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Inhibition of synaptically evoked cortical acetylcholine release by intracortical glutamate: involvement of GABAergic neurons

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Abstract

Cortical acetylcholine (ACh) has been shown to regulate diverse cognitive processes and its release can be regulated by neuromodulators that act presynaptically at cholinergic terminals. The neocortex receives dense glutamatergic input from thalamocortical and other fibres. The present study used *in vivo* microdialysis to examine, and pharmacologically characterize, the effect of glutamate on cortical ACh release evoked by electrical stimulation of the pedunculopontine tegmental nucleus in urethane-anaesthetized rats. All drugs were administered locally within the cortex by reverse dialysis. Application of glutamate had no detectable effect on spontaneous ACh release but reduced evoked cortical ACh efflux in a concentration-dependent manner. This effect was mimicked by the glutamate transporter blocker L-*trans*-pyrrolidine-2,4-dicarboxylic acid, as well as by the ionotropic glutamate receptor agonists N-methyl-D-aspartic acid and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid, and was blocked by the ionotropic glutamate receptor antagonists 6,7-dinitroquinoxaline-2,3-dione and (+/–)-3-(2-carboxypiperazin-4yl)-propyl-1-phosphonic acid. Glutamate application also increased extracellular adenosine levels but the simultaneous delivery of the broad-spectrum adenosine receptor antagonist caffeine failed to affect the inhibitory action of glutamate on evoked ACh release. However, the effect of glutamate was fully blocked by simultaneous delivery of the $GABA_A$ receptor antagonist bicuculline and partially blocked by the $GABA_B$ receptor antagonist phaclofen. These results suggest that ionotropic glutamate receptor activation by glutamate inhibits evoked cortical ACh release via an indirect pathway involving GABAergic neurons in the cortex.

Introduction

Cortical acetylcholine (ACh) has been implicated in attention (for a review, see Acquas *et al*., 1996; Sarter & Bruno, 2000), plasticity (Baskerville *et al*., 1997), facilitation of sensory information processing (Rasmusson, 1993), and cortical activation during wakefulness and rapid eye movement sleep (Kanai & Szerb, 1965; Szerb, 1967; Jasper & Tessier, 1971). Increases in cortical ACh efflux alter the properties of postsynaptic neurons and their responses to other inputs. Local delivery of ACh enhances the response of cortical neurons to appropriate modality-specific stimuli in somatosensory (Donoghue & Carroll, 1987; Metherate *et al*., 1987), auditory (Metherate & Weinberger, 1990) and visual cortices (Sillito & Kemp, 1983). This suggests that factors that regulate cortical ACh efflux could influence cognitive processes and behaviour by modulating the activity of cortical neurons.

Cortical ACh is derived from the terminals of cholinergic neurons whose cell bodies are located within the basal forebrain (for a review, see Semba, 2000). Therefore, the release of cortical ACh can be modulated at two levels. Firstly, cortical ACh release depends on the activity of cholinergic cells within the basal forebrain. Activation of cholinergic neurons by direct electrical stimulation (Rasmusson *et al*., 1992), stimulation of afferents to the basal forebrain (Rasmusson *et al*., 1994), or infusion of transmitters or analogs into the basal forebrain (Casamenti *et al*., 1986; Kurosawa *et al*., 1989; Bertorelli *et al*., 1991; Fadel *et al*., 2001) can alter cortical ACh release *in vivo*. A second mechanism for modulating ACh efflux is through activation of presynaptic receptors located on intracortical cholinergic terminals. Previous *in vivo* studies have suggested that ACh efflux is regulated presynaptically by a number of transmitters and modulators including serotonin (Crespi *et al*., 1997), adenosine (Materi *et al*., 2000), neurotensin (Lapchak *et al*., 1990), noradrenaline (Beani *et al*., 1986; Tellez *et al*., 1997), GABA (Giorgetti *et al*., 2000) and ACh (Marchi & Raiteri, 1985; Vannucchi & Pepeu, 1995).

In the light of the fact that the cortex receives dense glutamatergic inputs from a number of subcortical regions including the thalamus, and the increasing evidence for extrasynaptic spillover of glutamate (for a review, see Bergles *et al*., 1999; Mitchell & Silver, 2000), the possibility that this amino acid modulates cortical ACh release is worth considering. Studies using *in vivo* microdialysis in unanaesthetized rats demonstrated that intraperitoneal delivery of an NMDA receptor antagonist increased cortical ACh release, and this increase was antagonized by intracortical infusion of N-methyl-D-aspartate (NMDA; Hasegawa *et al*., 1993). This result suggests that activation of cortical NMDA receptors might inhibit ACh release. However, *in vitro* studies using rat cortical slices have demonstrated that NMDA administration evokes ACh release (Lodge & Johnston, 1985; Ulus *et al*., 1992). Because the level of cortical ACh efflux could have profound effects on behaviour, this discrepancy requires further investigation.

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To understand the role of glutamate in presynaptic modulation of cortical ACh release, the present study examined the effects of locally applied glutamate and specific receptor agonists and antagonists on ACh efflux using an established model of brainstem stimulation-induced cortical ACh release (Rasmusson *et al*., 1994). Preliminary results have been reported in abstract form (Materi & Semba, 1999).

Materials and methods

All experiments were performed in accordance with the guidelines of the Canadian Council on Animal Care. Data were collected from 125 male Wistar rats (300–500 g) anaesthetized with 1.4 g/kg (i.p.) urethane. The experimental protocol employed was as outlined in our previous study (Materi *et al*., 2000) with slight modifications. Briefly, a microdialysis probe (membrane length 2 mm, 0.5 mm outer diameter, molecular cutoff 30 kDa; Bioanalytical Systems (BAS), West Lafayette, Indiana, USA) was lowered vertically into the barrel field of somatosensory cortex (coordinates 1.4 mm posterior and 5.0 mm lateral to bregma, 2.8 mm ventral to the dural surface). The probe was continuously perfused at a rate of 2 μL/min with artificial cerebrospinal fluid to which atropine (10 μM; Sigma, Oakville, Ontario, Canada) and neostigmine methylsulphate (10 μM; Sigma) were added. The tip of a concentric bipolar stimulating electrode (250 μm tip diameter; Frederick Haer and Co., Brunswick, Maine, USA) was positioned within the pedunculopontine tegmental nucleus (PPT; coordinates 8.4 mm posterior and 2.0 mm lateral to bregma, 6.7 mm ventral to the pial surface). Sample collection commenced following a 90-min equilibration period.

As in our previous study (Materi *et al*., 2000), each sample was collected over a 20-min period. Following three or four baseline samples, one sample was collected during electrical stimulation of the PPT. The PPT was stimulated using a 1-s stimulus train (0.2 ms, 100 Hz, 400 μA) applied once per min for 20 min (Rasmusson *et al*., 1994). Following this, four samples were collected during which the amount of ACh present in the cortex returned to baseline levels. The dialysis perfusate was then switched to perfusate containing the drug being tested and this perfusate remained present for the remainder of the experiment. One sample collected in the presence of the drug was used to determine the effect of the drug on spontaneous ACh release. Following this, the PPT was stimulated using the same parameters as outlined above. This sample was used to determine the effect of the drug on synaptically evoked ACh release. Two or three poststimulation samples were then collected. Control animals were not exposed to any drugs.

Acetylcholine assay

Each sample was analysed for ACh content using high-performance liquid chromatography (HPLC) with electrochemical detection (Waters, Mississauga, Ontario, Canada) as described previously (Materi *et al*., 2000). Data were collected and analysed using Powerchrome software (Castle Hill, New South Wales, Australia). The system was calibrated for each experiment using standard solutions containing 1, 2 and 4 pmol of ACh. The detection limit was ≈0.1 pmol.

Adenosine assay

The samples derived from experiments examining the effects of glutamate alone or the simultaneous delivery of glutamate and caffeine were assayed for both ACh and adenosine. To obtain a sufficient volume for each assay, these samples were diluted with artificial cerebrospinal fluid (1 : 1 v/v), mixed vigorously, and then divided equally into two halves. One half was assayed for ACh content as outlined above. The second half was assayed for adenosine content using the protocol outlined by Pazzagli *et al*. (1994) with modifications. Briefly, for each microlitre of sample to be analysed for adenosine, 0.125 μL of chloroacetaldehyde (4.5%) was added. The adenosine/chloroacetaldehyde sample was then tightly sealed and placed in boiling water for 20 min. Following this, the samples were stored for up to one week at –20 °C. Each sample was assayed using HPLC with a Nova-Pak C_{18} column (4 μm; Waters) and ultraviolet fluorescence detection (Waters) at an excitation wavelength of 280 nm and a long-pass emission above 399 nm. The mobile phase consisted of a 50-mM acetate buffer (pH adjusted to 4.5 with acetic acid) containing 10% (v/v) acetonitrile and 2.4 mM 1-octanesulphonic acid sodium salt (Sigma) and was delivered at a rate of 0.7 mL/min. The amount of adenosine present in each sample was analysed by comparison of peak heights to adenosine standards of known concentration. The limit of detection was 60 nmol.

Histology

The placement of the microdialysis probe and stimulating electrode were examined histologically as described previously (Materi *et al*., 2000). Forebrain sections containing the microdialysis probe track were stained with cresyl violet, and brainstem sections containing the stimulating electrode track were processed histochemically for nicotinamide adenine dinucleotide phosphate (NADPH)-diaphorase activity, a selective marker for cholinergic neurons within the mesopontine tegmentum (Vincent *et al*., 1983).

Data analysis

Values were expressed as means ± SEM, and probabilities < 0.05 were considered statistically significant. Changes in spontaneous ACh release due to drugs delivered intracortically by reverse dialysis were examined by comparing the first sample collected during drug delivery without PPT stimulation to the sample immediately preceding it. Differences in the amount of ACh collected were compared using a paired Student's *t*-test.

To examine the amount of evoked release, the absolute amount of ACh detected in the sample immediately prior to PPT stimulation was subtracted from the absolute amount of ACh detected during stimulation for both stimulation trials (E1 and E2), and the mean ratio of E2 to E1 was calculated for each group. The effect of drug application on synaptically evoked ACh release was investigated by comparing the E2/E1 ratio of the control group, which was not exposed to any drug, to the same ratio calculated from each experimental group. The differences in the ratios between the control and experimental groups were assessed by analysis of variance. *Post hoc* comparisons were performed using Fisher's protected least significant difference test (PLSD).

Differences in cortical adenosine levels before and after delivery of glutamate, with or without caffeine, were determined using Student's *t*-test. Other specific comparisons are described in the results section.

Chemicals

The following chemicals were used: L-glutamic acid (Sigma); caffeine (BDH Chemicals, Toronto, Ontario, Canada); L-*trans*-pyrrolidine-2,4-dicarboxylic acid (L-*trans*-2,4-PDC; Tocris, Ballwin, MO, USA); N-methyl-D-aspartic acid (NMDA; Research Biochemicals International (RBI), Natick, MA, USA); α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA; Cambridge Research Biochemicals, Wilmington, DE, USA); 6,7-dinitroquinoxaline-2,3-dione (DNQX; RBI); (+/–)-3-(2-carboxypiperazin-4yl)-propyl-1-phosphonic acid (CPP; RBI); (–)-bicuculline methiodide (RBI);

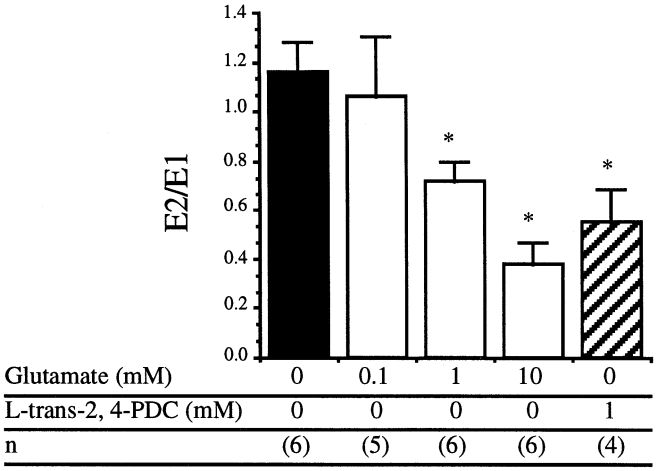


FIG. 1. The effects of glutamate and the glutamate transport blocker L-*trans*-2,4-PDC on PPT stimulation-evoked ACh release (white bars). Glutamate inhibited evoked cortical ACh efflux in a concentration-dependent manner. This effect was mimicked by infusion of L-*trans*-2,4-PDC (hatched bar). Mean ± SEM; **P* < 0.05 vs. control (black bar).

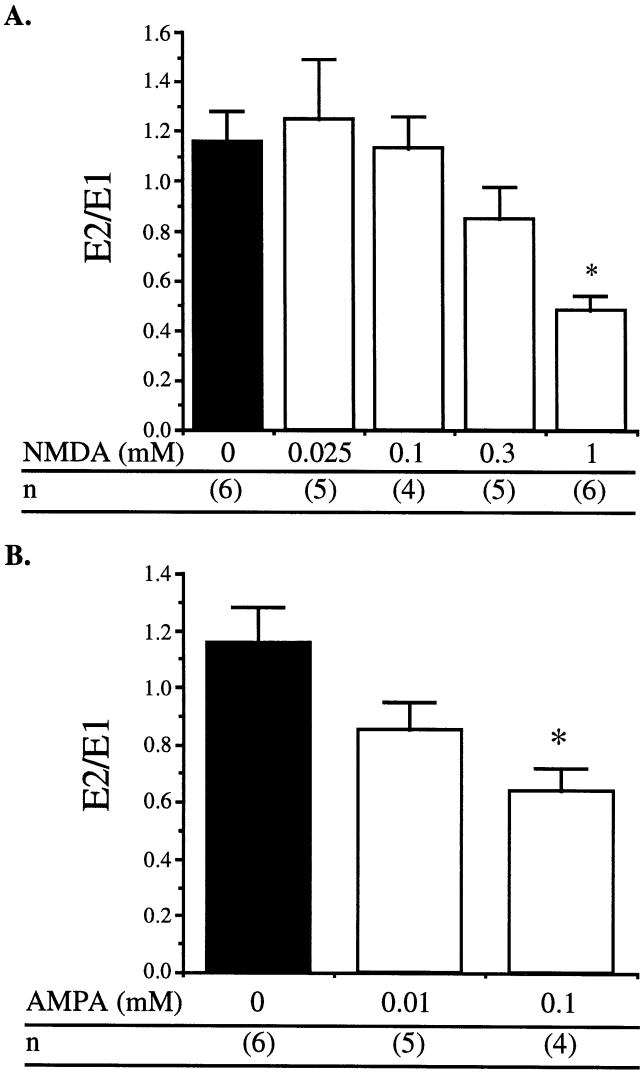


FIG. 2. The effects of selective ionotropic glutamate receptor agonists on evoked cortical ACh release. Delivery of (A) NMDA and (B) AMPA decreased evoked cortical ACh release in a concentration dependent manner. Mean ± SEM; **P* < 0.05 vs. control (black bar).

phaclofen (RBI); and 3-hydroxy-5-aminomethylisoxazole hydrobromide (muscimol; RBI). All of these drugs were dissolved in perfusate with the exception of DNQX which was dissolved initially in dimethylsulfoxide (DMSO; Sigma) and then diluted to the desired concentration with perfusate yielding a final DMSO concentration of 1%. We demonstrated in a previous study (Materi *et al*., 2000) that 1% DMSO has no effect on either spontaneous or PPT stimulation-evoked cortical ACh release. As in previous studies using microdialysis (e.g. Materi *et al*., 2000), concentrations used were approximately 10 × greater than those reported in *in vitro* studies measuring neurotransmitter release from brain slices. This was necessary due to the incomplete permeability of the microdialysis membrane and the poor diffusion of some analytes through tissue.

Results

Spontaneous vs. PPT stimulation evoked cortical acetylcholine release

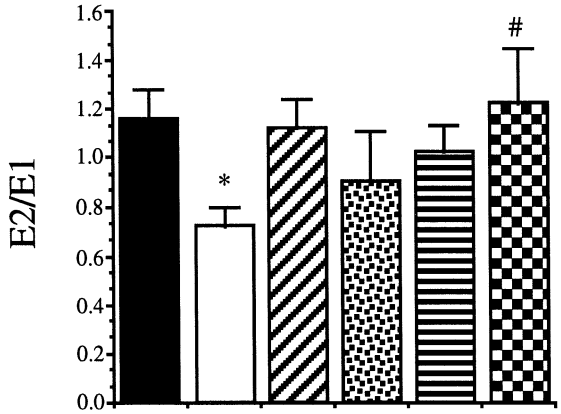
The mean basal amount of ACh present in the cortex prior to the first PPT stimulation was 0.49 ± 0.06 pmol per 20-min sample. During stimulation of the PPT, the amount of ACh collected from the cortex increased significantly (t_{124} = 12.21, $P < 0.0001$) to an average of 2.41 ± 0.20 pmol per 20-min sample. These values were comparable to those reported in previous studies (Rasmusson *et al*., 1994; Materi *et al*., 2000). This confirms that electrical stimulation of the PPT is a highly effective method for increasing cortical ACh release *in vivo*.

The effects of repeated PPT stimulation on cortical ACh release were examined in six control animals. The amount of cortical ACh released during the second stimulation was slightly, but not significantly, greater than the amount released during the first stimulation (E2/E1 = 1.16 ± 0.12). This suggests that repeated exposure to electrical stimulation does not damage the PPT or alter the ACh releasing ability of basal forebrain neurons. Any observed changes to the E2/E1 ratio in the experimental groups must therefore be due to local effects of the drugs delivered by reverse dialysis.

Glutamate inhibits evoked acetylcholine efflux in the cortex

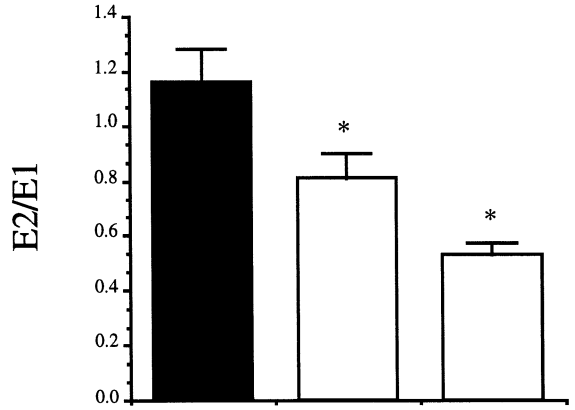
To examine the effect of the glutamate on spontaneous ACh release, the first sample collected during drug delivery was compared to the sample obtained immediately prior to it. Glutamate (0.1, 1 or 10 mM) had no significant detectable effect on spontaneous cortical ACh release (predrug vs. drug, mean ± SEM pmol: 0.1 mM glutamate, 0.55 ± 0.26 vs. 0.50 ± 0.17; 1 mM glutamate, 0.09 ± 0.06 vs. 0.12 ± 0.07; 10 mM glutamate, 0.05 ± 0.05 vs. 0.26 ± 0.10).

The effects of glutamate on PPT stimulation-evoked cortical ACh release were determined by comparing the E2/E1 ratios calculated from the groups exposed to different concentrations of glutamate to the same ratio calculated from the control group. As shown in Fig. 1, glutamate produced a concentration-dependent decrease in evoked cortical ACh release. Whilst application of 0.1 mM glutamate did not alter evoked ACh efflux, delivery of 1 and 10 mM glutamate yielded mean E2/E1 ratios that were 62 and 32% of the control value, respectively, representing a significant decrease in evoked ACh release ($F_{3,19}$ = 6.89, $P < 0.01$; Fisher's PLSD: control vs. 1 and 10 mM glutamate, respectively, $P < 0.05$). This suggests that



Glutamate (mM)	0	1	0	1	0	1
DNQX (mM)	0	0	0.1	0.1	0	0
CPP (mM)	0	0	0	0	0.1	0.1
n	(6)	(6)	(5)	(6)	(5)	(5)

FIG. 3. The effects of selective ionotropic glutamate receptor antagonists alone or in combination with glutamate. Neither DNQX nor CPP had a significant effect on evoked ACh release. Only CPP was able to fully reverse the inhibitory effect of glutamate on evoked ACh release in the cortex (checkered bar). Mean ± SEM; $^{*}P < 0.05$ vs. control (black bar); $^{\#}P < 0.05$ vs. 1 mM glutamate (white bar).



Glutamate (mM)	0	1	10
Caffeine (mM)	0	0.5	0.5
n	(6)	(5)	(4)

FIG. 4. The effects of simultaneous delivery of intracortical glutamate and caffeine on extracellular ACh levels. Simultaneous delivery of caffeine did not reverse the inhibitory effects of glutamate on evoked acetylcholine release. Mean ± SEM; $^{*}P < 0.05$ vs. control (black bar).

exogenously applied glutamate acts intracortically to inhibit evoked ACh release.

Inhibition of endogenous glutamate uptake reduces evoked ACh release

A previous microdialysis study demonstrated that infusion of the glutamate transport blocker L-*trans*-2,4-PDC increased cortical glutamate levels *in vivo* (Semba & Wakuta, 1998). We found that infusion of L-*trans*-2,4-PDC into the cortex did not alter spontaneous ACh release (predrug vs. drug, 0.40 ± 0.11 vs. 0.70 ± 0.20 pmol), but significantly reduced PPT stimulation-induced ACh release ($t_8 = 3.21$, $P < 0.05$; Fig. 1). The mean E2/E1 ratio calculated from the group exposed to the glutamate uptake blocker was 47% of the control value, comparable to the effects of exogenous glutamate described above. Thus, the effects of exogenous glutamate on cortical ACh efflux was mimicked by the glutamate uptake inhibitor L-*trans*-2,4-PDC.

Ionotropic glutamate receptors mediate the inhibitory effects of glutamate on evoked ACh release

To determine which receptors mediate glutamate-induced inhibition of ACh release, the effects of selective ionotropic glutamate receptor agonists and antagonists were examined. Spontaneous cortical ACh efflux was affected neither by NMDA (average ACh release in pmol predrug vs. drug: 0.025 mM NMDA, 0.56 ± 0.09 vs. 0.50 ± 0.08; 0.1 mM NMDA, 0.22 ± 0.08 vs. 0.27 ± 0.13; 0.3 mM NMDA, 0.26 ± 0.13 vs. 0.09 ± 0.06; 1 mM NMDA, 0.20 ± 0.08 vs. 0.20 ± 0.09) nor AMPA (10 μM AMPA, 0.38 ± 0.14 vs. 0.15 ± 0.12; 100 μM AMPA, 0.30 ± 0.14 vs. 0.15 ± 0.12). As shown in Fig. 2A, delivery of 1 mM NMDA (but not 0.025, 0.1 or 0.3 mM) by reverse dialysis significantly reduced evoked cortical ACh release ($F_{4,21} = 4.97$, $P < 0.01$; Fisher's PLSD: control vs. 1 mM NMDA, $P < 0.01$). AMPA at 100 μM, but not 10 μM, also significantly reduced cortical ACh release evoked by PPT stimulation ($F_{2,12} = 5.56$, $P < 0.05$; Fisher's PLSD: control vs. 100 μM AMPA, $P < 0.01$; Fig. 2B). This suggests that both NMDA and AMPA receptors are involved in the glutamate-induced inhibition of evoked ACh release.

To test this possibility further, the effect of glutamate (1 mM) on ACh efflux was tested in the presence of the non-NMDA receptor antagonist DNQX or the NMDA receptor antagonist CPP. Infusion of DNQX (0.1 mM) or CPP (0.1 mM) alone did not affect spontaneous (average release in pmol predrug vs. drug: 0.1 mM DNQX, 0.34 ± 0.21 vs. 0.16 ± 0.07; 0.1 mM CPP, 0.33 ± 0.23 vs. 0.19 ± 0.15) or evoked ACh release (Fig. 3).

Simultaneous delivery of DNQX and 1 mM glutamate produced a mean E2/E1 ratio that was 25% greater than the same ratio calculated from the group that received glutamate alone (Fig. 3). Whilst this difference was not statistically significant, this ratio was also not significantly different from the ratio calculated from the control group. Higher concentrations of DNQX were not used due to the possibility of nonspecific effects (Drejer & Honoré, 1988). These results suggest that infusion of 0.1 mM DNQX only partially antagonized the inhibitory effect of glutamate.

As shown in Fig. 3, CPP fully antagonized the inhibitory effects of 1 mM glutamate on evoked cortical ACh release. The E2/E1 ratio obtained from the group exposed to both CPP and glutamate was significantly greater than the same ratio obtained from the group exposed to glutamate alone, but not significantly different from the control value ($F_{2,14} = 3.78$, $P < 0.05$; Fisher's PLSD: 1 mM glutamate vs. 1 mM glutamate plus 0.1 mM CPP, $P < 0.05$).

The inhibitory effects of glutamate are not mediated by activation of adenosine receptors

Glutamate is known to increase extracellular adenosine concentrations in the cortex (Hoehn & White, 1989; Bennett *et al.*, 1999), and we recently demonstrated that adenosine inhibits evoked cortical ACh release in urethane-anaesthetized rats (Materi *et al.*, 2000). To determine if the inhibitory response to glutamate was due to an increase in extracellular adenosine levels and subsequent activation of adenosine receptors, the effect of glutamate in the presence of the

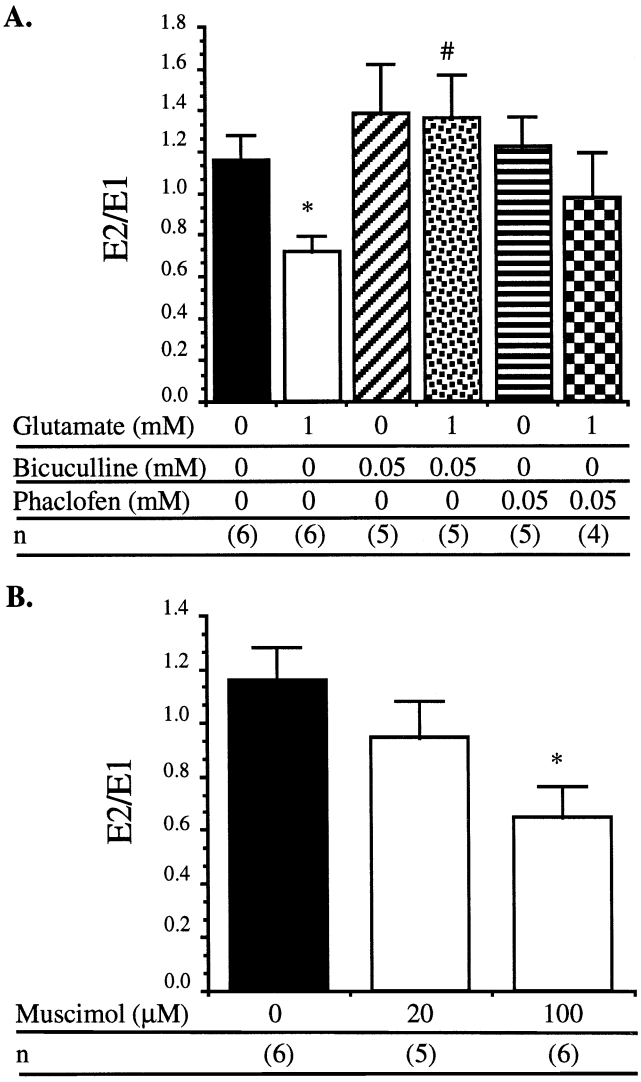


FIG. 5. The role of GABAergic receptors in mediating the effects of glutamate on evoked ACh release. (A) The effects of glutamate on evoked ACh release in the presence of GABA receptor antagonists. The inhibitory effects of glutamate were fully antagonized by the simultaneous delivery of bicuculline but not phaclofen. (B) The effects of muscimol on evoked ACh release. Application of the $GABA_A$ receptor agonist into the cortex by reverse diffusion inhibited synaptically evoked ACh efflux in a concentration-dependent manner. Mean ± SEM; $^*P < 0.05$ vs. control (black bar); $^{\#}P < 0.05$ vs. 1 mM glutamate.

broad spectrum adenosine receptor antagonist caffeine was examined. These samples were assayed for both ACh and adenosine content.

In addition to inhibiting evoked ACh release as described above, glutamate (1 and 10 mM) produced a significant increase in extracellular adenosine levels. Specifically, glutamate (1 mM) increased basal adenosine levels from 19.2 to 50.9 pmol per 20-min sample ($t_4 = 4.72$, $P < 0.01$). Similarly, samples collected prior to delivery of 10 mM glutamate contained an average of 19.8 pmol per 20-min sample and this increased significantly to 193.5 pmol per 20-min sample following drug delivery ($t_5 = 3.88$, $P < 0.05$).

Caffeine (0.5 mM) did not antagonize the inhibitory effects of glutamate (1 and 10 mM) on evoked ACh release (see Fig. 4). The E2/E1 ratios calculated from the groups exposed to both caffeine and glutamate were similar to those calculated from the groups exposed to glutamate alone (Fig. 1). Administration of caffeine did not block the significant increase in extracellular adenosine levels caused by glutamate (caffeine plus 1 mM glutamate: $t_4 = 5.04$, $P < 0.01$; caffeine plus 10 mM glutamate: $t_3 = 5.55$, $P < 0.05$).

Glutamate-mediated inhibition of evoked ACh release involves GABAergic systems

Local GABAergic inhibition of cortical ACh release was reported recently (Giorgetti *et al*., 2000). To determine if extracellular GABA in the cortex is involved in the inhibition of ACh release, the effects of glutamate (1 mM) on evoked cortical ACh release was tested in the presence of the $GABA_A$ receptor antagonist bicuculline or the $GABA_B$ receptor antagonist phaclofen.

The $GABA_A$ receptor antagonist bicuculline (0.05 mM) affected spontaneous ACh release. Application of bicuculline (0.05 mM) significantly increased spontaneous cortical ACh release, from an average basal release of 0.35 ± 0.05 to 0.91 ± 0.12 pmol per 20-min sample ($t_4 = 3.42$, $P < 0.05$). This suggests the presence of tonic inhibition of cortical ACh release by $GABA_A$-receptor activation under the present experimental conditions.

The simultaneous delivery of bicuculline (0.05 mM) and glutamate (1 mM) did not alter spontaneous ACh release (predrug vs. drug, 0.76 ± 0.27 vs. 1.66 ± 0.34 pmol) but did reverse the inhibitory effects of glutamate on evoked cortical ACh release (Fig. 5A). The ratio obtained from the group exposed to both bicuculline and glutamate was significantly greater than the ratio obtained from the 1 mM glutamate alone group ($F_{3,18} = 3.57$, $P < 0.05$; Fisher's PLSD: 1 mM glutamate vs. 1 mM glutamate and 0.05 mM bicuculline, $P < 0.05$) but not significantly different from the control group ratio.

Delivery of the $GABA_B$ receptor antagonist phaclofen (0.05 mM) alone or in combination with 1 mM glutamate did not have any effect on basal ACh release (in pmol predrug vs. drug: 0.05 mM phaclofen, 0.20 ± 0.09 vs. 0.21 ± 0.10; 0.05 mM phaclofen and 1 mM glutamate, 0.59 ± 0.15 vs. 1.22 ± 0.37). Simultaneous delivery of phaclofen and glutamate yielded a mean E2/E1 ratio that was 35% greater than that of the group exposed to glutamate alone but this was not statistically significant (Fig. 5A).

To examine the involvement of $GABA_A$ receptors further, the effects of the $GABA_A$ receptor agonist muscimol were tested. Application of muscimol by reverse dialysis did not alter spontaneous cortical ACh release (in pmol predrug vs. drug: 20 μM muscimol, 0.68 ± 0.52 vs. 0.20 ± 0.13; 100 μM muscimol, 0.52 ± 0.24 vs. 0.39 ± 0.15). However, as shown in Fig. 5B, 100 μM muscimol (but not 20 μM) significantly reduced synaptically evoked ACh efflux in the cortex ($F_{2,14} = 4.44$, $P < 0.05$; Fisher's PLSD: control vs. 100 μM muscimol, $P < 0.05$).

Histology

The placement of the microdialysis probe within the grey matter of the barrel field of the somatosensory cortex, and of the tip of the stimulating electrode within 0.2 mm of the PPT, was confirmed in every animal by histological examination.

Discussion

The main findings of this study are: (i) glutamate has no detectable effect on spontaneous cortical ACh release, but inhibits evoked ACh release in a concentration-dependent manner; (ii) this inhibition can be mimicked by the glutamate transporter blocker L-*trans*-2,4-PDC, as well as by the ionotropic glutamate receptor agonists NMDA and AMPA, and can be antagonized partially by DNQX and fully by CPP; and (iii) glutamate-induced inhibition of evoked cortical ACh efflux

was fully antagonized by the $GABA_A$ receptor antagonist bicuculline and partially by the $GABA_B$ receptor antagonist phaclofen. These findings suggest that glutamate can inhibit evoked cortical ACh release via an indirect circuit involving intracortical GABAergic receptors.

Technical considerations

Advantages and limitations of the use of urethane-anaesthetized animals, and the addition of atropine to perfusate, have been discussed previously (Rasmusson *et al*., 1992; Materi *et al*., 2000). Briefly, urethane-anaesthetized animals provide a steady baseline of ACh release upon which drug effects can be tested without confounds associated with behavioural changes in unanaesthetized animals. The use of anaesthetics, however, reduced the basal level of ACh, and it was necessary to add neostigmine and atropine to the perfusate (Rasmusson *et al*., 1992, 1994; Materi *et al*., 2000). Neostigmine is an acetylcholinesterase inhibitor and increases extracellular ACh levels. This increases activation of muscarinic, presumably M2, autoreceptors present on cholinergic terminals, and this would reduce ACh release (Moor *et al*., 1998). Atropine was therefore added to increase ACh recovery by blocking these autoreceptors. In the present study, atropine also served a second purpose. ACh is known to inhibit the release of glutamate (Hasselmo & Bower, 1992; Kimura & Baughman, 1997) and GABA (Hasselmo & Bower, 1992; Ferraro *et al*., 1997; Kimura & Baughman, 1997) in the cerebral cortex by activating presynaptic muscarinic receptors. Therefore, the inhibition of these feedback loops by atropine also made the results more straightforward to interpret.

Despite these advantages offered by the use of atropine, the possible interaction between muscarinic receptors and glutamate and GABA receptors should be considered. In the present study, none of the receptor agonists and antagonists, with the exception of bicuculline, had any detectable effect on basal ACh release. This suggests that, at least at the concentrations used, these drugs were unlikely to interact with muscarinic receptor activation directly. Furthermore, we are not aware of any report in the literature on direct modulation of muscarinic receptor activation by glutamate, GABA or their agonists. Therefore, the modulation of evoked ACh release by these drugs through their direct modulation at muscarinic receptors would seem unlikely, although it cannot be totally excluded.

One additional concern is the potential excitotoxic damage of intracortical neurons by glutamate and its analogues. However, Vanický *et al*. (1998) examined rat cerebral cortex 24 h following 20-min delivery by reverse dialysis, and found that NMDA caused lesions when administered at concentrations of 10 mM or higher. The highest concentration of NMDA used in the present study was 10-fold less and therefore damage due to NMDA is likely to be negligible.

Comparison with previous studies using ionotropic glutamate receptor agonists

As stated above, activation of cortical AMPA and NMDA receptors reduced synaptically evoked, but not spontaneous, cortical ACh efflux. The lack of inhibitory effect on spontaneous ACh release is likely to be due to already low basal ACh levels in anaesthetized rats, levels which can be close to the detection threshold. The present results with evoked release are consistent with those reported by Hasegawa *et al*. (1993) who demonstrated in unanaesthetized rats that local administration of NMDA to the cortex inhibited ACh efflux evoked by the systemic delivery of an NMDA receptor antagonist. In contrast, Lodge & Johnston (1985) reported that application of NMDA, quisqualate and kainate enhanced basal ACh efflux from cortical slices. Ulus *et al*. (1992) also reported the enhancement of basal ACh release from rat cortical slices following NMDA application. It is not immediately clear why the results should vary depending upon whether ACh is collected from cortical slices or from the cortex of an intact animal regardless of anaesthesia. However, it is possible that the absence of subcortical neuronal systems and other factors associated with *in vitro* conditions contributed to the discrepancy.

The inhibitory effects of glutamate on evoked ACh release is not mediated by adenosine

We tested the possibility that intracortical glutamate inhibited evoked ACh release via an indirect mechanism that involved other neuromodulatory agents, namely adenosine and GABA. Glutamate is known to increase extracellular cortical adenosine levels (Hoehn & White, 1989; Bennett *et al*., 1999) and our previous study (Materi *et al*., 2000) demonstrated that adenosine inhibits evoked cortical ACh release in a concentration-dependent manner in urethane-anaesthetized rats via activation of the A_1 adenosine receptor. In the present study, however, despite increased extracellular adenosine levels, simultaneous delivery of the broad-spectrum adenosine receptor antagonist caffeine did not reverse the effects of glutamate on PPT stimulation-evoked cortical ACh release. We have demonstrated previously that administration of 0.5 mM caffeine by reverse dialysis has no effect on spontaneous or PPT stimulation-evoked cortical ACh release but was able to antagonize the inhibitory effects of adenosine transporter inhibitors on evoked ACh release (Materi *et al*., 2000). In addition to this effect, caffeine antagonized the inhibitory actions of the selective A_1 receptor agonist N^6-cyclopentyladenosine (1 μM) on evoked ACh release (L.M. Materi, D.D. Rasmusson and K. Semba, unpublished observations). The activation of the A_1 adenosine receptor is known to inhibit adenylyl cyclase activity as well as calcium entry, and to increase potassium conductance (for a review, see Williams, 1987). The fact that caffeine did not have any effect on glutamate-induced inhibition of cortical ACh release suggests that glutamate-induced inhibition is independent of, and/or significantly more potent than, adenosine receptor-mediated inhibition.

Inhibition of ACh release by glutamate: involvement of GABAergic neurons

The observed inhibitory effect of glutamate on evoked cortical ACh release is likely to involve intracortical GABAergic receptors. This conclusion is based on the observation that the inhibitory effects of glutamate on evoked ACh release could be completely reversed by the simultaneous delivery of the $GABA_A$ receptor antagonist bicuculline. Furthermore, administration of bicuculline enhanced spontaneous ACh release suggesting the presence of a tonic, GABA-mediated inhibitory tone. These results are consistent with the findings of Giorgetti *et al*. (2000), who demonstrated GABA-induced inhibition of cortical ACh release in freely moving rats. Because glutamate had no effect on evoked ACh release in the presence of bicuculline, this suggests that glutamate does not act directly on intracortical cholinergic terminals. This interpretation is consistent with previous report that NMDA receptor-immunoreactive fibres are not particularly enriched in the rat cerebral cortex (Conti *et al*., 1997; Paquet & Smith, 2000).

The sensitivity of glutamate-induced inhibition of ACh release to GABA receptor antagonists suggests that the inhibition may be due to the effects of GABA released in response to activation of ionotropic glutamate receptors on GABAergic cortical neurons. Previous studies have shown that application of ionotropic glutamate receptor agonists increases GABA release *in vitro* from cultured cortical interneurons

(Drejer *et al*., 1987) and *in vivo* from the hippocampus and striatum of freely moving rats (Hata *et al*., 1997). In the cortex, GABA may originate from intracortical interneurons or from the terminals of GABAergic basal forebrain projection neurons (Freund & Meskenaite, 1992). However, presynaptic facilitation of GABA release from fibre terminals of GABAergic basal forebrain neurons (or terminals of GABAergic interneurons) by NMDA receptor activation seems unlikely because only sparse GABAergic terminals were immunoreactive for the NMDAR1 subunit of the NMDA receptor (DeBiasi *et al*., 1996; Conti *et al*., 1997; Paquet & Smith, 2000). Therefore, it is most likely that in the present study infusion of ionotropic glutamate receptor agonists increased cortical GABA levels by activating GABAergic cortical neurons, and GABA in turn inhibited ACh release via presynaptic $GABA_A$, and possibly $GABA_B$, receptors present on cholinergic terminals. Activation of the presynaptic $GABA_A$ receptors is known to be capable of reducing neurotransmitter efflux by hyperpolarizing nerve terminals via an increase in Cl^- conductance and thereby reducing the probability of neurotransmitter release (for a review, see MacDermott *et al*., 1999).

It is possible that $GABA_B$ receptors are also present on cholinergic terminals, and activation of these presynaptic receptors by GABA could open potassium channels and/or suppress calcium channel activation, thus inhibiting ACh release. However, this inhibition would not be as powerful as $GABA_A$ receptor-mediated inhibition because the $GABA_B$ antagonist only partially blocked glutamate-evoked inhibition of ACh release. It has been suggested that activation of $GABA_B$ receptors on GABAergic neurons participates in autoinhibition of GABA release from intracortical neurons and results in increased cortical ACh release (Giorgetti *et al*., 2000). This mechanism is unlikely to play a crucial role in the present experimental condition because administration of the $GABA_B$ receptor antagonist phaclofen partially reversed, rather than enhanced, the glutamate-induced inhibition of ACh release.

Previous *in vitro* studies reported that GABA has a facilitatory effect on cortical ACh release (Bianchi *et al*., 1982; Bonanno *et al*., 1991). However, our *in vivo* results demonstrate that GABA mediates not only a tonic inhibitory tone on cortical ACh release but is also responsible for the reduction in evoked cortical ACh release. This is consistent with the recent report by Giorgetti *et al*. (2000). The reason for the discrepancy between the *in vitro* and *in vivo* results is unclear.

That GABA can mediate the indirect inhibitory actions of other neurotransmitters on cortical ACh efflux has been suggested. Previous studies have shown that the inhibitory effects of serotonin (Ramírez *et al*., 1996), histamine (Giorgetti *et al*., 1997), and norepinephrine (Beani *et al*., 1986) on cortical ACh release are mediated by GABA. It has also been demonstrated that glutamate can regulate neurotransmitter efflux via a polysynaptic circuit involving GABAergic neurons. Becquet *et al*. (1990), using a push-pull cannula, demonstrated that glutamate-induced reduction in serotonin release from the caudate nucleus of rats was antagonized by bicuculline. A glutamatergic–GABAergic circuit within the septum of the rat has also been shown to regulate cortical ACh release (Giovannini *et al*., 1997). Specifically, increased cortical ACh efflux due to local administration of CPP to the septum was blocked by the simultaneous delivery of muscimol (Giovannini *et al*., 1997). The present results suggest that a similar circuit is present within the cerebral cortex at the terminal level of basal forebrain cholinergic neurons.

Functional implications

Within the cortex, the main effect of ACh is to increase the response of cortical neurons to specific input as demonstrated by an increase in the signal-to-noise ratio (for a review, see McCormick, 1990). Consistent with this notion, Metherate & Ashe (1995) have shown that in the rat auditory cortex *in vivo*, the spontaneously released ACh can preferentially depress responses to weak, compared to strong, inputs through activation of muscarinic receptors. Interestingly, the acetylcholinesterase inhibitor eserine depressed the non-NMDA receptor-mediated, but not the NMDA receptor-mediated, component of the response, whereas carbachol still depressed the latter. These results led the authors to suggest that the activation of NMDA receptors may reduce spontaneous ACh release, either by a postsynaptic mechanism and a diffusible messenger, or a direct presynaptic mechanism involving presynaptic NMDA receptors on cholinergic terminals. The present results are, first of all, consistent with this inhibitory effect of NMDA on synaptic responses and, in addition, suggest that this inhibition might involve an activation of GABAergic interneurons and subsequent release of GABA. Thus, synaptically released glutamate could activate GABAergic neurons to release GABA locally, and if GABA diffuses extrasynaptically in sufficient quantities, it could activate presynaptic $GABA_A$ receptors on cholinergic terminals, thus inhibiting ACh release. This action of glutamate via GABAergic interneurons might provide an indirect negative feedback to regulate the increased signal-to-noise ratio induced by ACh postsynaptically. A similar inhibitory action of NMDA has also been reported at systems and behavioural levels. Ludvig *et al*. (1992) have shown that administration of NMDA by reverse dialysis at concentrations equivalent to those used in the present study resulted in the depression of both behavioural and local cortical EEG activity.

Whilst less is known about specific mechanisms, ACh has also been shown to induce long lasting changes in the response properties of cortical neurons to a variety of sensory inputs, including touch (Metherate *et al*., 1987), audition (Metherate & Weinberger, 1990) and vision (Sillito & Kemp, 1983). It is also suggested that ACh mediates deafferentation-induced synaptic plasticity (Sachdev *et al.*, 1998) and reorganization (Kilgar & Merzenich, 1998). It is possible that ACh provides a feedback that serves to regulate and optimize these functional adaptations. Such regulation might be mediated by the glutamatergic inputs to GABAergic neurons from pyramidal neurons, excitatory intrinsic neurons and/or thalamocortical afferents (Douglas & Martin, 1998).

Increasing evidence suggests that neurotransmitters, including glutamate and GABA, can spill over and act at extrasynaptic receptors away from the original site of release (Bergles *et al*., 1999; Mitchell & Silver, 2000). Consistent with this notion, $GABA_A$ receptors are found both synaptically and extrasynaptically, and these receptors appear to have different pharmacokinetics (Nusser *et al*., 1995; Brickley *et al*., 1999). Recent evidence suggests that tonic activation of extrasynaptic $GABA_A$ receptors plays an important role in optimizing neuronal excitability for salient input (Brickley *et al*., 2001). It is possible therefore that the inhibitory effect of glutamate on evoked ACh release reflects an inhibitory feedback mechanism for maintaining optimal levels of ACh release. In the light of the findings that GABA mediates the inhibitory effects of glutamate, histamine and noradrenaline on cortical ACh release (the present study; Beani *et al*., 1986; Ramírez *et al*., 1996; Giorgetti *et al*., 1997), GABAergic interneurons in the cortex appear to be at a strategic site for optimizing cortical ACh release to regulate cortical neuronal excitability for adaptive behaviour.

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Abbreviations

ACh, acetylcholine; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; CPP, (+/–)-3-(2-carboxypiperazin-4yl)-propyl-1-phosphonic acid; DMSO, dimethylsulfoxide; DNQX, 6,7-dinitroquinoxaline-2,3-dione; HPLC, high-performance liquid chromatography; L-*trans*-2,4-PDC, L-*trans*-pyrrolidine-2,4-dicarboxylic acid; NMDA, N-methyl-D-aspartic acid; PLSD, protected least significant difference test; PPT, pedunculopontine tegmental nucleus.

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