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$GABA_A$ Receptors Inhibit Acetylcholine Release in Cat Pontine Reticular Formation: Implications for REM Sleep Regulation

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Vazquez, Jacqueline and Helen A. Baghdoyan. $GABA_A$ receptors inhibit acetylcholine release in cat pontine reticular formation: implications for REM sleep regulation. *J Neurophysiol* 92: 2198–2206, 2004. First published June 22, 2004; 10.1152/jn.00099.2004. This study used in vivo microdialysis in cat ($n = 12$) to test the hypothesis that gamma aminobutyric acid A ($GABA_A$) receptors in the pontine reticular formation (PRF) inhibit acetylcholine (ACh) release. Animals were anesthetized with halothane to hold arousal state constant. Six concentrations of the $GABA_A$ receptor antagonist bicuculline (0.03, 0.1, 0.3, 1, 3, and 10 mM) were delivered to a dialysis probe in the PRF, and endogenously released ACh was collected simultaneously. Bicuculline caused a concentration dependent increase in ACh release (maximal increase = 345%; $EC_{50} = 1.3$ mM; $r^2 = 0.997$). Co-administration of the $GABA_A$ receptor agonist muscimol prevented the bicuculline-induced increase in ACh release. In a second series of experiments, the effects of bicuculline (0.1, 0.3, 1, and 3 mM) on ACh release were examined without the use of general anesthesia. States of wakefulness, rapid-eye-movement (REM) sleep, and non-REM sleep were identified polygraphically before and during dialysis delivery of bicuculline. Higher concentrations of bicuculline (1 and 3 mM) significantly increased ACh release during wakefulness (36%), completely suppressed non-REM sleep, and increased ACh release during REM sleep (143%). The finding that ACh release in the PRF is modulated by $GABA_A$ receptors is consistent with the interpretation that inhibition of GABAergic transmission in the PRF contributes to the generation of REM sleep, in part, by increasing pontine ACh release.

INTRODUCTION

Contemporary studies of sleep neurobiology seek to understand how multiple neurotransmitters, neuromodulators, and brain regions interact to regulate levels of arousal (for reviews, see Baghdoyan and Lydic 2002; Hobson and Pace-Schott 2002; Lydic and Baghdoyan 2003; McCarley 1999; Pace-Schott and Hobson 2002; Reinoso-Suarez et al. 2001; Saper et al. 2001). Cholinergic neurotransmission in the pontine reticular formation is a principle component of the neuronal network generating the rapid eye movement (REM) stage of sleep (Steriade and McCarley 1990). Gamma aminobutyric acid (GABA) is the major inhibitory neurotransmitter in brain, and many drugs used clinically to produce sleep or anesthesia enhance GABAergic transmission at $GABA_A$ receptors (Campagna et al. 2003; Lancel 1999). The present study focused on the REM sleep-related interaction between pontine cholinergic and GABAergic neurotransmission based on the following rationale.

The pontine reticular formation does not contain cholinergic neurons and receives cholinergic projections from the laterodorsal and pedunculopontine tegmental (LDT/PPT) nuclei (reviewed in Steriade and McCarley 1990). LDT/PPT neurons release acetylcholine (ACh) in the pontine reticular formation (Lydic and Baghdoyan 1993), and during REM sleep, ACh release is significantly increased in the pontine reticular formation over wakefulness and non-REM (NREM) sleep levels (Kodama et al. 1990; Leonard and Lydic 1995). Direct administration of cholinomimetics into the pontine reticular formation causes a concentration dependent enhancement of REM sleep that is blocked by atropine, suggesting that cholinergic transmission at pontine reticular formation muscarinic receptors contributes to REM sleep generation (reviewed in Baghdoyan 1997). Further support for the role of pontine cholinergic neurotransmission in REM sleep generation comes from studies showing that the discharge rate of putatively cholinergic LDT/PPT neurons is significantly increased prior to and during REM sleep (El Mansari et al. 1989; Kayama et al. 1992; Steriade et al. 1990), electrical stimulation of LDT/PPT neurons increases REM sleep (Thakkar et al. 1996), and neurotoxic lesions of the LDT/PPT decrease REM sleep in amounts proportional to the loss of cholinergic neurons (Webster and Jones 1988). Taken together, these data provide strong support for the role of ACh, the LDT/PPT, and the pontine reticular formation in generating REM sleep.

GABAergic neurons are distributed throughout cholinergic (LDT/PPT) and noncholinergic cholinoceptive regions of the pons, including the pontine reticular formation (Ford et al. 1995; Mugnaini and Oertel 1985). GABAergic neurons in several nuclei of the pons, including the LDT/PPT, have been shown to be active during REM sleep based on expression of the protein c-Fos (Maloney et al. 1999; Torterolo et al. 2000–2002). The $GABA_A$ receptor ligands muscimol (agonist) and bicuculline (antagonist) have opposite effects on sleep and wakefulness when microinjected into the same pontine reticular formation sites where cholinomimetics enhance REM sleep. Bicuculline triggers a REM sleep-like state with a short latency, whereas muscimol increases wakefulness and suppresses sleep (Xi et al. 1999).

The mechanisms by which blocking transmission at $GABA_A$ receptors in the pontine reticular formation enhances REM sleep are unknown but may involve removing GABAergic inhibition of ACh release from LDT/PPT terminals. GABA inhibits the release of ACh in other brain regions (Giorgetti et al. 2000; Materi and Semba 2001; Moor et al. 1998; Vazquez

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and Baghdoyan 2003b), and enhancing cholinergic transmission in the pontine reticular formation increases REM sleep (reviewed in Baghdoyan 1997). Therefore this study tested the hypothesis that blocking GABAergic transmission in the pontine reticular formation by dialysis administration of bicuculline increases ACh release in the pontine reticular formation. Preliminary reports of these data have been presented (Baghdoyan et al. 2002; Vazquez and Baghdoyan 2003a).

METHODS

Animals and surgery

All procedures using animals were approved by the University of Michigan Committee on Use and Care of Animals and were conducted in accordance with the U. S. Department of Agriculture Animal Welfare Act and the American Physiological Society's *Guiding Principles in the Care and Use of Animals* (http://www.theaps.org/publications/journals/guide.htm#note2). The rationale for performing these studies in cat has been reviewed recently (Vazquez and Baghdoyan 2003b). One advantage of cat over rat or mouse is the comparatively long duration of cat NREM sleep and REM sleep epochs, allowing collection of dialysis samples during periods comprised entirely of each sleep stage (Kodama et al. 1990; Leonard and Lydic 1995, 1997; Marrosu et al. 1995; Vazquez and Baghdoyan 2001; Vazquez et al. 2002). Second, the large size of the cat brain stem (Berman 1968) provides a high level of anatomic resolution and facilitates accurate placement of microdialysis probes. Third, bicuculline-induced REM sleep enhancement was discovered in cat (Xi et al. 1999).

Sterile surgical procedures that have been described previously (Vazquez and Baghdoyan 2003b) were used to implant cats ($n = 12$) with standard recording electrodes for objectively identifying states of sleep and wakefulness (Ursin and Sterman 1981). A permanent craniotomy was created over the cerebellum by surgically removing a small portion of the skull and replacing the bone with sterile Bone Wax (Ethicon, Sommerville, NJ) (Baghdoyan et al. 1998; Leonard and Lydic 1997; Lydic and Baghdoyan 2002). The craniotomy provided access to the pontine reticular formation during subsequent dialysis experiments. Dental acrylic was used to fix the electrodes to the skull and to secure two stainless steel sleeves that attach to a Kopf 880 semi-chronic head holder (David Kopf Instruments, Tujunga, CA). The Kopf 880 device permits the placement of an animal in stereotaxic position without tissue contact. The head holder and the absence of pain receptors in the brain makes it possible to place dialysis probes in specific brain regions of an unanesthetized animal (Leonard and Lydic 1997; Lydic and Baghdoyan 1993, 2002; Vazquez and Baghdoyan 2001; Vazquez et al. 2002).

Chemicals and microdialysis probes

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). The $GABA_A$ receptor antagonist bicuculline methiodide and the $GABA_A$ receptor agonist muscimol were dissolved in Ringer solution [which contained (in mM)147 NaCl, 2.4 $CaCl_2$, and 4.0 KCl and 10 μM neostigmine bromide; pH 5.8–6.2] and were made fresh prior to each experiment. Concentrations of bicuculline used to perfuse the dialysis probe were 0.03, 0.1, 0.3, 1, 3, and 10 mM. Muscimol (0.1 mM) was delivered to the probe either alone or with bicuculline (0.3 mM).

Microdialysis probes (CMA Microdialysis, North Chelmsford, MA) had a shaft length of 70 mm and a polycarbonate dialysis membrane (20-kDa cutoff, 2-mm length, 0.5 mm diam). A CMA/100 dialysis pump was used for continuous perfusion (3 μl/min) of dialysis probes with Ringer solution. Immediately prior to placing a dialysis probe in the brain and immediately after removal of a dialysis probe from the brain the percent of ACh recovered by the probe was determined in vitro (Baghdoyan et al. 1998; Lydic and Baghdoyan 2002; Tanase et al. 2003; Vazquez and Baghdoyan 2003b). Pre- and postexperimental probe recovery values were compared by *t*-test. Results from an experiment were included in the final data set only if there was no significant change in probe recovery during the experiment. Average ± SD of ACh recovered was 7.8 ± 2.0%, which is typical for this type of dialysis probe (Tanase et al. 2003; Vazquez and Baghdoyan 2003b).

Dialysis during general anesthesia

ACh release in the dorsal tegmental field (Kodama et al. 1990) and the medial pontine reticular formation (Leonard and Lydic 1995, 1997) increases significantly during REM sleep compared with NREM sleep and wakefulness. Therefore the first part of this study used general anesthesia to eliminate REM sleep and hold arousal state constant. This procedure has been shown to be particularly useful for studies involving microdialysis delivery of drugs to the pontine reticular formation during simultaneous collection of endogenous ACh (Baghdoyan et al. 1998; Bernard et al. 2003). Inhalation anesthesia is ideal for this purpose because end tidal anesthetic concentration can be monitored continuously using spectrophotometry. As described previously in detail (Baghdoyan et al. 1998; Tanase et al. 2003; Vazquez and Baghdoyan 2003b), every experiment began by mask induction with halothane (3–4% in oxygen). The trachea was intubated, the cat was mechanically ventilated and fixed in stereotaxic position, and monitors were placed to measure delivered and end tidal concentrations of halothane, CO_2, core body temperature, blood-oxygen saturation, and heart rate. Continuous monitoring of autonomic parameters and end tidal halothane concentration helped ensure adequate depth of anesthesia. End tidal halothane was maintained at 1.4–1.5% throughout the dialysis experiments.

A microdialysis probe was aimed stereotaxically for the pontine reticular formation, located within the gigantocellular tegmental field (Berman 1968). Dialysis samples (30 μl) were collected every 10 min and ACh content was quantified as described in the following text. Stable levels of ACh release were observed within 30 min after probe placement in the brain. Five sequential dialysis samples were collected during perfusion of the dialysis probe with Ringer solution to determine control levels of ACh release (Baghdoyan et al. 1998; Vazquez and Baghdoyan 2003b). A liquid switch (CMA/110) then was turned to deliver Ringer solution containing bicuculline, muscimol, or a mixture of bicuculline and muscimol. Five sequential dialysis samples were obtained during drug administration. After collection of the last sample, the dialysis probe was removed from the brain, and the craniotomy was closed with sterile bone wax. Halothane administration was discontinued, the ventilator was turned off, and the animal was extubated when it demonstrated regular breathing. Animals were kept under continuous observation in the laboratory until fully recovered from anesthesia and were returned to their home cages in the Unit for Laboratory Animal Medicine.

Only one concentration of bicuculline was administered per experiment. A minimum of 7 days was allowed between experiments in the same animal. Dialysis probe aim sites in the same animal were separated by ≥1 mm in the rostrocaudal and mediolateral planes, and the same dialysis site was used only once to avoid possible alterations in ACh release caused by probe-induced glial scarring (Moore et al. 1995).

Dialysis and polygraphic recordings during sleep and wakefulness

The effects of bicuculline on ACh release also were studied in unanesthetized cats that displayed spontaneous epochs of wakefulness, NREM sleep, and REM sleep. For every experiment, a Grass Model 7D polygraph (Grass Instruments, West Warwick, RI) was

used to record the cortical electroencephalogram (EEG), electrooculogram (EOG), electromyogram (EMG), ponto-geniculo-occipital (PGO) waves, and respiratory rate. These physiological measures were used for objectively scoring states of sleep and wakefulness according to standard, previously described criteria (Lydic and Baghdoyan 2002; Ursin and Sterman 1981; Vazquez and Baghdoyan 2001; Vazquez et al. 2002).

An experiment began by placing an animal in stereotaxic position using the Kopf 880 semi-chronic head holder. A stainless steel guide tube was then stereotaxically aimed for the pontine reticular formation. A dialysis probe was inserted into the guide tube while the animal was awake. Dialysis samples (30 μl) were collected during polygraphically defined states of wakefulness, NREM sleep, and REM sleep while the dialysis probe was perfused with Ringer solution to obtain control levels of ACh release for each of the three states (Leonard and Lydic 1997; Vazquez and Baghdoyan 2001; Vazquez et al. 2002). A CMA/110 liquid switch then was activated to deliver Ringer containing bicuculline, and dialysis samples continued to be collected during objectively defined states of sleep and wakefulness. After collection of the last dialysis sample, the dialysis probe and guide tube were removed from the brain, the craniotomy was closed, and the animal was returned to its home cage.

Quantification of ACh

ACh content of the dialysis samples was quantified using a Bioanalytical Systems (BAS, West Lafayette, IN) high-performance liquid chromatography and electrochemical detection (HPLC/EC) system and a standard curve generated prior to every experiment. As previously described (Baghdoyan et al. 1998; Vazquez and Baghdoyan 2003b; Vazquez et al. 2002), dialysis samples were injected into the HPLC/EC system immediately after collection. Samples were transported through the system (1 ml/min) by a Na_2HPO_4 mobile phase (50 mM, pH 8.5). A column containing acetylcholinesterase produced H_2O_2 in amounts proportional to the ACh in the sample, and H_2O_2 was detected by a platinum electrode (500 mV applied potential) referenced to a Ag^+/AgCl electrode. A continuous record of the chromatograms was obtained using a flatbed recorder. Chromatograms were simultaneously digitized and stored to disk with ChromGraph software (BAS). ChromGraph also was used to calculate the amount of ACh in each dialysis sample, expressed as pmol/10 min.

Histological confirmation of dialysis probe placement in the pontine reticular formation

Stereotaxic coordinates used to aim dialysis probes for the pontine reticular formation ranged from posterior (P) 1.5 to P3.0, lateral (L) 1.0 to L2.5, and horizontal (H) −4.0 to H-6.0 (Berman 1968) on either side of the brain stem. Probe placement was confirmed histologically, as described previously (Baghdoyan et al. 1998; Leonard and Lydic 1997; Tanase et al. 2003). Animals were anesthetized and brains were perfused in situ with 10% formalin, removed, and soak-fixed in formalin. The brain stem then was cryoprotected in formalin containing 30% sucrose. Serial, sagittal, brain stem sections (40 μm thick) were cut on a freezing microtome, float mounted onto chrome alum coated glass slides, and stained with cresyl violet. All tissue sections containing a dialysis probe-induced lesion were digitized and the stereotaxic coordinates of the dialysis sites were defined according to a cat brain stem atlas (Berman 1968).

Statistical analyses

Data analysis included descriptive and inferential statistics. Significant drug effects on pontine ACh release were determined by one-way ANOVA, post hoc multiple comparisons test (Dunnett's or Tukey-Kramer), and *t*-test. The ANOVA model was adjusted for unequal variance when needed as determined by Levene's test of equality of error variances. The degrees of freedom for the *F* test were adjusted to compensate for unequal variance, resulting in a noninteger value for the denominator degrees of freedom. Dunnett's T3, which takes into account unequal variances between groups, was used for post hoc multiple comparisons with the adjusted ANOVA model. Concentration response data were fit to a sigmoid curve using nonlinear regression analysis (GraphPad Prism version 4.0a for Macintosh, GraphPad Software, San Diego, CA) with the following equation: ACh release = basal release + (maximal release-basal release)/[1 + 10^(log EC_{50} − *X*)], where *X* is the logarithm of the bicuculline concentration. The coefficient of determination (r^2) and concentration of bicuculline that produced a 50% increase in ACh release (EC_{50}) were calculated using this equation.

RESULTS

Bicuculline delivered to the pontine reticular formation during general anesthesia caused a concentration dependent increase in ACh release that was prevented by co-administration of muscimol

Figure 1*A* schematizes simultaneous collection of ACh and drug delivery using the same dialysis probe. Figure 1*B* illustrates a typical histological section used to localize microdialysis sites. Probe placement ranged from approximately P1 to P3, L1 to L2.5, and H-5 to H-7 (the H coordinate indicates the deepest part of the 2-mm-long dialysis membrane). These stereotaxic coordinates (based on the sagittal plates in Berman 1968) provide confirmation that measures of ACh were obtained from the pontine reticular formation. Figure 1*C* shows an example of chromatograms used to quantify ACh during control (Ringer solution) dialysis (Fig. 1*C*, *left*) and during dialysis with Ringer solution containing bicuculline (Fig. 1*C*, *right*).

Figure 2 illustrates that each experiment conducted during general anesthesia quantified ACh release during five sequential control samples (dialysis with Ringer solution) followed by five sequential samples obtained during dialysis delivery of bicuculline. Control levels of ACh release (Fig. 2, ▨) were stable across the 50-min period of dialysis with Ringer solution. The lowest concentration of bicuculline (0.03 mM, Fig. 2*A*, ■) did not alter ACh release. A 10-fold greater concentration of bicuculline (0.3 mM, Fig. 2*B*, ■) caused a clear enhancement of ACh release after 10 min of delivery. Delivering 3 mM bicuculline to the dialysis probe increased ACh release within the first 10 min (Fig. 2*C*, ■).

Figure 3 depicts ACh release as a function of bicuculline concentration. ANOVA based on measures of ACh release obtained during dialysis with Ringer solution (control) and six concentrations of bicuculline revealed a significant main effect of bicuculline concentration on ACh release ($F = 34.2$; df = 6, 26.9; $P < 0.001$). Dunnett's T3 post hoc multiple comparisons test identified the lowest concentration of bicuculline to significantly ($P < 0.05$) increase ACh above control levels as 0.1 mM. Nonlinear regression analysis of the Fig. 3 data indicated that 99% of the variance in ACh release was accounted for by the concentration of bicuculline ($r^2 = 0.997$). The calculated EC_{50} for bicuculline was 1.3 mM.

Having demonstrated that the bicuculline-induced increase in ACh release was concentration dependent, this study next sought to ascertain whether the enhancement of ACh release by bicuculline could be prevented with the $GABA_A$ receptor agonist muscimol. Figure 4 summarizes the effects on ACh

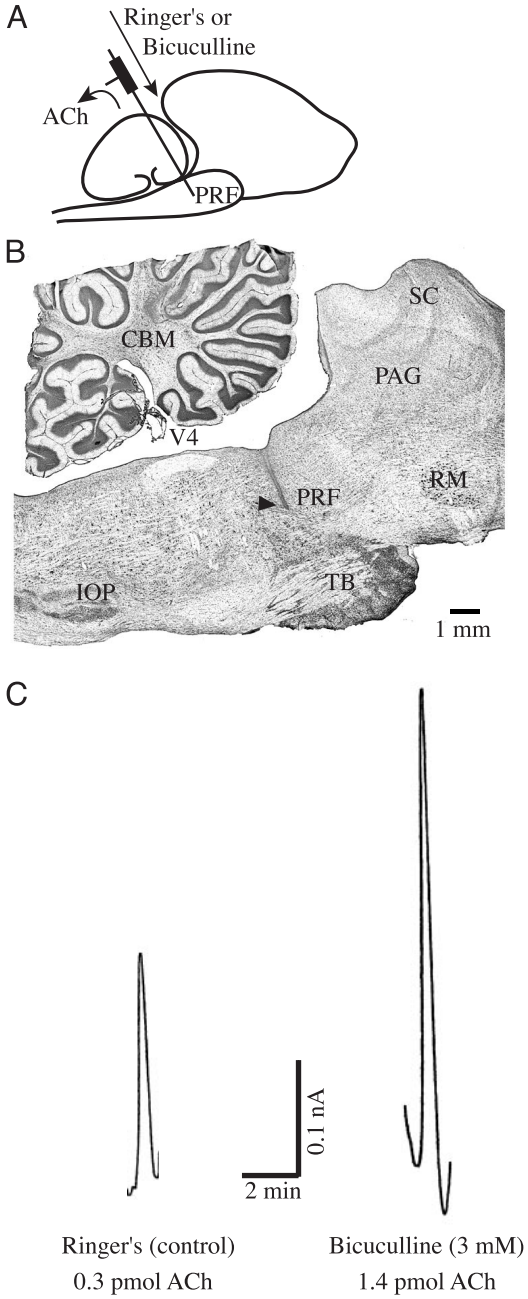

FIG. 1. In vivo microdialysis, histological localization of a microdialysis site, and chromatography for quantification of acetylcholine (ACh). *A*: drawing schematizes a sagittal view of the cat brain with a microdialysis probe placed in the pontine reticular formation (PRF). Ringer solution or Ringer containing a drug was pumped into the probe through the inflow port (straight arrow). Drugs were delivered to the pontine reticular formation, and endogenous ACh was collected from the pontine reticular formation via passive diffusion through the dialysis membrane comprising the tip of the probe. Ringer solution containing ACh was collected from the outflow port on the dialysis probe (curved arrow). *B*: a representative cresyl violet-stained sagittal section of cat brain stem at ~1.4 mm from the midline (according to the sagittal plates in Berman 1968). The arrowhead points to the bottom of the 2-mm-long microdialysis site, which is localized to approximately P-1 and H-6. CBM, medial cerebellar nucleus; IOP, principle nucleus of the inferior olive; PRF, pontine reticular formation; PAG, periaqueductal gray; RM, red nucleus, magnocellular division; SC, superior colliculus; TB, trapezoid body; V4, fourth ventricle. *C*: typical chromatographic peaks generated by ACh after injection of dialysis samples into the high-performance liquid chromatography and electrochemical detection (HPLC/EC) system. Both peaks are from the same experiment in a halothane anesthetized cat. Each peak represents the ACh content of 1 30-μl sample obtained during PRF dialysis with Ringer solution (*left*) or bicuculline (*right*).

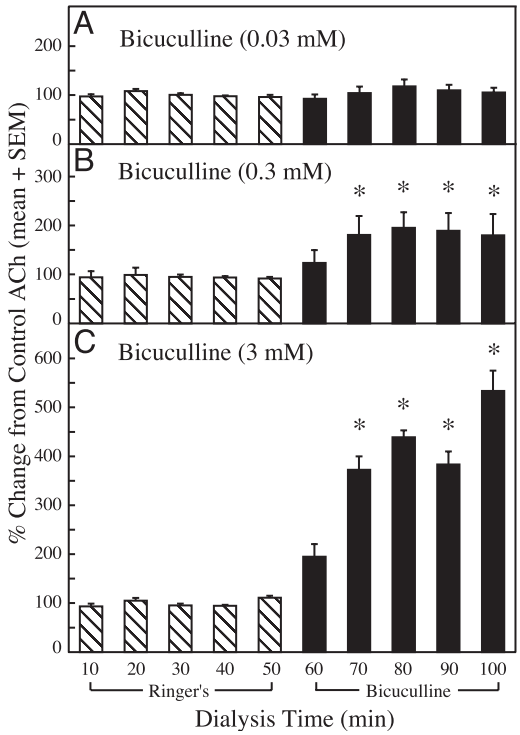

FIG. 2. Sequential dialysis samples collected every 10 min from the pontine reticular formation during anesthesia show the time course and concentration dependence of bicuculline-induced ACh release. Control levels of ACh release were established during 50 min of dialysis with Ringer solution (▧). Bicuculline was delivered for 50 min (■). Each bar represents the mean of 3 experiments. *, significant ($P < 0.05$) increases over average Ringer (control) levels of ACh release. These data were obtained from 9 dialysis sites in 8 animals.

release of dialyzing the pontine reticular formation simultaneously with bicuculline plus muscimol or with muscimol alone. ANOVA revealed a significant drug effect on ACh release (Fig. 4*A*: F = 6.65; df = 2, 58; $P = 0.003$). The Tukey-Kramer multiple comparisons test showed that the 36% increase in ACh release caused by bicuculline was blocked by

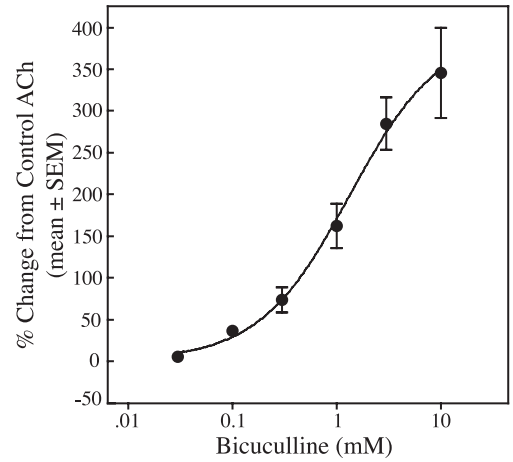

FIG. 3. The bicuculline-induced increase in ACh release was concentration dependent. Six concentrations of bicuculline were administered to the pontine reticular formation by dialysis during anesthesia. The number (n) of dialysis samples comprising the means is listed for every concentration of bicuculline: 0.03 mM (15); 0.1 mM (15); 0.3 mM (15); 1 mM (14); 3 mM (15); and 10 mM (5). These data were obtained from 16 dialysis sites in 11 animals.

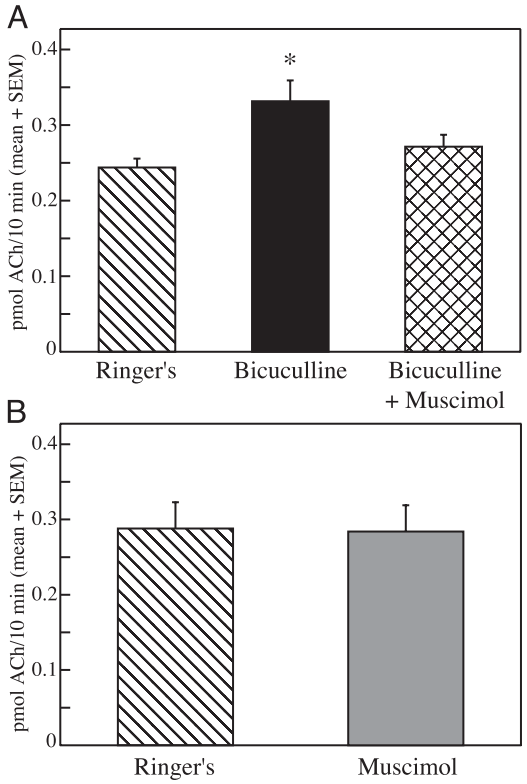

FIG. 4. Muscimol blocked the bicuculline-induced increase in ACh release. *A*: ACh release in the pontine reticular formation was quantified during dialysis with Ringer solution (control; $n = 30$ dialysis samples), during dialysis administration of bicuculline (0.3 mM; $n = 15$), and during co-administration of bicuculline (0.3 mM) and muscimol (0.1 mM; $n = 14$). *, bicuculline caused a significant ($P < 0.01$) increase in ACh release over control levels. *B*: muscimol alone (0.3 mM, $n = 14$ dialysis samples) had no effect on ACh release in the pontine reticular formation relative to Ringer control ($n = 15$). Each treatment condition (bicuculline, bicuculline + muscimol, and muscimol) was tested in 3 anesthetized animals. These data were obtained from 6 dialysis sites in 3 animals.

co-administration of muscimol. Dialysis delivery of muscimol alone (Fig. 4*B*) did not alter ACh release (*t*-test).

Bicuculline delivered to the pontine reticular formation of unanesthetized cat increased ACh release

The foregoing data obtained during general anesthesia established that pharmacological antagonism of $GABA_A$ receptors in the pontine reticular formation increased ACh release in the pontine reticular formation. Because volatile anesthetics have direct actions on the $GABA_A$ receptor (Campagna et al. 2003), the effects of bicuculline on ACh release next were studied without the use of halothane to rule out possible confounding effects of the anesthetic. Polygraph recordings were used to distinguish among wakefulness (Fig. 5*A*), NREM sleep (*B*), and REM sleep (*C*). Figure 5*D* shows that REM sleep during dialysis delivery of bicuculline (REM_{BIC}) appears to be indistinguishable from spontaneously occurring REM sleep (Fig. 5*C*), consistent with previous reports (Xi et al. 1999). ACh chromatograms to the right of the polygraph records illustrate typical ACh measures obtained during each arousal state.

During dialysis with Ringer solution (control), mean ± SD levels of ACh release (pmol/10 min) were 0.32 ± 0.12 during wakefulness ($n = 33$ dialysis samples), 0.23 ± 0.08 during NREM sleep ($n = 26$), and 0.51 ± 0.17 during REM sleep ($n = 19$), similar to previous reports (Leonard and Lydic 1995, 1997). ANOVA demonstrated a significant effect of arousal state on ACh release ($F = 28.0$; df = 2, 77; $P < 0.001$). ACh release was significantly ($P < 0.01$) greater during REM sleep than during NREM sleep or wakefulness (Tukey-Kramer multiple comparisons test).

The effects of four bicuculline concentrations on ACh release during sleep and wakefulness are summarized by Fig. 6. ANOVA revealed a significant concentration main effect of bicuculline on ACh release during wakefulness (Fig. 6*A;* $F = 7.5$; df = 4, 16.3; $P = 0.001$) and during REM sleep (Fig. 6*C;*

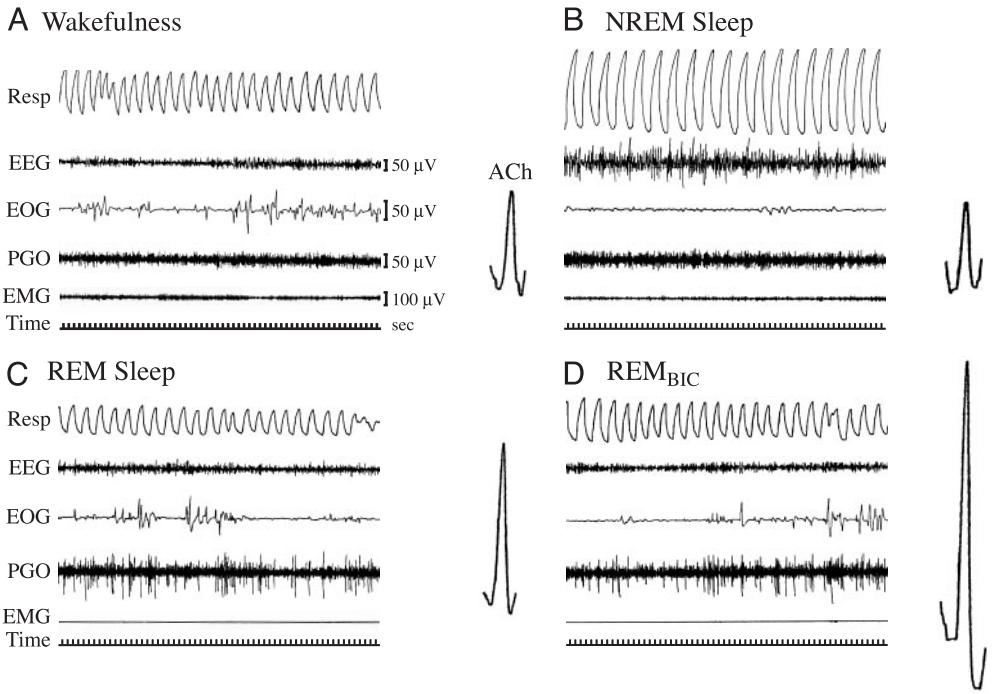

FIG. 5. Identification of sleep and wakefulness based on polygraph recordings. *A–D*: typical 1-min recordings from the same experiment in 1 cat. Recordings labeled wakefulness, NREM sleep, and REM sleep were obtained during dialysis of the pontine reticular formation with Ringer solution (control). The recording labeled REM_{BIC} was obtained during dialysis of the pontine reticular formation with bicuculline (3 mM). An ACh chromatogram obtained during each arousal state is shown to the right of the corresponding polygraph recording. Note the increased peak height during dialysis administration of bicuculline (*bottom right*) relative to ACh peak height during natural REM sleep (*bottom left*). The amount of ACh (pmol/10 min) in each of the four representative samples is *A*: wakefulness (0.15), *B*: NREM sleep (0.13), *C*: REM sleep (0.27), *D*: REM_{BIC} (0.48). Resp, respiratory rate; EEG, cortical electroencephalogram; EOG, electrooculogram; PGO, ponto-geniculo-occipital waves; EMG, electromyogram.

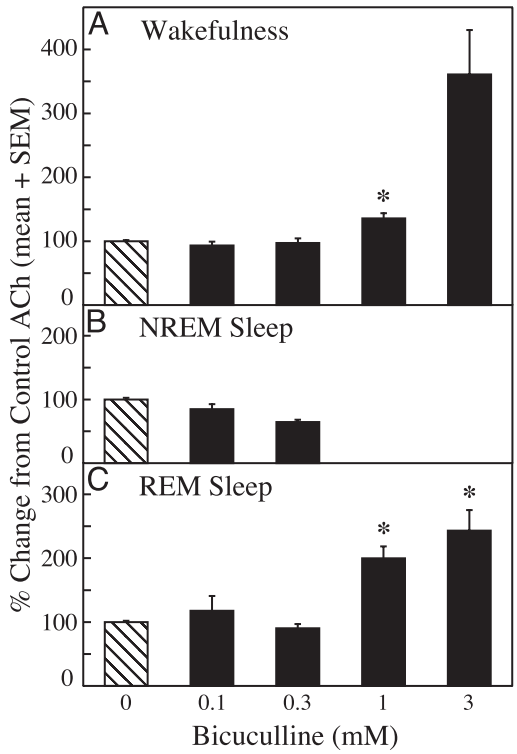

FIG. 6. Bicuculline caused a concentration dependent increase in ACh release during wakefulness and REM sleep. *, concentrations of bicuculline that significantly ($P < 0.01$) increased ACh release over control (▨) levels. Each concentration of bicuculline was tested in ≥3 animals. The number of dialysis samples (n) that contributed to each mean follows the concentration of bicuculline. *A*: wakefulness: 0 mM (33); 0.1 mM (17); 0.3 mM (18); 1 mM (20); 3 mM (4). *B*: NREM sleep: 0 mM (26); 0.1 mM (4); 0.3 mM (3). *C*: REM sleep: 0 mM (19); 0.1 mM (3); 0.3 mM (3); 1 mM (3); 3 mM (8). These data were obtained from 13 dialysis sites in 4 animals.

$F = 9.7$; df = 4, 5.8; $P = 0.01$). Dunnett's T3 post hoc multiple comparisons test showed that perfusion of the dialysis probe with 1 mM bicuculline significantly ($P < 0.01$) increased ACh release during wakefulness (Fig. 6*A*). Although 3 mM bicuculline increased ACh release during wakefulness by 261% over control levels, this increase was not statistically significant because of the small sample size ($n = 4$) and large variability (coefficient of variation = 39%). The amount of time spent in NREM sleep was reduced by lower concentrations of bicuculline (0.1 and 0.3 mM), and NREM sleep was completely suppressed by higher bicuculline concentrations (1 and 3 mM). Thus ANOVA was not applied to the ACh measures obtained during NREM sleep (Fig. 6*B*) due to the small sample size. During REM sleep (Fig. 6*C*), Dunnett's T3 indicated that dialysis with 1 and 3 mM bicuculline significantly ($P < 0.01$) increased ACh release.

Sagittal brain stem sections were used to determine the location of all dialysis sites tested during sleep and wakefulness. Histological analyses confirmed that measures of ACh release across the sleep cycle were obtained from the same part of the pontine reticular formation that was studied during general anesthesia. Dialysis sites studied in unanesthetized animals ranged from approximately P1.5 to P3, L1 to L2, and H-5 to H-7.

Behavioral effects of bicuculline

Dialysis administration of higher bicuculline concentrations to the unanesthetized cat caused a number of behavioral effects, including REM sleep enhancement, motor activation with circling behavior, and motor atonia during wakefulness. Animals that received higher concentrations of bicuculline during general anesthesia also exhibited circling behavior on recovery from anesthesia. Systematic examination of these effects was outside the scope of this study.

DISCUSSION

The data show for the first time that dialysis administration of the $GABA_A$ receptor antagonist bicuculline to the pontine reticular formation of halothane anesthetized cat causes a concentration dependent increase in ACh release that is prevented by co-administration of the $GABA_A$ receptor agonist muscimol. Additionally, the data demonstrate that bicuculline increases ACh release when delivered to the pontine reticular formation of unanesthetized cat. These findings reveal that $GABA_A$ receptors in the pontine reticular formation modulate ACh release and suggest that GABA tonically inhibits ACh release in the pontine reticular formation. These results fit well with previous data showing that microinjecting bicuculline into the pontine reticular formation of cat causes REM sleep enhancement and microinjecting muscimol increases wakefulness and suppresses sleep (Xi et al. 1999). The present findings support the interpretation that one mechanism by which pontine administration of bicuculline enhances REM sleep includes increasing ACh release in the pontine reticular formation. The results are discussed in the following text within the context of existing knowledge about the role of pontine GABA and ACh in REM sleep generation.

GABAergic regulation of REM sleep

Sedative hypnotics that enhance the actions of GABA, such as benzodiazepines and barbiturates, generally increase NREM sleep and decrease REM sleep when administered systemically to humans or animals (Lancel 1999). However, when delivered intracranially to experimental animals, the effects of GABA on sleep and wakefulness vary significantly as a function of brain region. GABA has been shown to promote wakefulness, NREM sleep, or REM sleep, depending on the brain region of action (reviewed in Baghdoyan and Lydic 2002). In the locus coeruleus, GABAergic inhibition of waking-active neurons has been proposed to promote REM sleep generation (Kaur et al. 1997; Mallick et al. 2001). This proposal is supported by the findings that GABA tonically inhibits locus coeruleus neurons during sleep (Gervasoni et al. 1998) and that endogenous GABA levels in the locus coeruleus are greater during REM sleep than during wakefulness or NREM sleep (Nitz and Siegel 1997). GABAergic neurons in the locus coeruleus and dorsal raphe nucleus have been shown to be active during recovery REM sleep after sleep deprivation (Maloney et al. 1999). These findings are consistent with the concept that GABAergic inhibition of wakefulness-promoting monoaminergic neurons contributes to the generation of REM sleep (Jones 1990b).

In contrast to the locus coeruleus, pontine reticular formation GABA is thought to promote wakefulness and inhibit REM sleep. Early studies in rat noted that pontine reticular

formation microinjection of muscimol increased wakefulness and suppressed NREM sleep, whereas microinjection of bicuculline into the same pontine reticular formation sites produced a nonsignificant decrease in the latency to onset of both NREM sleep and REM sleep (Camacho-Arroyo et al. 1991). Compelling evidence that pontine reticular formation GABA promotes wakefulness and inhibits REM sleep has come from microinjection studies in cat. These data show that pontine reticular formation administration of bicuculline causes a REM sleep-like state. Bicuculline significantly increases the amount of time spent in the REM sleep-like state and decreases the latency to onset of the REM sleep-like state (Xi et al. 1999). Microinjection of muscimol increases wakefulness and suppresses sleep (Xi et al. 1999).

The discovery that bicuculline injected into the rostral portion of the pontine reticular formation of cat significantly increased REM sleep time and decreased REM sleep latency (Xi et al. 1999) has prompted similar studies in rat and mouse focused on homologous regions of the pontine reticular formation. The first study to report bicuculline-induced REM sleep enhancement in rat used iontophoresis and localized an effective region to the subdorsolateral nucleus (Boissard et al. 2002), also named the dorsal subcoeruleus nucleus (Paxinos and Watson 1998). Unilateral microinjection of bicuculline into the dorsal subcoeruleus nucleus also has been reported to produce a REM sleep-like state (Pollock and Mistlberger 2003). Microinjection studies in rat demonstrated REM sleep enhancement caused by bicuculline bilaterally administered into the oral part of the rat pontine reticular formation (reticularis pontis oralis) (Sanford et al. 2003). Bicuculline-induced REM sleep enhancement did not occur after microinjections into the caudal part of the rat pontine reticular formation (reticularis pontis caudalis) (Sanford et al. 2003). Preliminary data indicate that microinjection of bicuculline into the rostral pontine reticular formation of C57BL/6J mouse evokes a REM sleep-like state (Falgout et al. 2003). Dialysis sites used in the present study were in the rostral and medial portion of the cat pontine reticular formation, homologous to the pontine reticular formation, oral part, in rat (Paxinos and Watson 1998) and mouse (Franklin and Paxinos 2001).

The $GABA_B$ antagonist phaclofen has been shown to significantly increase REM sleep when microinjected into cat pontine reticular formation, although the enhancement was less robust than with the $GABA_A$ antagonist bicuculline (Xi et al. 2001). Similar to muscimol, the $GABA_B$ agonist baclofen increased wakefulness and decreased NREM sleep after microinjection into the rostral pontine reticular formation (Xi et al. 2001). The effects of phaclofen and baclofen on pontine reticular formation ACh release remain to be investigated. Most recently, baclofen has been shown to inhibit REM sleep when microinjected into rat PPT (Ulloor et al. 2004). Baclofen also blocked the discharge of PPT neurons that selectively increase their firing rates during REM sleep (putatively cholinergic REM-on cells) (Ulloor et al. 2004). Cholinergic PPT neurons project to the pontine reticular formation (Jones 1990a; Mitani et al. 1988; Semba 1993; Shiromani et al. 1988) where they release ACh (Lydic and Baghdoyan 1993). Thus it is likely that PPT administration of baclofen inhibits ACh release in the pontine reticular formation.

Evocation of a REM sleep-like state or REM sleep traits during dialysis administration of bicuculline

Microinjection of bicuculline into the rostral pontine reticular formation of cat (Xi et al. 1999), rat (Sanford et al. 2003), and mouse (Falgout et al. 2003) significantly increases REM sleep. Dialysis administration of bicuculline in the present study enhanced REM sleep but not as consistently or with the same magnitude as has been reported after microinjection delivery of bicuculline in cat (Xi et al. 1999, 2001). Potential reasons for these differences include different routes of bicuculline delivery (microinjection verses microdialysis) and different sites of bicuculline administration within the pontine reticular formation. Different routes of administration would cause differences in the amount of bicuculline delivered to the tissue, and bicuculline-induced REM sleep enhancement has been shown to be concentration dependent (Sanford et al. 2003; Xi et al. 2001). Furthermore, bicuculline-induced REM sleep enhancement is site dependent within the pontine reticular formation. Effective microinjection sites in cat pontine reticular formation (Xi et al. 1999, 2001) are located more dorsally than most of the dialysis sites used for the present study. Iontophoretic studies mapping dorsal portions of the rat pontine reticular formation showed that the effective region for enhancing REM sleep with bicuculline was restricted to the subdorsolateral nucleus (Boissard et al. 2002). Microinjection studies in rat have shown that sites in the rostral pontine reticular formation (Sanford et al. 2003) or dorsal subcoeruleus nucleus (Pollock and Mistlberger 2003) were more effective for enhancing REM sleep with bicuculline than sites in the caudal pontine reticular formation (Pollock and Mistlberger 2003; Sanford et al. 2003).

Bicuculline caused motor atonia during wakefulness. Microinjection of cholinomimetics into cat pontine reticular formation can evoke individual REM sleep traits, such as motor atonia or PGO waves, dissociated from the state of REM sleep. These state-trait dissociations have been shown to be dependent on the intrapontine site of cholinomimetic administration (reviewed in Baghdoyan 1997). The dissociated REM sleep trait of motor atonia observed during wakefulness in the present study may be accounted for, in part, by the site of bicuculline administration. A systematic mapping study is required to investigate this possibility.

The evocation of motor activation and circling by dialysis administration of bicuculline is consistent with previous reports of REM sleep disruption due to circling following microinjection of bicuculline into the caudal part of the rat pontine reticular formation (Pollock and Mistlberger 2003; Sanford et al. 2003) and by microinjecting higher concentrations of bicuculline to the rostral part of the rat pontine reticular formation (Sanford et al. 2003). Motor activation also has been reported in rat after iontophoretic application of bicuculline to the dorsal subcoeruleus nucleus (Boissard et al. 2002). The neural circuits mediating bicuculline-induced circling have been discussed in detail (Sanford et al. 2003).

Limitations and conclusions

The data presented in this report suggest that $GABA_A$ receptors in cat pontine reticular formation modulate ACh release. This conclusion is limited, however, because the only

drug tested was bicuculline methiodide. In thalamic slices from young rats, bicuculline methiodide has been shown to alter cell excitability by actions that are independent of $GABA_A$ receptor antagonism (Debarbieux et al. 1998). Future studies can test additional more selective $GABA_A$ antagonists, such as picrotoxin, for their ability to increase ACh release in vivo in cat pontine reticular formation. Testing the effects of $GABA_B$ antagonists and GABA uptake inhibitors on ACh release can also address the present limitations.

The synaptic location of the pontine reticular formation $GABA_A$ receptors modulating ACh release remains to be identified. Pontine reticular formation neurons are not cholinergic, and anatomical and functional data demonstrate that cholinergic input to the pontine reticular formation arises from the LDT/PPT (Jones 1990a; Lydic and Baghdoyan 1993; Mitani et al. 1988; Semba 1993; Shiromani et al. 1988; Steriade and McCarley 1990). The $GABA_A$ receptors that modulate ACh release could be localized presynaptically on LDT/PPT terminals in the pontine reticular formation or postsynaptically on pontine reticular formation neurons that project back to the LDT/PPT.

One implication of the present findings is that GABA levels in the pontine reticular formation are significantly greater during wakefulness than during REM sleep. A preliminary report of studies in rat supports this prediction (Marks et al. 2003). A second implication of the current findings is that increasing ACh release in the pontine reticular formation is one mechanism by which blockade of pontine $GABA_A$ receptors enhances REM sleep. Cholinergic neurotransmission at pontine reticular formation muscarinic receptors has been proposed to be the final common pathway through which a variety of neuroactive molecules contribute to REM sleep generation (reviewed in Baghdoyan and Lydic 2002; Lydic and Baghdoyan 2003).

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