NO formation from SNAP and SIN-1 (200 µmol/l in CSF at 22° C) were analyzed by determining the nitrite formation. The rate of nitrite formation from SIN-1 (0.64 ± 0.09 µmol/l.10 min, $n = 8$) was lower than that from SNAP (1.51 ± 0.38 µmol/l.10 min, $n - 10$). At a concentration of 200 µmol/l, a linear increase in nitrite concentration over 25 min was observed (not shown). The formation rate of nitrate was found to be negligible.

Superfusion of the ventral striatum with SIN-1 (100 or 200 µmol/l) caused a concentration-dependent increase in acetylcholine release. Superfusion with 200 µmol/l induced a pronounced enhancement which lasted for 30 min, whereas 100 µmol/l tended to cause a short-lasting increase of acetylcholine release. The effect of SIN-1 was prevented by superfusion with 1 µmol/l tetrodotoxin (Fig. 2).

Like SIN-1, superfusion with SNAP (200 µmol/l) for 10 min increased striatal acetylcholine release. The SNAP-induced effect was also abolished by 1 µmol/l tetrodotoxin (Fig. 3).

Superfusion of the ventral striatum with SIN-1C (200 µmol/l) or penicillamine (200 µmol/l), the decomposition products of SIN-1 and of SNA respectively, was ineffective (not shown).

Effects of guanylyl cyclase inhibitors

Since NO can exert its effects via stimulation of cGMP synthesis, we tested the effect of two guanylyl cyclase inhibitors on acetylcholine release. Superfusion with LY83583 (100 µmol/l) lowered acetylcholine output to about 50% of the basal value. On the other hand, superfusion with 50 µmol/l methylene blue for 40 min reduced the release rate in only 4 out of 6 experiments, with the mean results showing no decrease (Fig. 4).

Methylene blue was also used to determine whether the effect of SIN-1 depends on cGMP formation. In control experiments, in which the striatum was superfused with CSF, addition of 200 µmol/l of SIN-1 for 20 min led to a pronounced increase in acetylcholine release. Presuperfusion with methylene blue (50 µmol/l for 40 min (not shown) abolished the effect of SIN-1 on the release of acetycholine (Fig. 5).

Effects of NO synthase inhibitors

To investigate whether the basal acetylcholine release in the striatum is under influence of a continuous NO output, the ventral striatum was superfused with 3 different inhibitors of NO synthase for 40 min. Superfusion of the ventral striatum with LNA or LNAME

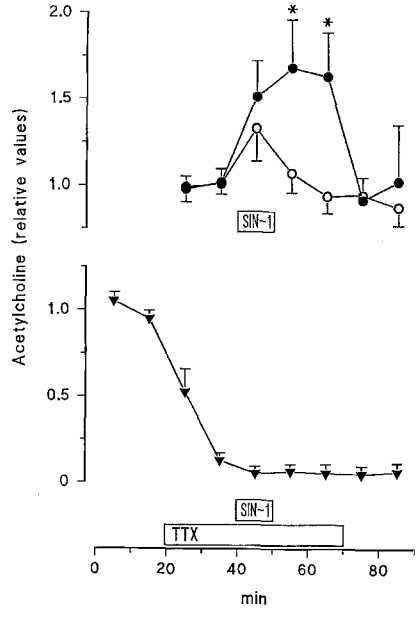

Fig. 2 Effect of superfusion with SIN-1, or with SIN-1 in the presence of tetrodotoxin, on the release of acetylcholine in the ventral striatum. *Open circles*: Superfusion with 100 µmol/l SIN-1 ($n = 6$); *Solid circles*: Superfusion with 200 µmol/l SIN-1 ($n = 7$) . *Solid triangles*: Superfusion with 1 µmol/l tetrodotoxin (TTX) and 200 µmol/l SIN-1 ($n = 4$) . *Horizontal bars* indicate begin and durations of superfusions with drugs. The mean release rates in the first two samples of each group (SIN-1 100 µmol/l 335 ± 93 fmol/min; SIN-1 200 µmol/l 559 ± 69 fmol/min; SIN-1 and TTX 409 ± 81 fmol/min mean values ± SEM) were taken as 1.0. Mean values and SEM as *vertical bars*. $^*P < 0.05$

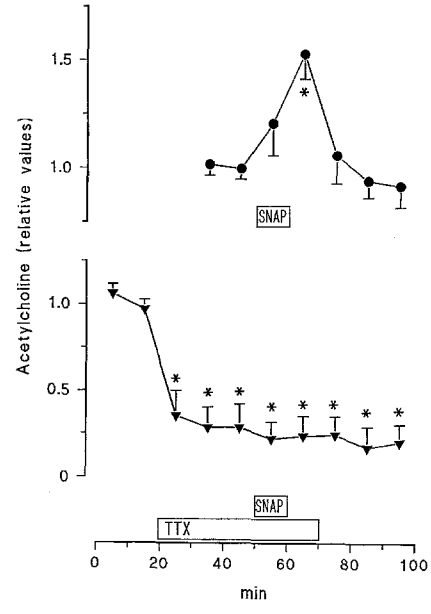

Fig. 3 Effect of superfusion with SNAP, or of superfusion with SNAP in the presence of tetrodotoxin, on acetylcholine release in the ventral striatum. *Open circles*: Superfusion with SNAP (200 µmol/l, $n = 7$); *solid circles*: Superfusion with tetrodotoxin (TTX; 1 µmol/l) and SNAP (200 µmol/l, $n = 5$). *Horizontal bars* indicate begin and duration of superfusions with drugs. The mean release rates in the first two samples of each group (SNAP 230 ± 99 fmol/min TTX and SNAP 286 ± 96 fmol/min mean values ± SEM) were taken as 1.0. Mean values and SEM as *vertical bars*. $^*P < 0.05$

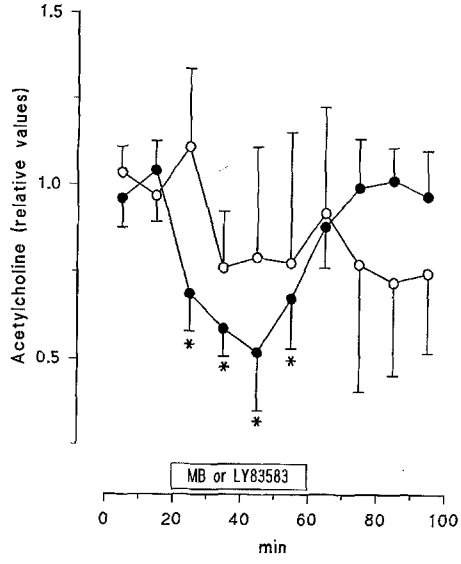

Fig. 4 Effect of superfusion with methylene blue or LY83583 on acetylcholine release in the ventral striatum. *Open circles*: Superfusion with methylene blue (MB, 50 µmol/l, $n = 6$); *Solid circles*: Superfusion with LY83583 (100 µmol/l, $n = 8$). *Horizontal bar* indicates begin and duration of superfusions with drugs. The mean release rates in the first two samples of each group (MB 453 ± 57 fmol/min; LY83583 430 ± 69 fmol/min; mean values ± SEM) were taken as 1.0. Mean value and SEM as *vertical bars*. $*P < 0.05$

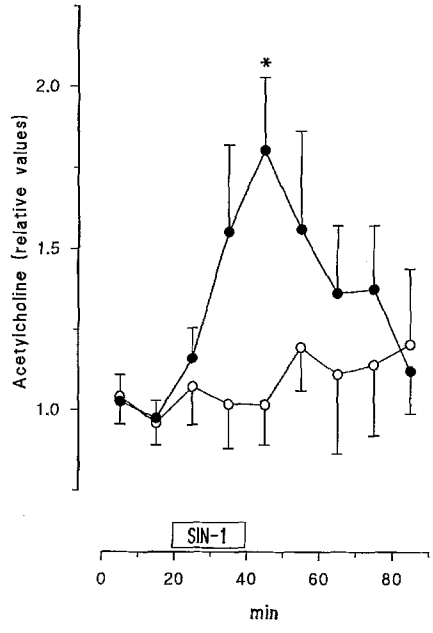

Fig. 5 Effect of presuperfusion with methylene blue on the SIN-1-induced enhancement of acetylcholine release in the ventral striatum. *Solid circles*: Superfusion with SIN-1 (200 µmol/l, $n = 7$). *Open circles*: The striatum was presuperfused with methylene blue (MB; 50 µmol/l) for 40 min (not shown); superfusion with CSF (control values) started 20 min prior to superfusion with SIN-1 (200 µmol/l, $n = 5$). *Horizontal bar* indicates time of superfusion with SIN-1. The mean release rates in the two samples before perfusion with SIN-1 of each group (SIN-1 303 ± 39 fmol/min; SIN-1 after MB 289 ± 45 fmol/min; mean values ± SEM) were taken as 1.0. Mean values and SEM as *vertical bars*. $*P < 0.05$ versus control values

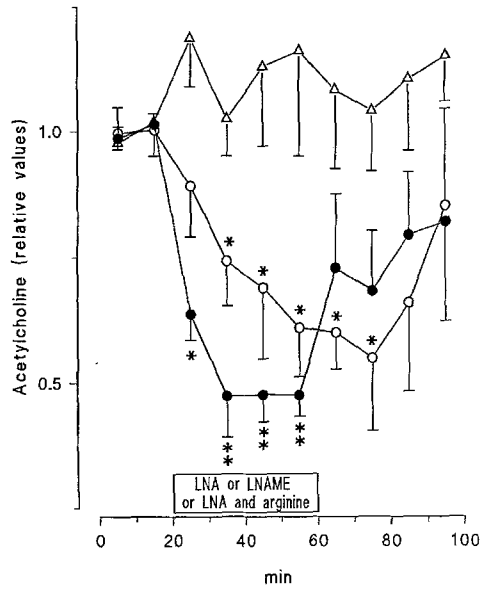

Fig. 6 Effect of superfusion with L-N^G-nitroarginine methyl ester, with L-N^G-nitroarginine or simultaneously with L-N^G-nitroarginine and L-arginine on acetylcholine release in the ventral striatum. *Solid circles*: Superfusion with L-N^G-nitroarginine methyl ester (LNAME, 100 µmol/l, $n = 10$). *Open circles*: superfusion with L-N^G-notroarginine (LNA, 100 µmol/l, $n = 10$). *Triangles*: Superfusion with LNA (100 µmol/l) and L-arginine (500 µmol/l; $n = 6$). *Horizontal bar* indicates begin and duration of superfusions with drugs. The mean values in the first two samples of each group (LNAME 258 ± 19 fmol/min; LNA 311 ± 35 fmol/min; LNA and L-arginine 295 ± 23; mean values ± SEM) were taken as 1.0. Mean values and SEM as *vertical bars*. $*P < 0.05$, $**P < 0.01$

(100 µmol/l each) led to a pronounced and sustained decrease in acetylcholine release. LNAME seemed to be more effective, but the inhibition elicited by LNA lasted longer than that of LNAME. After termination of superfusion with the drugs, the release rate of acetylcholine gradually returned to basal level. When the ventral striatum was superfused simultaneously with LNA (100 µmol/l) and with L-arginine (500 µmol/l), the LNA-induced decrease was abolished (Fig. 6).

Superfusion with LMA (50 or 100 µmol/l) was ineffective (not shown).

Discussion

When acoustic or other exogenous stimuli were carefully avoided, the release rate of acetylcholine in the striatum of the freely moving rat was fairly constant for at least 6 h. Under these conditions, spontaneous changes in the release rate were negligible. These experimental conditions seem to be substantial when pharmacological or behavioural influences are investigated in the conscious, freely moving rat.

Superfusion of the striatum with tetrodotoxin reduced the acetylcholine output by at least 75%, indicating its

high dependency on action potentials and demonstrating the suitability of the push-pull technique for monitoring the in vivo release of acetylcholine.

Before starting superfusion experiments with NO donors, it was necessary to study the kinetics of their decomposition in CSF at room temperature because about 12 min elapsed between preparing SIN-1 or SNAP solutions and superfusion of the striatum with the drugs. Due to the low decomposition rate, only a negligible amount of the compounds decomposed before reaching the superfused tissue. The higher rate of nitrite formation of SNAP indicates that this donor generated NO at a higher rate than SIN-1.

When slices of the cerebellum are perfused, concentrations of 100 μmol/l of SIN-1 or of 20 μmol/l of SNAP are required to induce considerable cGMP synthesis (SIN-1: EC_{50} = 200 μmol/l, SNAP: EC_{50} = 50 μmol/l, maximum effective concentrations above 1 mmol/l; Southam and Garthwaite 1991). Moreover, as a consequence of the rapid decay of NO, 100 to 200 μmol/l of the NO donors in the extracellular environment seem to be necessary to produce NO concentrations in the range of endogenous NO in cerebellum and in smooth muscle (Southam and Garthwaite 1991). From these findings it might be concluded that superfusion with SIN-1 and SNAP at concentrations of 200 μmol/l is a rather physiological tool to study the significance of NO in distinct brain regions.

Superfusion of the ventral striatum with 200 μmol/l of SIN-1 or SNAP increased acetylcholine release. SIN-1 exerted a more pronounced and longer lasting effect, a finding which is not easy to interprete when the lower NO formation rate of SIN-1 is considered. In slices of the cerebellum, SNAP stimulates cGMP synthesis more effectively than SIN-1 (Southam and Garthwaite 1991). Possibly, the higher in vivo-effectiveness of SIN-1 might be due to SIN-1 and SNAP having different physico-chemical properties of these molecules. By the push-pull technique, the NO-dependency of SIN-1- or SNAP-induced effects cannot be shown by the use of hemoglobin as a scavenger, because hemoglobin diffuses at a very low rate into the tissue, thus reaching only a part of the NO donor-influenced area. However, it was proven by presuperfusion with methylene blue that the effect of SIN-1 depended on guanylyl cyclase activity.

Indeed, our results demonstrate that even when a high and prolonged enhancement of acetylcholine release is induced by superfusion with SIN-1, this strong effect is clearly abolished by presuperfusion with methylene blue. Hence, the mechanism of action of SIN-1 and, very probably, SNAP, appears to be based on stimulation of cGMP synthesis by NO.

Interestingly, the enhanced release of acetylcholine by SIN-1 and SNAP was abolished by the neurotoxin tetrodotoxin. This observation demonstrates that the effect of NO on acetylcholine release depends on action potentials.

Very recently it was published that, in the anaesthetized rat, perfusion of the striatum with NO-saturated Ringer's solution or with 1 mmol/l SNAP increases acetylcholine release in a calcium-dependent manner (Guevara-Guzman et al. 1994). Though results in anaesthetized animals should be interpreted carefully, these findings are consistent with our own and support the idea that NO enhances cholinergic transmission (Prast and Philippu 1992a). Thus, at least cholinergic neurons do not show a NO-induced, calcium-independent mechanism of release as proposed by Meffert et al. (1994).

LY83583 is the most selective inhibitor of guanylyl cyclase that is presently available (Malta et al. 1988; Schmidt et al. 1985). In contrast to the NO donor compounds, LY83583 diminished basal acetylcholine release. This result further supports the idea that neuronal messengers which stimulate cGMP synthesis also enhance the striatal acetylcholine output. It is not clear, why methylene blue did not influence basal acetylcholine release. However, besides different physico-chemical properties, methylene blue is less selective than LY83583, and might additionally influence acetylcholine release via further pathways (Matsuoka et al. 1987; Okamura et al. 1990).

The results obtained with the NO synthase inhibitors demonstrate differences in their in vivo effectiveness. The ineffectiveness of LMA might be attributed to its relatively high IC_{50} (15 μmol/l), compared to those of LNA (0.7 μmol/l) and L-NAME (2.8 μmol/l) (Heinzel et al. 1992). In addition, LMA might be metabolized to citrulline, thereby acting as a partial agonist which, at high concentrations, even increases NO synthesis (Archer and Hampl 1992). In contrast to LMA, LNA and LNAME decreased basal acetylcholine release. The higher potency of LNAME to decrease acetylcholine release is probably based on its high lipophilic property. The fast response of the release rate to LNAME might indicate that this effect is exerted locally in the striatum, and not at remote structures reached by diffusion. The tendency of the release rate to return to basal values after termination of superfusion with LNA and LNAME suggests that in vivo inhibition of NO synthesis by these compounds is reversible. Indeed, it has been recently reported that LNA and LNAME are reversible antagonists of NO synthase (Klatt et al. 1994; Mayer et al. 1993), thus contrasting earlier findings (Dwyer et al. 1991). Simultaneous superfusion of the striatum with LNA and L-arginine prevented the LNA-induced decrease of the acetylcholine output, indicating that the effect of LNA on cholinergic transmission depends exclusively on inhibition of NO synthase.

Recently, LNAME was found to be a muscarinic receptor antagonist (Buxton et al. 1993). However, if the inhibitor would exert a significant atropine-like influence on cholinergic neurons, then the acetylcholine release should be increased (Prast and Philippu 1992b).

Hence, the observed inhibition of acetylcholine release by LNAME cannot be attributed to blockade of muscarinic receptors. Obviously LNAME seems to be a valuable tool to inhibit NO synthesis in this experimental setup.

It might be argued that the ability of LNA inhibitors to decrease basal acetylcholine release is an artifact due to pro-inflammatory processes and expression of inducible NO synthase II. However, the push-pull cannula was not implanted chronically but acutely inserted (see methods). In addition, the concentration of LNA used (100 μmol) was too low to inhibit NO synthase II (Lambert et al. 1991).

It is possible that superfusion of striatal tissue with SIN-1 or SNAP causes a local dilatation of vessels, while superfusion with inhibitors of NO synthase and guanylate cyclase might cause vasoconstriction. It has been reported that cholinergic neurons of the striatum innervate neurons which possess NO synthase (Phelps et al. 1985) and that acetylcholine released from cholinergic neurons induces a vasodilatation that is, at least in part, mediated by NO (Moncada et al. 1991). However, in this functional context, there is no plausible explanation why the cholinergic neurons should respond to vasodilation with increase, and to vasoconstriction with decrease in acetylcholine release. Furthermore, LNAME does not decrease striatal acetylcholine release in the urethane anaesthetized rat (unpublished observation). Thus, the effects of LNA and LNAME very probably indicate that, in the striatum of awake rats, endogeneous NO which is continuously released enhances acetylcholine outflow. The continuous NO release does not seem to be a unique feature of the striatum. Very recently, a permanent release of NO has also been detected in rat frontal cortex (Cespuglio et al. 1994).

In this connection it should be noted that NO might not be able to act within cholinergic neurons. Data from several histochemical studies indicate that cholinergic and NO synthase containing neurons are similarly distributed throughout the striatum, but are separate populations. The cholinergic neurons lack NO synthase and the cGMP system (Review: Vincent and Kimura 1992). This suggests that the influence of NO on acetylcholine release might be an indirect one. We have shown that superfusion of the ventral striatum with LNAME lowers, whereas superfusion with SIN-1 increases the release rate of glutamate (Prast et al. 1994). Besides the acetylcholine and glutamate release, NO seems to influence the in vivo release rate of dopamine and other classical transmitters in the striatum of the anaesthetized rat (Guevara-Guzman et al. 1994). Further investigations will show whether the enhancement of glutamate release, or a decrease in dopamine output by NO, is essential for the effect of NO on acetylcholine release.

In conclusion, our findings indicate that NO synthesized in the striatum acutely modulates acetylcholine release. The effects of NO synthase inhibitors demonstrate that, in the ventral striatum, continuously synthesized NO leads to a permanent enhancement of acetylcholine release. It remains to be elucidated whether the change in acetylcholine release rate is a regulatory event occurring secondary to modulation of other transmitters and/or messengers, or whether NO influences directly cholinergic neurons.

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