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Muscarinic and $GABA_A$ receptors modulate acetylcholine release in feline basal forebrain

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Abstract

Acetylcholine (ACh) release within the basal forebrain changes significantly as a function of sleep and wakefulness, hence identifying the neurochemical modulators of basal forebrain ACh release will contribute to a mechanistic understanding of sleep cycle regulation. This study tested the hypothesis that muscarinic and gamma aminobutyric $acid_A$ ($GABA_A$) receptors modulate basal forebrain ACh release. Cats were anaesthetized with halothane to hold arousal state constant and a microdialysis probe was aimed stereotaxically for the substantia innominata region of the basal forebrain. Four concentrations of the muscarinic antagonist scopolamine (0.1, 0.3, 1.0, and 10 nM) and five concentrations of the $GABA_A$ antagonist bicuculline (3, 10, 30, 100, and 300 μM) were delivered by reverse dialysis from the same probes used to collect ACh. These results are based on 27 experiments in nine animals. Scopolamine and bicuculline each caused a concentration dependent enhancement of ACh release. Scopolamine increased ACh by 118% above control levels whereas bicuculline was more effective, causing a 287% increase in ACh release. Scopolamine was more potent ($EC_{50} = 0.16$ nM) than bicuculline ($EC_{50} \geq 90$ μM) for increasing ACh release. The results support the hypothesis that substantia innominata ACh release is modulated by muscarinic autoreceptors and inhibited by $GABA_A$ receptors. These findings are consistent with the interpretation that inhibition of basal forebrain cholinergic neurotransmission by GABA contributes to the generation of sleep.

Introduction

Basal forebrain cholinergic neurons provide widespread innervation to the neocortex in many species, including rat (Mesulam *et al*., 1983; Rye *et al*., 1984), cat (Fisher *et al*., 1988), and human (Mesulam & Geula, 1988). Acetylcholine (ACh) released from basal forebrain projection neurons plays a pivotal role in learning and memory (Winkler *et al*., 1995; Baxter & Chiba, 1999; Miranda & Bermudez-Rattoni, 1999), attention (Sarter & Bruno, 2000), and the cortical activation characteristic of wakefulness and rapid eye movement (REM) sleep (Semba, 2000). Loss of basal forebrain cholinergic neurons contributes to the memory impairments of Alzheimer's disease (Muir, 1997; Perry *et al*., 1999), and current therapies for the treatment of neurodegenerative diseases such as Alzheimer's include cholinomimetics (Buccafusco & Terry, 2000). Regulation of cortical ACh release has been studied extensively (reviewed in Sarter & Bruno, 1997, 2000). The mechanisms modulating cholinergic neurotransmission within the basal forebrain, however, have only recently begun to be investigated (Vazquez *et al*., 2002).

In addition to cholinergic neurons, the basal forebrain is comprised of gamma aminobutyric acid (GABA) containing neurons (Mugnaini & Oertel, 1985). Basal forebrain GABAergic neurons outnumber cholinergic neurons by approximately two-to-one in the cat (Gritti *et al*., 1993). Electron microscopic and immunohistochemical studies have demonstrated that GABAergic terminals contact cholinergic cell bodies in rat basal forebrain (Zaborszky *et al*., 1986; Ingham *et al*., 1988). Immunohistochemistry in combination with *in vitro* electrophysiology has revealed a GABAergic inhibition of basal forebrain cholinergic neurons that is mediated by $GABA_A$ receptors (Khateb *et al*., 1998). GABAergic neurons in the ventrolateral preoptic area (VLPO) of the anterior hypothalamus are selectively active during sleep (Sherin *et al*., 1996, 1998; Szymusiak *et al*., 1998) and microinjection of the $GABA_A$ agonist muscimol into rat basal forebrain increases non-REM (NREM) sleep and decreases wakefulness (Manfridi *et al*., 2001). Taken together with early lesion and stimulation studies demonstrating a role for the basal forebrain in sleep cycle control (reviewed in Szymusiak, 1995), these findings suggest that sleep and wakefulness are regulated, in part, by an interaction between basal forebrain cholinergic and GABAergic neurons.

The aforementioned evidence provided the rationale for the present investigation aiming to characterize two receptor mechanisms regulating basal forebrain ACh release. This study tested the hypothesis that muscarinic autoreceptors and $GABA_A$ receptors modulate ACh release within the substantia innominata region of cat basal forebrain. Portions of these data have been presented as a preliminary report (Vazquez & Baghdoyan, 2002).

Materials and Methods

Surgical preparation and animal model

Cat was the ideal experimental animal for these studies as much of what is currently known about the neurophysiological mechanisms underlying sleep cycle control was derived from experiments using cat (Steriade & McCarley, 1990; Lydic & Baghdoyan, 1999). For microdialysis studies aiming to characterize changes in transmitter release as a function of sleep, cat is a logical choice because of the long duration NREM sleep and REM sleep epochs compared to rat or mouse. REM

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sleep epochs in cat last, on average, five to eight min (Ursin, 1968; Baghdoyan *et al*., 1984; Lydic *et al*., 1987; Vazquez *et al*., 1998), thus permitting collection of dialysis samples during periods comprised entirely of REM sleep. Recent studies using cat have demonstrated that ACh release in the substantia innominata region of the basal forebrain is significantly greater during REM sleep than during wakefulness and NREM sleep (Vazquez & Baghdoyan, 2001). The choice of cat for the present study was reinforced by data identifying nitric oxide as a molecular modulator of ACh release in the substantia innominata (Vazquez *et al*., 2002). Therefore, cat was used for the present study designed to test the hypothesis that basal forebrain ACh release is modulated by muscarinic and $GABA_A$ receptors.

The cat NREM/REM cycle (and parallel oscillations in neurotransmitters that modulate the sleep cycle) occurs with a polycyclic rhythm throughout the 24 h day (Tobler & Scherschlicht, 1990). In contrast to rodent, cat exhibits only weak circadian modulation of its sleep–wake cycle (Tobler & Scherschlicht, 1990). Cats used for the study were housed in constant illumination to minimize the probability that the results would be confounded by circadian factors.

All animal procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences Press, Washington, DC, 1996) and were approved by the University of Michigan Committee on Use and Care of Animals. Surgery was performed under sterile conditions in a dedicated animal operating room. The surgical goal was to create a craniotomy that provided access to the substantia innominata (Vazquez & Baghdoyan, 2001; Vazquez *et al*., 2002). Adult male cats ($n = 9$) were anaesthetized with isoflurane (2–3% in oxygen) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) equipped with a Kopf 880 semichronic head holder. An acrylic head cap was built to surround and protect the craniotomy. Two plastic sleeves fitted to the Kopf 880 head holder were positioned above the skull and embedded within the acrylic head cap. These sleeves allowed placement of the animal in stereotaxic position without the use of ear bars during subsequent dialysis experiments. Cats were given a three-week postsurgical recovery period prior to being used for experiments.

Drug preparation

The muscarinic cholinergic antagonist scopolamine methyl bromide and the $GABA_A$ antagonist bicuculline methiodide were purchased from Sigma-Aldrich Corp. Drugs were dissolved in Ringer's solution in (mM) NaCl, 147; $CaCl_2$, 2.4; KCl, 4.0; plus 10 μM neostigmine bromide; pH 5.8–6.2. Neostigmine, a cholinesterase inhibitor, prevents the degradation of ACh and is used routinely for microdialysis (Quirion *et al*., 1994; Billard *et al*., 1995; Moor *et al*., 1998a; Giorgetti *et al*., 2000; Materi *et al*., 2000; Fadel *et al*., 2001). Drug solutions were made on the day of each experiment and serially diluted to final concentrations using Ringer's solution. Administered concentrations were 0.l, 0.3, 1.0, and 10 nM scopolamine, and 3, 10, 30, 100, and 300 μM bicuculline. The pH was maintained at 5.8–6.2 for all drug concentrations.

Experimental procedure

In many brain regions, including basal forebrain, ACh release changes significantly across the sleep–wake cycle (Jasper & Tessier, 1971; Kodama *et al*., 1990, 1992; Williams *et al*., 1994; Marrosu *et al*., 1995; Leonard & Lydic, 1997; Vazquez & Baghdoyan, 2001). Thus, in order to conclude that changes in ACh release were caused by drug administration, and not caused by changes in behavioural state, arousal state was held constant with general anaesthesia. This technique has been used successfully to distinguish between effects of drugs and arousal state on transmitter release in cat pontine reticular formation (Baghdoyan *et al*., 1998), rat cortex (Materi *et al*., 2000), and mouse cortex (Douglas *et al*., 2001).

All experiments were conducted at the same time of day. Sample collection began between 11:30 and 13:00 h and experiments lasted 3.5–4 h. Each experiment began by anaesthetizing a cat with halothane (3–4% in O_2) administered via a mask. The vocal cords were sprayed with 1% lidocaine, the trachea was intubated with a cuffed paediatric endotracheal tube, and the animal was mechanically ventilated using an SAV 2500 Anaesthesia Ventilator (SurgiVet, Waukesha, WI). The animal then was placed in stereotaxic position and several physiological parameters were measured continuously throughout each experiment. Systemic blood pressure and mean arterial pressure were monitored noninvasively using a Dinamap (Critikon, Tampa, FL). Oxygen saturation and heart rate were quantified using a Biox 3700 Pulse Oximeter (Datex-Ohmeda, Madison, WI). Core body temperature was measured with a rectal thermometer and maintained at 37 °C using a T500 T/Pump Heat Therapy System (Gaymar Industries, Inc., Orchard Park, NY). End tidal concentrations of CO_2 and halothane were maintained at 30 mm Hg and 1.4–1.5%, respectively, using a Rascal II spectrometer (Datex-Ohmeda, Madison, WI).

Once all autonomic signs were stable, a microdialysis probe was aimed stereotaxically for the substantia innominata (Berman & Jones, 1982) as previously described (Vazquez & Baghdoyan, 2001; Vazquez *et al*., 2002). Stereotaxic coordinates for the dialysis probe aim sites ranged from anterior (A) 14.5 to A16.0, lateral (L) 3.0 to L5.5, and horizontal (H) −2.0 (Berman & Jones, 1982). Aim sites were separated by at least 1 mm. Each dialysis site was used for only one experiment in order to avoid potential changes in ACh release due to repeated microdialysis sampling (Moore *et al*., 1995). A minimum of seven days elapsed between experiments using the same animal.

In vivo *microdialysis and high performance liquid chromatography with electrochemical detection (HPLC/EC)*

Microdialysis and HPLC/EC procedures have been previously described in detail (Leonard & Lydic, 1997; Baghdoyan *et al*., 1998; Vazquez & Baghdoyan, 2001; Vazquez *et al*., 2002). The dialysis probe tip was comprised of a polycarbonate membrane with a 20 kDa cut-off, 0.5 mm diameter, and 2 mm length (CMA Microdialysis, North Chelmsford, MA). The probe was perfused continuously with Ringer's solution at a rate of 3 μL/min using a CMA/100 pump and dialysis samples were collected on ice every 10 min. Stable levels of ACh release always were observed within 30 min following probe insertion. Five sequential dialysis samples then were collected to establish baseline levels of ACh release. A CMA/110 liquid switch was then turned to deliver Ringer's containing either scopolamine or bicuculline to the dialysis probe. Nine sequential dialysis samples (90 min) were obtained during drug administration and only one concentration of drug was administered per experiment. Immediately following collection, each dialysis sample was injected into the HPLC/EC system (Bioanalytical Systems (BAS), Inc., West Lafayette, IN) for quantification of ACh. Samples were carried in a 50 mM Na_2HPO_4 mobile phase (pH 8.5) through an analytical column that separated ACh and choline, then through an immobilized enzyme reactor column that produced hydrogen peroxide in amounts proportional to the ACh and choline. A platinum electrode with an applied potential of 500 mV was referenced to an Ag^+/AgCl electrode and detected hydrogen peroxide generated by the enzyme column. The chromatogram was digitized and stored to disk using ChromGraph® software (BAS). Area under the chromatographic peak for each dialysis sample was compared to a standard curve generated from known ACh amounts in order to quantify the ACh content (pmol/10 min) in each sample. A standard curve was created prior to every experiment.

In vitro *analysis of percent ACh recovery from the dialysis probes*

Before beginning each experiment, the microdialysis probe was placed into a known concentration of ACh. Five dialysis samples were collected during perfusion of the probe with Ringer's solution and the samples were injected into the HPLC/EC system for quantification of ACh. Once the experiment was terminated, the microdialysis probe was removed from the brain, placed into the same known concentration of ACh, and five dialysis samples again were collected for analysis of ACh. Percent recovery of ACh obtained before and after the experiment was compared by *t*-test. The data presented in this report were obtained from experiments that showed no statistically significant change in probe recovery. This procedure demonstrated that measured changes in ACh resulted from drug administration and were not due to mechanical alterations of the dialysis probe. These probe recovery data also were used to compare ACh recovery from probes that delivered scopolamine with ACh recovery from probes that delivered bicuculline. Comparison by *t*-test ensured that differences in the amount of ACh release evoked by the two drugs did not result from differences in probe recovery of ACh. Mean ± standard deviation recovery for all probes used in this study was 8.5 ± 2.1%.

Statistical analyses

Data were analysed by descriptive and inferential statistics. ACh levels are reported as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was used to determine the effect of scopolamine or bicuculline on basal forebrain ACh release. Post hoc comparisons were made using Dunnett's multiple comparison test. The probability (P) value for statistical significance was $P < 0.05$. Nonlinear regression analysis of the concentration response data was performed using the following sigmoidal equation: ACh Release = Basal + (Maximal–Basal) / $1 + 10^{(\log EC50 - X)}$, where X is the logarithm of the antagonist concentration. Curve fitting was carried out with GraphPad Prism version 3.0a for Macintosh (GraphPad Software Inc., San Diego CA). These analyses provided coefficients of determination (r^2) and identified the concentration of each drug that produced 50% of the maximal increase in ACh release (EC_{50}).

Histology

Following the last dialysis experiment, cats were deeply anaesthetized with pentobarbital (35–40 mg/kg i.p.) and perfused transcardially with saline followed by 10% formalin. Brains were removed and fixed in formalin for two to three weeks. The forebrain block then was placed in 30% sucrose and 10% formalin for seven days and serial, coronal sections were cut on a freezing microtome. Alternate sections (40 µm thick) were stained with cresyl violet or processed for glial fibrillary acidic protein (GFAP) immunohistochemistry (Benevento & McCleary, 1992; Miasnikov *et al*., 1999). All microdialysis sites were localized by comparison of the tissue sections with a stereotaxic atlas (Berman & Jones, 1982).

Results

Scopolamine increased ACh release in substantia innominata

The effects of scopolamine on ACh release were based upon 1660 min of microdialysis sampling in 12 experiments using six cats. Figure 1A schematizes scopolamine delivery through the same dialysis probe used to collect ACh. Representative chromatograms show peaks due to ACh obtained during control (Ringer's) dialysis (Fig. 1B) and during dialysis administration of scopolamine (Fig. 1C). Each individual ACh peak was quantified and expressed as pmol ACh/10 min Fig. 2 summarizes sequential ACh measures obtained during control (Ringer's) dialysis and during dialysis with four concentrations of scopolamine. Control ACh levels were similar between experiments delivering different concentrations of scopolamine (compare open bars across Fig. 2A–D). Control levels of ACh also were relatively stable across time (compare open bars from 10–50 min within Fig. 2A–D). Small increases in ACh release were apparent within 40 min of delivering 0.1 nM (Fig. 2A) and 0.3 nM (Fig. 2B) of scopolamine. Larger increases in ACh release occurred within 20 min of administering 1 nM scopolamine (Fig. 2C), and after 10 min of delivering 10 nM scopolamine (Fig. 2D).

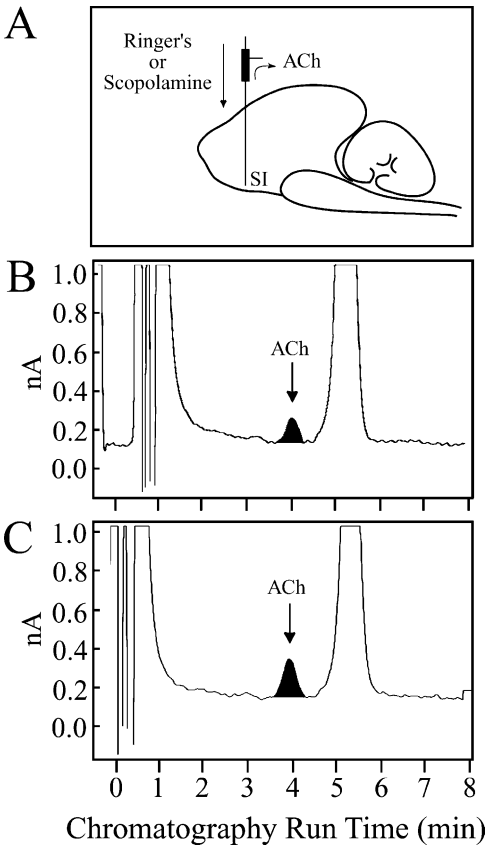


FIG. 1. Microdialysis administration of scopolamine and chromatography for quantifying ACh. (A). Sagittal schematic of cat brain illustrating a dialysis probe in the substantia innominata (SI) region of the basal forebrain. Scopolamine was delivered through the dialysis probe and ACh was collected simultaneously. (B). A typical chromatogram produced by one sample obtained during control (Ringer's) dialysis. Filled peak (arrow) represents 0.2 pmol ACh. (C). A representative chromatogram produced by a single dialysis sample obtained during delivery of 0.3 nM scopolamine. Filled peak (arrow) represents 0.4 pmol ACh. For parts (B) and (C) the scale of the ordinate in nanoamperes is arbitrary and is set, in part, according to the sensitivity of the electrochemical detector.

One goal of these studies was to determine the minimum concentration of scopolamine that produced a significant increase in ACh release (Billard *et al*., 1995). Toward that end, control ACh levels were averaged across time for all experiments and compared statistically with average ACh levels obtained during treatment with scopolamine. Figure 3 shows the concentration main-effect of scopolamine on ACh release ($F = 18.6$; df = 4, 165; $P < 0.0001$). Dunnett's multiple comparison test showed that 0.3 nM scopolamine was the minimum concentration that significantly ($P < 0.01$) increased ACh above control levels.

Bicuculline increased ACh release in substantia innominata

The effects of bicuculline on ACh release were based on 2100 min of microdialysis sampling in 15 experiments using six cats. Figure 4 plots

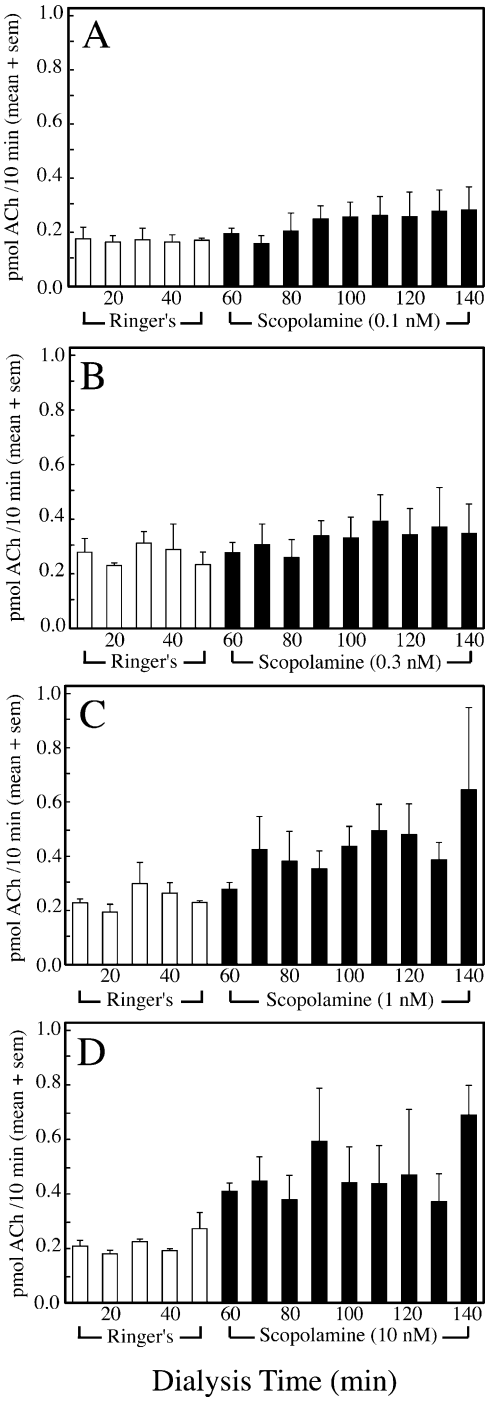


FIG. 2. Time-course of the scopolamine-induced increase in ACh release. Open bars indicate control levels of ACh during dialysis with Ringer's solution. Filled bars plot ACh during dialysis with Ringer's containing scopolamine. Average ACh values at each 10 min time point are based on samples obtained from three experiments. A–D show results using different concentrations of scopolamine.

the time course of ACh release during dialysis of the substantia innominata with Ringer's (control) and five concentrations of bicuculline. Lower concentrations of bicuculline (3 and 10 μM, Fig. 4A,B) did not markedly increase ACh release. Within 30 min of delivering 30 μM bicuculline (Fig. 4C) ACh release was elevated. At bicuculline concentrations of 100 and 300 μM (Fig. 4D,E) sustained increases in ACh release were observed within 20 min of drug administration. Figure 5

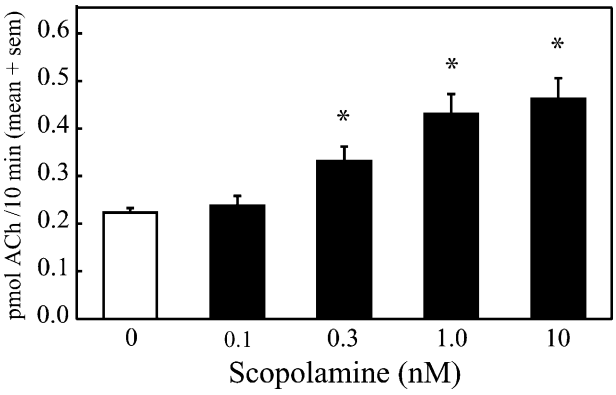


FIG. 3. Scopolamine caused a concentration dependent increase in ACh release. The number of dialysis samples (*n*) follows each scopolamine concentration: 0 nM (59); 0.1 nM (27); 0.3 nM (27); 1.0 nM (27), and 10 nM (26). Asterisks indicate a significant ($P < 0.01$) increase over control (0 nM) levels.

shows ACh release averaged across time, revealing the concentration dependent effect of bicuculline on ACh release ($F = 28.9$; df = 5, 209; $P < 0.0001$). The minimum ACh releasing concentration for bicuculline was 30 μM (Dunnett's test, $P < 0.01$).

Scopolamine and bicuculline showed different efficacy and potency for causing increased ACh release

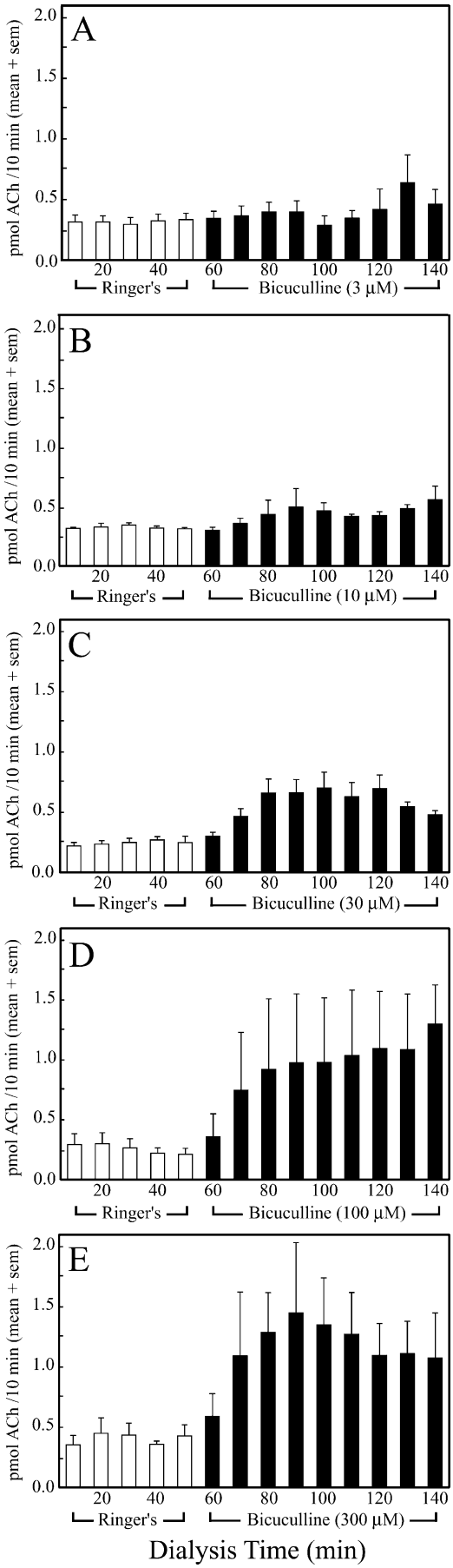
Figure 6 demonstrates the efficacy and relative potency of scopolamine and bicuculline for enhancing ACh release. Over the range of concentrations tested, bicuculline caused a greater increase in ACh release (287%) than scopolamine (118%). Bicuculline raised ACh levels to an average of 1.15 ± 0.12 pmol/10 min, resulting in an increase over control of 0.84 pmol/10 min. Scopolamine caused a maximal ACh level of 0.46 ± 0.04 pmol/10 min, which was an increase over control of 0.22 pmol/10 min. Comparison by *t*-test showed no significant difference between control levels of ACh release for scopolamine experiments (0.24 ± 0.03 pmol/10 min, Fig. 3 open bar) and bicuculline experiments (0.31 ± 0.03 pmol/10 min, Fig. 5 open bar). The EC_{50} values for scopolamine and bicuculline were 0.16 nM and approximately 90 μM, respectively, demonstrating that scopolamine was more potent than bicuculline in stimulating ACh release (Fig. 6). The bicuculline-induced increase in ACh release did not reach saturation.

Histological confirmation of dialysis probe placement in substantia innominata

Figure 7 shows a representative histological section of the basal forebrain with a microdialysis probe-induced lesion localized to the substantia innominata (Berman & Jones, 1982). Examination of histological sections from the nine animals used for this study confirmed that all dialysis probes had been placed in the substantia innominata. Figure 8 uses templates from a cat stereotaxic atlas (Berman & Jones, 1982) to indicate the caudo-rostral and medio-lateral extent of the basal forebrain region that was dialysed. Dialysis probe sites for scopolamine experiments ranged from A14.0 to A16.0 and L3.0 to L5.5. Dialysis probes from bicuculline experiments were localized to sites ranging from A14.0 to A16.0 and L4.0 to L5.0.

Discussion

These results provide the first demonstration that microdialysis delivery of the muscarinic receptor antagonist scopolamine or the $GABA_A$ receptor antagonist bicuculline to the substantia innominata region of cat basal forebrain significantly increased substantia innominata ACh



release. Increases in ACh release were dependent on the concentration of scopolamine or bicuculline, confirming the hypothesis that muscarinic and $GABA_A$ receptors modulate cholinergic neurotransmission in the substantia innominata. Over the range of concentrations tested, scopolamine evoked less of an increase in ACh release than did bicuculline. Scopolamine was more potent than bicuculline in enhancing that ACh release. Taken together, these results suggest that both autoreceptor and heteroreceptor mechanisms for regulating ACh release exist within the substantia innominata. Following an overview of methodological considerations, these new findings are discussed relative to their implications for basal forebrain mechanisms regulating ACh release and arousal states.

Methodological considerations: use of acetylcholinesterase inhibitors for microdialysis studies of ACh

In vivo microdialysis is a technique that has revolutionized neurochemistry by permitting near real-time measures of neurotransmitter release from behaving animals and conscious humans (Robinson & Justice, 1991). ACh has been collected from brain using *in vivo* microdialysis for more than 15 years (Westerink *et al*., 1987), and routine measurement of ACh is made possible by use of an acetylcholinesterase inhibitor. Cholinesterase inhibitors allow the detection of ACh over relatively short collection intervals (10–12.5 min) using probes with small tip sizes (1–2 mm in length). However, the presence of neostigmine in the dialysis solution causes a concentration dependent increase in basal levels of cortical ACh (Himmelheber *et al*., 1998). This increased basal ACh level may attenuate autoreceptor function (de Boer *et al*., 1990), alter the responses of cholinergic neurons to stimulation, or may change the way cholinergic neurons respond to drugs (for a detailed discussion see Himmelheber *et al*., 1998).

Given that most HPLC/EC systems currently used to quantify ACh during relatively short sampling periods cannot detect ACh in brain dialysis samples without the presence of an acetylcholinesterase inhibitor, it has been necessary to work around this limitation. Many *in vivo* studies have demonstrated that autoreceptors are functional even when a cholinesterase inhibitor is used for dialysis. For example, in the presence of neostigmine, muscarinic antagonists cause concentration dependent increases in ACh release in cat pontine reticular formation (Roth *et al*., 1996; Baghdoyan *et al*., 1998), rat striatum (Billard *et al*., 1995), hippocampus (Moor *et al*., 1995; Kitaichi *et al*., 1999) and medial septal area (Moor *et al*., 1995), and mouse prefrontal cortex (Douglas *et al*., 2001). In cat pontine reticular formation (Baghdoyan *et al*., 1998) and in rat hippocampus and medial septal area (Moor *et al*., 1995), muscarinic autoreceptors respond to agonist stimulation by decreasing ACh release. Thus, in the presence of elevated ACh levels due to neostigmine, muscarinic autoreceptors are not maximally inhibited and respond, as predicted, by increasing ACh release in the presence of antagonists and by decreasing ACh release in the presence of agonists.

Several *in vivo* microdialysis studies have also demonstrated that behavioural state-dependent changes in ACh release occur in the presence of cholinesterase inhibitors. ACh release measured with the use of neostigmine changes significantly across the sleep cycle in cortex and hippocampus (Marrosu *et al*., 1995), substantia innominata (Vazquez & Baghdoyan, 2001; Vazquez *et al*., 2002), thalamus (Williams *et al*., 1994), pontine reticular formation (Kodama *et al*.,

FIG. 4. Time-course of the bicuculline-induced increase in ACh release. Open bars indicate control ACh levels obtained during dialysis with Ringer's solution. Filled bars plot average ACh during dialysis administration of bicuculline. ACh values at each 10 min time point are based on three dialysis samples. A–E show results using different concentrations of bicuculline.

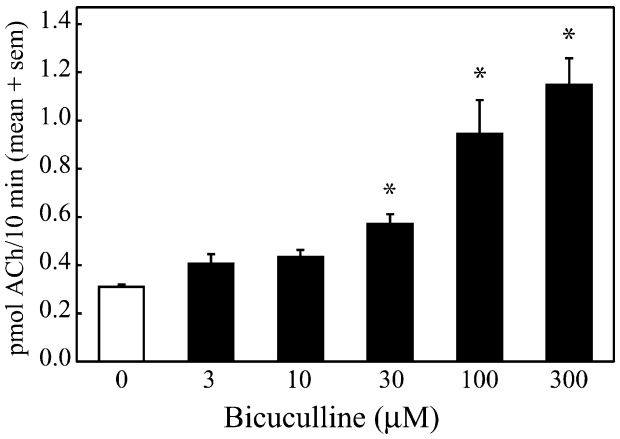


FIG. 5. Bicuculline caused a concentration dependent increase in ACh release. The number of dialysis samples (n) follows each bicuculline concentration: 0 μM (75); 3 μM (27); 10 μM (27); 30 μM (27); 100 μM (27); and 300 μM (27). Asterisks indicate a significant ($P < 0.01$) increase over control (0 μM) levels.

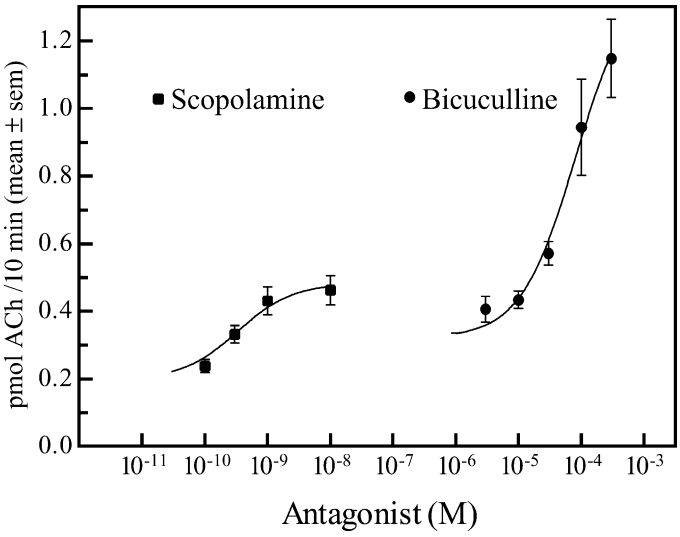
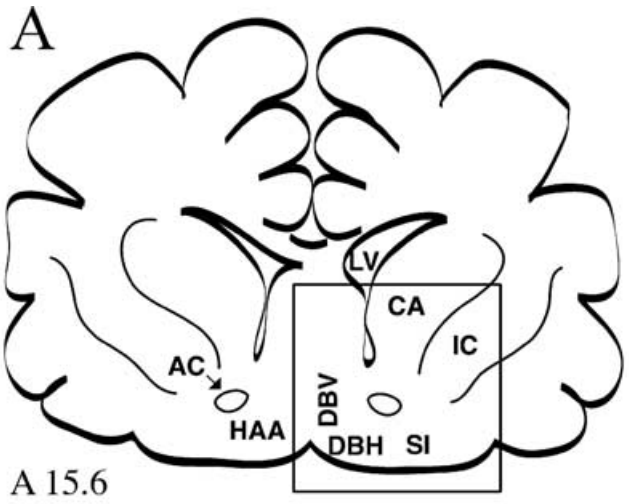


FIG. 6. Concentration response curves for ACh release evoked by the muscarinic antagonist scopolamine and the $GABA_A$ receptor antagonist bicuculline. Curves were fit using nonlinear regression analysis, which demonstrated that 99% of the variation in ACh release was accounted for by the concentration of scopolamine ($r^2 = 0.9938$) or bicuculline ($r^2 = 0.9898$).



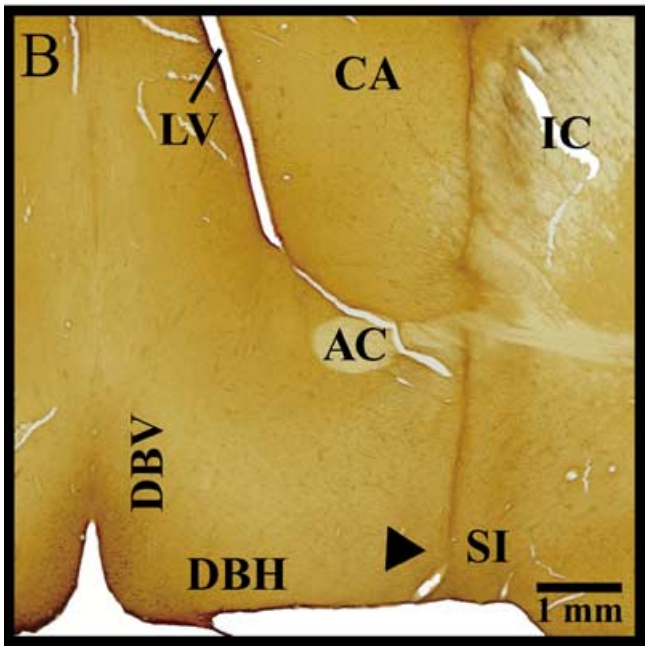


FIG. 7. Histological localization of one dialysis site. (A). Schematic of a coronal cat brain section at 15.6 mm anterior to stereotaxic zero (modified from Berman & Jones, 1982). Boxed area indicates the location of the histological section enlarged below. (B). This coronal section shows one representative microdialysis site in the substantia innominata. GFAP immunohistochemistry was used to visualize glial formation caused by the microdialysis probe. The arrowhead points to the bottom of the track, indicating the deepest part of the 2 mm long dialysis membrane. All microdialysis sites were localized histologically. Abbreviations: AC, anterior commissure; CA, caudate nucleus; DBH, diagonal band of Broca, horizontal division; DBV, diagonal band of Broca, vertical division; HAA, anterior hypothalamic area; IC, internal capsule; LV, lateral ventricle; SI, substantia innominata.

1990; Leonard & Lydic, 1997), and medullary reticular formation (Kodama *et al*., 1992). Within the state of wakefulness, cortical ACh release varies significantly with changes in attention (Sarter & Bruno, 1997; Himmelheber *et al*., 2000), and the magnitude of increased cortical ACh release in response to behavioural stimulation is not influenced by the concentration of neostigmine (Himmelheber *et al*., 1998). Thus, numerous studies provide confidence that ACh release data obtained in the presence of cholinesterase inhibitors are reliable.

Methods do exist for measuring ACh in brain dialysis samples without the use of acetylcholinesterase inhibitors (de Boer *et al*., 1990; Ichikawa *et al*., 2002). These techniques can help to characterize how cholinesterase inhibitors influence measures of brain ACh. The relatively long sampling periods required for dialysis without cholinesterase inhibitors, however, have so far limited applicability of this approach for measuring ACh release during sleep states.

Evidence for muscarinic autoreceptor modulation of ACh release in the substantia innominata

The lowest concentration of a muscarinic antagonist to cause a significant increase in transmitter release has been defined as the minimum ACh releasing concentration (Billard *et al*., 1995). Comparison of antagonist minimum releasing concentration with antagonist affinity (K_i) for the five muscarinic receptor subtypes has been used to make inferences about the subtype which functions as an autoreceptor in

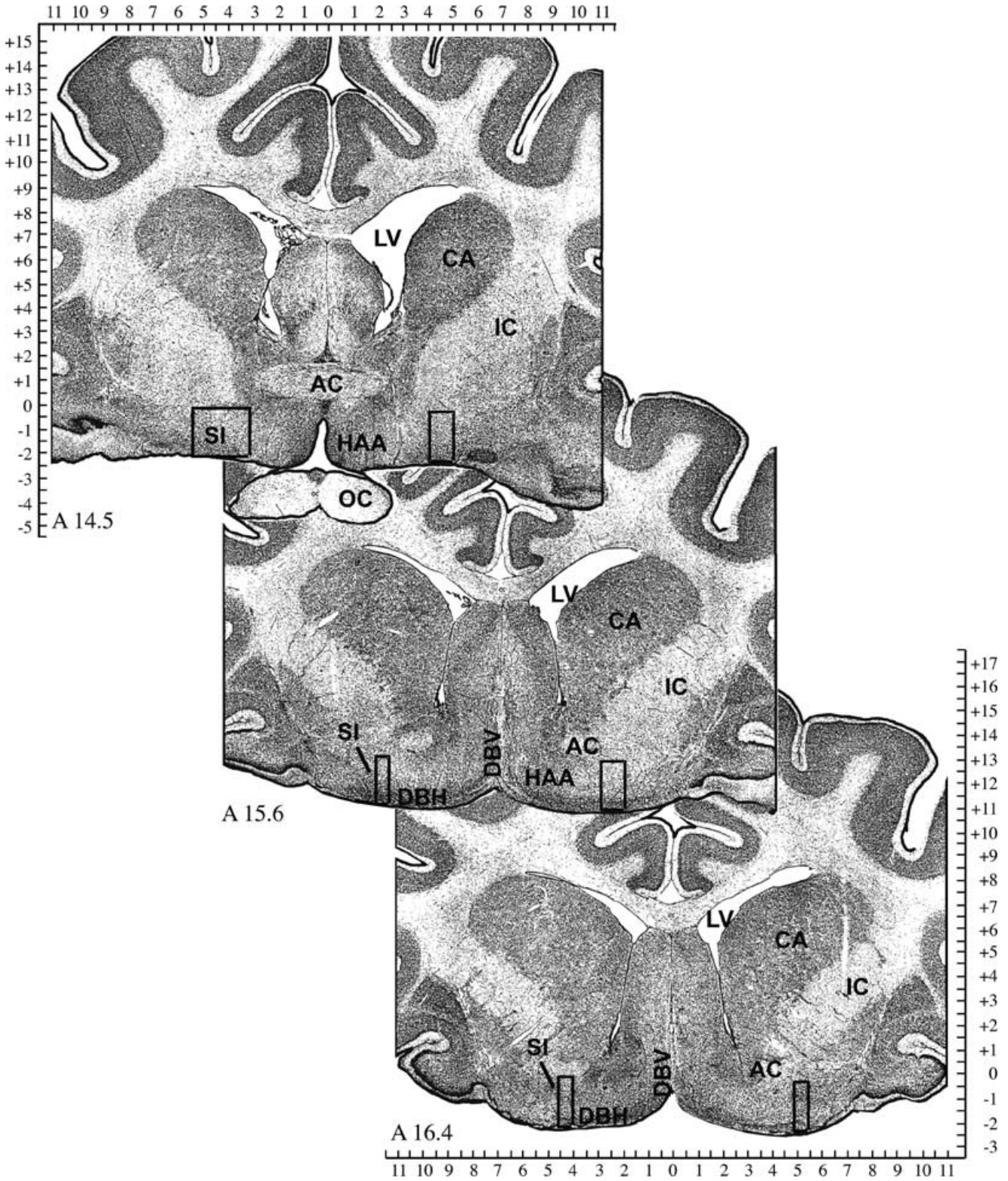


FIG. 8. Schematic representation of the basal forebrain region studied by dialysis. Coronal atlas plates at 14.5, 15.6 and 16.4 mm anterior (A) to stereotaxic zero are from Berman & Jones (1982) and the perspective is from caudal (top) to rostral (bottom). In the bottom portion of each of the three plates, boxed areas indicate the extent of the substantia innominata region that was dialysed. Twenty-seven microdialysis sites in nine cats were localized within the substantia innominata. At the most caudal level (A14.5), three dialysis probe sites were localized within the box drawn on the left side of the brain and five sites were localized within the box drawn on the right side of the brain. At the level of A15.6, boxed areas contained four probe sites on the left and 11 probe sites on the right. At the most rostral level (A16.4), boxed areas contained two probe sites on the left and two probe sites on the right. Axes scales are in mm. Abbreviations: AC, anterior commissure; CA, caudate; DBH, diagonal band of Broca, horizontal division; DBV, diagonal band of Broca, vertical division; HAA, anterior hypothalamic area; IC, internal capsule; LV, lateral ventricle; OC, optic chiasm; SI, substantia innominata.

specific brain regions (Billard *et al*., 1995; Baghdoyan *et al*., 1998; Douglas *et al*., 2001). Scopolamine is selective for muscarinic *versus* nicotinic cholinergic receptors, but scopolamine does not distinguish between muscarinic receptor subtypes. The minimum ACh releasing concentration of scopolamine in cat substantia innominata (0.3 nM, Fig. 3) was similar to the affinity of scopolamine for all muscarinic receptor subtypes ($K_i = 0.1–1$ nM, Billard *et al*., 1995). The Fig. 3 data are consistent with the conclusion that scopolamine acted at muscarinic receptors to increase ACh release. The EC_{50} for scopolamine (0.16 nM, Fig. 6) lends additional support to this conclusion.

Future studies using muscarinic antagonists with relative selectivity for different subtypes will be required to identify the muscarinic receptor subtype functioning as an autoreceptor in cat substantia innominata. M2 muscarinic receptors have been localized to cholinergic terminals in basal forebrain of rat (Levey *et al*., 1995) and monkey (Smiley *et al*., 1999), suggesting that M2 muscarinic receptors function to modulate ACh release in these brain regions. No comparable anatomical data are available for cat. *In vitro* electrophysiological studies using cultured neurons from rat basal forebrain have shown that ACh release is inhibited by activation of M2 muscarinic receptors, consistent with the functional role of an autoreceptor (Allen & Brown, 1996; Allen, 1999). The present minimum releasing concentration of scopolamine in cat substantia innominata (0.3 nM, Fig. 3) was similar to that reported for other brain regions in cat (1 nM by Baghdoyan *et al*., 1998), rat (1 nM by Billard *et al*., 1995), and mouse (3 nM by Douglas *et al*., 2001). In all three of these studies the M2 subtype was pharmacologically identified as an autoreceptor.

The present data do not prove that scopolamine increased ACh release by antagonism of an autoreceptor. Scopolamine blocks all muscarinic receptor subtypes, and under the conditions of this study scopolamine would have blocked muscarinic heteroreceptors as well as autoreceptors. Because muscarinic receptor activation hyperpolarizes basal forebrain cholinergic neurons (Khateb *et al*., 1997), blockade of postsynaptic muscarinic receptors with scopolamine could have disinhibited basal forebrain cholinergic neurons and caused the observed increase in ACh release from local dendrites or axons. In addition, *in vitro* electrophysiological studies using whole-cell patch-clamp recordings in rat brain slices have shown that excitation of magnocellular basal forebrain neurons by glutamate was reduced by activating presynaptic muscarinic receptors (Sim & Griffith, 1996). Thus, presynaptic muscarinic heteroreceptors also may have contributed to the presently observed scopolamine-induced increase in ACh release.

Two possible sources of ACh measured within the substantia innominata include terminals from brainstem cholinergic neurons in the laterodorsal and pedunculopontine tegmental nuclei (LDT/PPT) and, as mentioned above, axon collaterals from local basal forebrain cholinergic neurons (Jones & Mühlethaler, 1999; Semba, 1999). The existence of cholinergic projections from LDT/PPT to basal forebrain in cat has not been evaluated systematically using retrograde tracing in combination with immunohistochemistry. In rat, one study reported that less than 1% of cholinergic LDT/PPT neurons project to the basal forebrain (Jones & Cuello, 1989), whereas another study found that about 40% of cholinergic LDT neurons project to the basal forebrain (Semba *et al*., 1988). Slightly different basal forebrain injection sites of the retrograde tracers may account for the difference between the results of these two studies (Semba *et al*., 1988; Jones & Cuello, 1989). In support of the latter report (Semba *et al*., 1988), *in vivo* microdialysis studies in rat have shown that LDT/PPT neurons release ACh in the basal forebrain (Consolo *et al*., 1990; Bertorelli *et al*., 1991). Further studies are needed to identify the source of ACh released in cat substantia innominata.

GABA inhibits cholinergic transmission in the substantia innominata via $GABA_A$ receptors

The minimum ACh releasing concentration of bicuculline was 30 μM (Fig. 5). The pharmacological properties of $GABA_A$ receptors depend on their subunit composition, which varies markedly across brain regions (Fritschy & Mohler, 1995). Thus, it is not possible to compare the presently determined minimum ACh releasing concentration of bicuculline with the affinity of bicuculline for $GABA_A$ receptors. Furthermore, the $GABA_A$ receptor isoforms present in cat substantia innominata are unknown. The minimum ACh releasing concentration of bicuculline determined in this study was similar to concentrations of bicuculline that were effective for increasing ACh release from rat cortex (10–25 μM, Giorgetti *et al*., 2000; 50 μM, Materi & Semba, 2001), striatum (10–25 μM, Anderson *et al*., 1993; de Boer & Westerink, 1994), and septo-hippocampal system (5–10 μM, Moor *et al*., 1998a, 1998b). This similarity supports the conclusion that bicuculline acted at $GABA_A$ receptors to increase substantia innominata ACh release. Two additional classes of GABA receptors, $GABA_B$ and $GABA_C$, are found within the central nervous system (Bormann, 2000). The roles these GABA receptors play in the regulation of substantia innominata ACh release remain to be identified. $GABA_A$ and $GABA_B$ agonists recently have been shown to differentially alter sleep and wakefulness when microinjected directly into rat basal forebrain (Manfridi *et al*., 2001).

The concentration-dependent increase in ACh release evoked by bicuculline did not reach saturation (Fig. 6). Thus, the actual EC_{50} for bicuculline may be greater than 90 μM. No previous studies have determined an EC_{50} for bicuculline-induced ACh release in any brain region.

Sources of GABA in the substantia innominata region of the basal forebrain include intrinsic GABAergic interneurons (Gritti *et al*., 1993), basal forebrain GABAergic projection neurons (Gritti *et al*., 1997, 1998) which may release GABA from axon collaterals, and terminals arising from GABAergic neurons in the nucleus accumbens (Zaborszky & Cullinan, 1992). GABAergic terminals have been shown to make synaptic contact with magnocellular cholinergic neurons in rat basal forebrain (Zaborszky *et al*., 1986; Ingham *et al*., 1988). One mechanism by which bicuculline may have increased ACh release in the present study is by disinhibiting basal forebrain cholinergic neurons. For example, it been suggested based on *in vivo* microdialysis studies that cholinergic input from LDT/PPT neurons activates basal forebrain GABAergic neurons, which would inhibit basal forebrain cholinergic neurons (Bertorelli *et al*., 1991). As has been noted previously (Zaborszky & Duque, 2000), an understanding of the mechanisms by which $GABA_A$ receptors modulate basal forebrain ACh release will be advanced by additional information about the synaptic connections between physiologically and neurochemically identified basal forebrain neurons.

Differences in efficacy between scopolamine and bicuculline

Basal levels of ACh release, percent ACh recovery from dialysis probes, and the stereotaxic coordinates of the anatomical regions dialysed were not significantly different between experiments that delivered scopolamine (Figs 1–3) and experiments that delivered bicuculline (Figs 4 and 5) to the substantia innominata. Thus, these factors cannot account for the finding that bicuculline caused a greater increase in ACh release than scopolamine (Fig. 7). Scopolamine and bicuculline are both relatively small molecules and it can be assumed that both drugs had similar diffusion characteristics with respect to the dialysis membrane and the surrounding brain tissue (discussed in detail in Billard *et al*., 1995). One likely explanation for the differences in efficacy between scopolamine and bicuculline is that scopolamine

increased ACh release by blocking autoreceptors whereas bicuculline increased ACh release by blocking heteroreceptors. The relatively high efficacy of bicuculline is consistent with the interpretation that GABA exerts a powerful inhibitory effect on cholinergic neurotransmission within the substantia innominata.

Functional considerations

Mounting evidence supports a role for basal forebrain cholinergic and GABAergic projection neurons in the regulation of sleep and wakefulness (reviewed in Sarter & Bruno, 2000; Semba, 2000; Baghdoyan & Lydic, 2002). Putatively cholinergic neurons of cat substantia innominata (Szymusiak & McGinty, 1986, 1989) and rat magnocellular basal forebrain (Szymusiak *et al*., 2000) discharge at their fastest rates during wakefulness and REM sleep. This discharge pattern correlates with cortical ACh levels, which are greatest during wakefulness and REM sleep and lowest during NREM sleep (Jasper & Tessier, 1971; Marrosu *et al*., 1995). In contrast, putatively GABAergic neurons localized to the VLPO region of the basal forebrain discharge at their greatest rates during sleep (Szymusiak *et al*., 1998). GABAergic VLPO neurons also show increased immediate early gene expression during recovery sleep following prolonged wakefulness (Sherin *et al*., 1996, 1998). Microinjection of a $GABA_A$ agonist into the basal forebrain increases NREM sleep and decreases wakefulness (Manfridi *et al*., 2001), whereas basal forebrain microinjection of a cholinergic agonist increases wakefulness and suppresses sleep (Baghdoyan *et al*., 1993). The present finding that substantia innominata ACh release is powerfully inhibited by a $GABA_A$ receptor antagonist supports the concept that inhibition of basal forebrain cholinergic neurotransmission by GABA contributes to the generation of sleep.

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Abbreviations

AC, anterior commissure; ACh, acetylcholine; ANOVA, analysis of variance; CA, caudate nucleus; DBH, diagonal band of Broca, horizontal division; DBV, diagonal band of Broca, vertical division; GABA, gamma aminobutyric acid; GFAP, glial fibrillary acidic protein; HAA, anterior hypothalamic area; HPLC/EC, high performance liquid chromatography with electrochemical detection; IC, internal capsule; LV, lateral ventricle; NREM, non-REM; OC, optic chiasm; REM, rapid eye movement; SI, substantia innominata; VLPO, ventrolateral preoptic area.

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