M2 Muscarinic Receptor Subtype in the Feline Medial Pontine Reticular Formation Modulates the Amount of Rapid Eye Movement Sleep

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Abstract: Rapid eye movement (REM) sleep is generated, in part, by activating muscarinic cholinergic receptors (mAChRs) in the medial pontine reticular formation (mPRF). Molecular cloning has identified five mAChR subtypes, and this study tested the hypothesis that the M2 subtype in the mPRF modulates the amount of REM sleep. This hypothesis cannot be tested directly, due to lack of subtype selective muscarinic agonists. However, the amount of REM sleep can be enhanced by mPRF microinjection of a muscarinic agonist, and the relative potencies of muscarinic antagonists to block the REM sleep enhancement can be determined. Two muscarinic agonists, methoctramine and 4-DAMP, were studied. Six concentrations of each antagonist were microinjected into the mPRF of conscious cat 15 min prior to the agonist bethanechol. Nonlinear regression analysis was used to calculate the dose of antagonist that caused a 50% inhibition (ID50) of bethanechol-induced REM sleep. Bethanechol significantly increased (442%) the amount of time spent in REM sleep. Both methoctramine and 4-DAMP significantly blocked the bethanechol-induced REM sleep increase, with an ID50 of 1.8 µM and 0.6 µM, respectively. The ID50 ratio for methoctramine-to-4-DAMP (3.0) was similar to the affinity ratio of methoctramine-to-4-DAMP only at the M2 subtype (3.5), suggesting that the M2 subtype in the mPRF modulates the amount of REM sleep. This study also tested the null hypothesis that sleep-dependent respiratory depression evoked by mPRF cholinomimetics would not be antagonized by pretreatment of the mPRF with muscarinic antagonists. Neither methoctramine nor 4-DAMP antagonized the bethanechol-induced decrease in respiratory rate.

Key words: Intracerebral microinjection; bethanechol; methoctramine; 4-DAMP; pons

INTRODUCTION

ACTIVATION OF MUSCARINIC, CHOLINERGIC RECEPTORS (mAChRs) in the medial pontine reticular formation (mPRF) is known to be an important step in the generation of rapid eye movement (REM) sleep. Direct administration of cholinomimetics into the mPRF causes a REM sleep-like state and sleep-dependent respiratory depression that can be blocked by pontine administration of the muscarinic antagonist atropine.1,2 Natural REM sleep is decreased by direct administration of muscarinic antagonists into the pontine reticular formation.3-5 In humans, mAChR activation appears to play a role in REM sleep generation based on the finding that intravenous administration of the muscarinic agonist arecoline during non-REM (NREM) sleep shortens the latency to onset of REM sleep.6,7

Five mAChR subtypes have been identified by molecular cloning.8,9 mAChR subtypes also can be distinguished pharmacologically on the basis of differential antagonist binding affinities.10,11 No single muscarinic antagonist, however, shows both high affinity for one cloned mAChR subtype and low affinity for the other four molecularly identified subtypes. This fact, combined with the complete lack of subtype selective muscarinic agonists,12 has severely hampered efforts to identify functional roles of mAChR subtypes in vivo. The effects of relatively subtype selective mAChR antagonists on REM sleep have been studied in an attempt to identify the sleep-related roles of individual mAChR subtypes. To date, it has been suggested that M1,13 M2,3,14,15 and M316 receptors are of primary importance in generating REM sleep.

In vitro autoradiography has demonstrated that M2 mAChRs comprise the predominant receptor subtype in the mPRF of cat17 and in the homologous nucleus pontis oralis (PnO) of rat.18 In vivo microinjection studies have shown that mPRF administration of drugs that inhibit a signal transduction pathway activated by M2/M4 mAChR subtypes blocks cholinergic REM sleep generation.19,20 Most recently, in vitro autoradiographic studies have demonstrated that mAChR stimulation causes a dose-dependent, atropine sensitive activation of guanine nucleotide binding...
proteins (G proteins) in REM sleep-related brain stem nuclei. The foregoing findings encouraged the present study testing the hypothesis that in the feline mPRF, the M2 subtype of mAChR modulates the amount of REM sleep. This study also quantified the effects of muscarinic antagonists on cholinergically induced respiratory rate depression.

METHODS

A novel approach was used to determine which mAChR subtypes in the feline mPRF contribute to cholinergic REM sleep generation. Two different series of experiments were performed on each animal in order to circumvent limitations imposed by the lack of subtype-selective muscarinic agonists. First, the REM sleep-like state was evoked by mPRF administration of the muscarinic agonist bethanechol. These data were used to quantify the REM sleep enhancement caused by bethanechol at each mPRF injection site. In a second series of experiments, muscarinic antagonists with different affinities for different mAChR subtypes were microinjected into the mPRF 15 min prior to bethanechol to determine the antagonist dose that significantly inhibited the bethanechol-evoked REM sleep-like state. The two antagonists studied were methoctramine and 4-diphenyl acetoxy-methyl piperidine methiodide (4-DAMP). The affinities of these antagonists for the five mAChR subtypes are provided in Table 1. By microinjecting a range of concentrations of each antagonist into the mPRF, it was possible to determine the dose of each antagonist that caused a 50% inhibition of the REM sleep-like state. Pharmacological studies commonly use the 50% inhibitory dose (ID₅₀) as a quantitative measure of potency. The potencies for REM sleep inhibition (ID₅₀) then were compared with the affinities (Kᵢ) of the antagonists for each mAChR subtype (Table 1) to draw conclusions about the mAChR subtype in the feline mPRF mediating cholinergic REM sleep generation.

Surgical Preparation of Animals

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (7th edition, National Academy of Sciences Press, Washington, DC, 1996), and all the procedures used in the present study have been described previously. Seven adult, male cats were anesthetized with halothane (1-3% in O₂) and placed in a stereotaxic frame. Electrodes for recording the cortical electroencephalogram (EEG), nuchal electromyogram (EMG), electrooculogram (EOG), and the geniculate component of ponto-geniculo-occipital (PGO) waves were inserted and fixed to the skull with dental acrylic. Bilateral, stainless steel guide tubes (24 gauge) were aimed stereotaxically for the mPRF (posterior:P = 2.0, lateral:L = 1.5, horizontal H=-5; theta=30°; coordinates according to Berman) and implanted approximately 5 mm above the target to ensure minimal damage of the injection sites. Also embedded in the acrylic were two stainless steel sleeves that allowed subsequent placement of unanesthetized animals in a head-stable position. Animals were given one month to recover from surgery and adapt to sleeping in the laboratory.

Intracranial Drug Administration, Experimental Design, and Polygraphic Recordings

To begin each experiment, a cat was placed in the recording apparatus, a thermister was positioned at the...
nares to provide a non-invasive measure of respiratory rate,24,26 and the implanted electrodes were connected to the Grass polygraph by a shielded cable. The guide tubes and the lack of pain receptors in the brain permitted direct access to the mPRF in the conscious animal. Drugs were dissolved in sterile saline and administered unilaterally through a 31 gauge injection cannula when the animals were in a state of quiet wakefulness. A manual microdrive was used to inject at a rate of approximately 0.25 µl/30 sec. Drugs administered, injection weight/volume, concentration, and number of injections (n) included carbamyl-β-methylcholine chloride (bethanechol; 4.3 µg/0.25 µl; 87

**Figure 1.** Polygraphic recordings of wakefulness, sleep, and breathing. The nuchal electromyogram (EMG), the electrooculogram (EOG), the lateral geniculate body component of ponto-geniculo-occipital (PGO) waves, and the cortical electroencephalogram (EEG) were used to objectively identify and quantify wakefulness, rapid eye movement (REM) sleep, non-REM (NREM) sleep, and the REM sleep-like state (REM-Beth) produced by microinjecting bethanechol into the medial pontine reticular formation. For the respiratory recordings, upward going peaks correspond to the inspiratory phase of the respiratory cycle. The amplitude of the respiratory rate trace is arbitrary and does not represent tidal volume. Each panel shows a 1 minute recording from the same cat.
mM; n=28), methoctramine (0.5 µl of six concentrations ranging from 10^{-12} M to 10^{-2} M; see Table 2) 15 minutes prior to bethanechol (4.3 µg/0.25 µl; n = 49). Three animals each received, in randomized order, three vehicle injections (0.9% saline, 0.25 µl; n=9) followed by polygraphic recordings and three control (no vehicle injection) recordings. Saline caused no significant changes in any measure of REM sleep. A previous study demonstrated that microinjecting saline (0.5 Ml) into the mPRF had no significant effect on sleep or wakefulness for four hours post injection. 27 Taken together, these data provided the present rationale for making control recordings (n=28) that were not preceded by vehicle (saline) injections, in order to minimize tissue damage at the injection site. Only one experiment was performed per day in the same animal, and three to five days elapsed between experiments using the same animal.

Continuous polygraphic recordings of behavioral state and breathing were obtained for two hours following onset of the bethanechol injection. As previously described, the bethanechol-induced REM sleep-like state (REM-Beth) was behaviorally and polygraphically similar to natural REM sleep.4,28 The many similarities between spontaneous REM sleep and cholinergically induced REM sleep have been reviewed recently.1,2 States of wakefulness (W), non-REM (NREM) sleep, REM sleep, and REM-Beth were scored in one minute bins in real time according to standard criteria29,30 (Figure 1.) Dependent measures of behavioral state included the percent of total recording time spent in W, NREM sleep, REM sleep, and REM-Beth; and the total number of minutes spent in REM sleep or REM-Beth. Values are reported as mean±standard error of the mean (s.e.m.). Descriptive statistics, one way analysis of variance (ANOVA), and Tukey’s multiple comparison test were used to evaluate the effects of bethanechol, methoctramine + bethanechol, and 4-DAMP + bethanechol on the amount of time spent in sleep and wakefulness (GB-STAT™, Dynamic Microsystems).

**Determining the Relative Potencies of Methoctramine and 4-DAMP**

The major goal of this study was to determine the potency with which methoctramine and 4-DAMP blocked...
bethanechol-induced REM sleep enhancement. To accomplish this goal, nonlinear regression analysis was used to determine the dose of muscarinic antagonist (independent variable) needed to inhibit the amount (min) of bethanechol-induced REM sleep (dependent variable) by 50% (ID$_{50}$). The data were fitted to the equation $Y = R + \frac{(L - R)}{1 + (X/D)^S}$, where $R$ is the lower limit for the dependent variable (i.e., the Right end of the curve), $L$ is the upper limit for the dependent variable (i.e., the Left end of the curve), $X$ is the concentration of antagonist microinjected, $D$ is the ID$_{50}$, and $S$ corresponds to the slope of the curve. This equation is used commonly for fitting data to a sigmoid curve, and is related to the Hill equation.\textsuperscript{31} For curve fitting, the value for $L$ was fixed at the mean minutes spent in REM sleep following microinjection of bethanechol alone. The value for $R$ was fixed at the mean minutes of REM sleep during control recordings. Curve fitting was carried out with the Scientist program (MicroMath Scientific Software).

**Analysis of Respiratory Rate Data**

From a subset of microinjection experiments, respiratory rate (breaths/min) was counted manually from the polygraph records (Fig. 1) for up to 10 minutes of W, NREM sleep, REM sleep, and REM-Beth. For control and bethanechol microinjection experiments, ANOVA and Tukey’s multiple comparison test were used to quantify a behavioral state main effect on respiratory rate. The independent variable (behavioral state) had three levels: W, NREM sleep, and REM sleep or REM-Beth. Independent $\tau$-test was used to determine the effect of bethanechol on respiratory rate within each state. For antagonist pretreatment experiments, ANOVA and Dunnett's multiple comparison test were used to determine the effect of pretreating the mPRF with mAChR antagonists on the bethanechol-induced respiratory rate depression. The independent variable (drug dose) had four levels: bethanechol alone, and bethanechol plus three concentrations of muscarinic antagonist (10$^{-6}$ M, 10$^{-4}$ M, and 10$^{-2}$ M).

### RESULTS

**Histological Localization of Microinjection Sites**

Upon completion of the microinjection protocol, cats were deeply anesthetized with pentobarbital (40 mg/kg i.p.) and perfused transcardially with heparinized saline followed by 10% formalin. Brains were removed and soaked in 10% formalin for a minimum of two weeks. Brain stems then were dehydrated in 30% sucrose-formalin prior to freezing and sectioning at 40 $\mu$m on a sliding microtome. Sagittal brain stem sections were float mounted, stained with cresyl violet, and compared with the sagittal plates in a brain stem atlas\textsuperscript{25} to identify the stereotaxic coordinates corresponding to the injection site.

**Table 3.** Minutes (mean ± s.e.m.) of the two hr recording period spent in wakefulness, NREM sleep, or REM sleep under control conditions (n = 28 recordings) and following bethanechol (4.3 $\mu$g/25 $\mu$l, n = 28 recordings) microinjected into the mPRF during quiet wakefulness. Asterisks (*) indicate a significant (p<0.05) change from control. These data agree with results from previous reports showing that mPRF administration of bethanechol increased REM sleep percent\textsuperscript{4, 28}.

<table>
<thead>
<tr>
<th>State</th>
<th>Control</th>
<th>Bethanechol</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wakefulness</td>
<td>39.5 ± 3.6</td>
<td>21.9 ± 2.5 *</td>
<td>-45%</td>
</tr>
<tr>
<td>NREM Sleep</td>
<td>65.4 ± 3.2</td>
<td>16.5 ± 2.1 *</td>
<td>-75%</td>
</tr>
<tr>
<td>REM Sleep</td>
<td>15.1 ± 1.3</td>
<td>81.8 ± 3.0 *</td>
<td>442%</td>
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NREM sleep and 68.1±13.0% of time in REM sleep. The percent of total sleep time (NREM sleep + REM sleep) occupied by REM sleep also was enhanced by bethanechol. The percent of total sleep time comprised of REM sleep was 19% under control conditions and 83% following bethanechol administration.

Figure 3 plots the occurrence and duration of sleep and wakefulness for 120 consecutive min following six different treatment conditions. The Fig. 3 histograms show individual experiments and are representative of the average group data for each treatment condition. Compared to a two hour recording session which was not preceded by a micro injection (Fig. 3A), delivery of saline (Fig. 3B) into the mPRF had no effect on dependent measures of sleep and wakefulness. Another important control illustrated by Fig. 3 is that the bethanechol-induced REM sleep-like state (Fig. 3C) was not quantitatively altered by mPRF microinjection of saline 15 minutes prior to bethanechol (Fig. 3D). The bethanechol-induced REM sleep-like state was blocked by pre-treating the mPRF with 0.036 µg (10^-4 M) methoctramine (Fig. 3E) or 2.3 µg (10^-2 M) 4-DAMP (Fig. 3F). The group data for all injections and all conditions that correspond to the examples in Fig. 3 are discussed below.

The effects of methoctramine or 4-DAMP pretreatment on the bethanechol-induced REM sleep-like state are sum-
The effects of mPRF bethanechol administration (Fig. 7, solid bars). Asterisks indicate that bethanechol microinjection caused a further reduction of respiratory rate below control levels (p < 0.01) during NREM sleep (✝) and during REM sleep (✝). This decrease occurred during control recordings (Fig. 7, hatched bars) and following mPRF bethanechol administration (Fig. 7, solid bars).

DISCUSSION

The present study determined ID₅₀ values for the muscarinic antagonists methoctramine (1.8 µM) and 4-DAMP (0.6 µM) to block cholinergically generated REM sleep. The results are interpreted to support the conclusion that the M2 subtype in the mPRF modulates the amount of REM sleep. This interpretation is based on comparing the potency ratio of methoctamine-to-4-DAMP(ID₅₀METH/ID₅₀4-DAMP) to the affinity ratio of methoctamine-to-4-DAMP(KᵢMETH/Kᵢ4-DAMP) at the five mAChR subtypes (Table 1, bottom row). The KᵢMETH/Kᵢ4-DAMP ratio is similar to the ID₅₀METH/ID₅₀4-DAMP ratio only at the M2 subtype. In other words, the three-fold difference in potency to block REM sleep between methoctamine and 4-DAMP approximates the three and one half-fold difference in affinities of methoctamine and 4-DAMP at the M2 subtype. Taken together with data showing that M2 is the predominant mAChR subtype in the mPRF,17 and that pharmacological antagonism of the M2/M4-coupled signal transduction pathway in the mPRF inhibits cholinergically generated REM sleep,19,20 the present findings are consistent with the interpretation that in the feline mPRF, the M2 mAChR sub-

bethanechol is shown for animals that received either methoctramine (Fig. 5A) or 4-DAMP (Fig. 6A). There was no difference between animals that received methoctramine and animals that received 4-DAMP with regard to either control amounts of REM sleep or the percent enhancement of REM sleep produced by bethanechol. The ID₅₀ values for methoctamine and 4-DAMP are shown in Fig. 5B and Fig. 6B, respectively. The calculated ID₅₀ values for methoctamine and 4-DAMP were 1.8 µM and 0.6 µM, respectively. The potency ratio of methoctamine-to-4-

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One purpose of this study was to determine the dose of methoctamine and 4-DAMP needed to produce a 50% inhibition (ID₅₀) of bethanechol-induced REM sleep. Results of the ID₅₀ analyses are presented in Figures 5 and 6. The mean number of minutes spent in REM sleep under control conditions and following mPRF microinjection of

Figure 4. Effects of mPRF bethanechol and methoctramine or 4-DAMP on the percent total recording time spent in REM sleep. Asterisks (*) and crosses (✝) indicate values that are significantly (p < 0.05) different from control or bethanechol, respectively. A. Percent of total recording time spent in REM sleep under control conditions (diagonally hatched bar) and following mPRF microinjection of bethanechol alone (solid bar) or bethanechol 15 min after mPRF microinjection of methoctramine (cross hatched bars). Methoctramine pretreatment at 10⁻⁴ M (0.036 µg) and 10⁻² M (3.6 µg) significantly blocked the bethanechol-induced REM sleep-like state and returned percent REM sleep to control levels. The number of trials (n) for each treatment condition was: control (n=11), bethanechol alone (n=12), bethanechol + methoctramine 10⁻¹² M (n=8), 10⁻¹⁰ M (n=8), 10⁻⁸ M (n=8), 10⁻⁶ M (n=8), 10⁻⁴ M (n=8), 10⁻² M (n=6), and 10⁻¹ M (n=6). B. Percent of total recording time spent in REM sleep under control conditions (diagonally hatched bar) and following mPRF microinjection of bethanechol alone (solid bar) or bethanechol 15 min after mPRF microinjection of 4-DAMP (cross hatched bars). 4-DAMP pretreatment at 10⁻⁶ M (2.3 pg), 10⁻⁴ M (0.023 µg), and 10⁻² M (2.3 µg) significantly reduced the bethanechol-induced REM sleep-like state. 4-DAMP pretreatment at 10⁻² M returned REM sleep to control levels. The number of trials (n) for each treatment condition was: control (n=17), bethanechol alone (n=16), bethanechol + 4-DAMP 10⁻¹² M (n=8), 10⁻¹⁰ M (n=9), 10⁻⁸ M (n=8), 10⁻⁶ M (n=8), 10⁻⁴ M (n=8), 10⁻² M (n=8), and 10⁻¹ M (n=8).

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DAMP (ID₅₀METH/ID₅₀4-DAMP) was 3.0.

The effects of mPRF bethanechol on breathing are summarized by Figure 7. Respiratory rate changed significantly (p < 0.01) as a function of arousal state, both under control conditions (Fig. 7, hatched bars; F=21.32; d.f. =2, 371) and following mPRF administration of bethanechol (Fig. 7, solid bars; F=52.47; d.f. =2, 371). Tukey’s post hoc comparison test revealed that, compared to W, respiratory rate was significantly (p < 0.05) decreased during NREM sleep (✝) and during REM sleep (✝). This decrease occurred during control recordings (Fig. 7, hatched bars) and following mPRF bethanechol administration (Fig. 7, solid bars). Asterisks indicate that bethanechol microinjection caused a further reduction of respiratory rate below control levels (p < 0.01) during NREM sleep (✝) and during REM sleep (✝). ANOVA revealed that pre-

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type modulates the amount of cholinergically generated REM sleep.

This study also quantified the effects on respiratory rate of administering bethanechol, methoctramine, and 4-DAMP into the mPRF. The data revealed that mPRF administration of bethanechol caused sleep-dependent respiratory depression that mimicked the naturally occurring sleep-dependent decrease in respiratory rate (Fig. 7). The bethanechol-induced decrease in respiratory rate could not be dissociated from REM sleep by pretreating the mPRF with muscarinic antagonists. This finding suggests that the respiratory rate depression characteristic of cholinergically induced REM sleep occurs as a function of the sleep-induced alterations in respiratory rhythm generating networks.

The lack of subtype-selective muscarinic agonists, the moderate subtype-selectivity of muscarinic antagonists, and the fact that many brain regions express multiple mAChR subtypes, impose limitations on all current efforts to identify the functional roles of mAChR subtypes. After reviewing those limitations, the subsequent discussion compares the present results to previous findings, and concludes by suggesting that the M2 mAChR subtype in the feline mPRF generates cholinergically evoked REM sleep.

Muscarinic Receptor Nomenclature and Pharmacology: Relevance for Sleep Neurobiology

The existence of mAChR subtypes first was revealed pharmacologically with the demonstration in 1980 that the mAChR antagonist pirenzepine showed different binding affinities in different tissues, and subsequently four mAChR subtypes were distinguished pharmacologically based on the binding affinities of different muscarinic antagonists. In 1986 and 1987, genes coding for five mAChR subtypes were cloned. Genetically identified subtypes were named m1-m5 and pharmacologically identified subtypes were designated M1-M4. This convention of differential nomenclature was developed because there are no exclusively subtype-specific muscarinic ligands. In other words, there are no muscarinic agonists or antagonists that show a high affinity for one subtype and a low affinity for the other four subtypes (Table 1). Subtypes of mAChRs can be distinguished pharmacologically by comparing the affinity profiles of several muscarinic antagonists. For example, pharmacologically defined M1 receptors have a relatively high affinity for pirenzepine and 4-DAMP and a relatively low affinity for methoctramine; M2 receptors have a relatively high affinity for methoctramine and a relatively low affinity for pirenzepine and 4-DAMP; and pharmacologically defined M3 receptors have a relatively high affinity for 4-DAMP and a relatively low affinity for pirenzepine and methoctramine (Table 1). Because there is now good evidence that pharmacologically defined M1-M5 mAChRs correspond to molecularly identified m1-m5 subtypes, it recently was recommended that the upper case nomenclature (M1-M5) be used to describe both the pharmacologically and molecularly identified mAChR subtypes. The present report adheres to this recommendation.

How are the above features of mAChR pharmacology relevant for mechanistic studies of sleep neurobiology? One implication is that experiments seeking to demonstrate a role for a specific mAChR subtype in mediating a behavioral state must make use of multiple antagonists (discussed in detail in Caulfield). Second, dose-response studies are essential because the ability of muscarinic
antagonists to selectively antagonize different mAChR subtypes is concentration dependent. Conclusions about the sleep-related role of a particular mAChR subtype cannot be based on the results of testing one dose of one muscarinic antagonist. Third, it is not correct to assign a particular muscarinic antagonist to a particular mAChR subtype, meaning statements such as "4-DAMP is an M3 antagonist" or "pirenzepine is an M1 antagonist" are not accurate. Fourth, it is important to use multiple techniques in an attempt to identify the mAChR subtype(s) mediating a behavioral state.

**ID\textsubscript{50} Determination: Limitations for In Vivo Studies**

All studies aiming to identify functional roles of mAChR subtypes must contend with the considerable restrictions imposed by the lack of ligands showing selectivity for a single mAChR subtype. In addition, in vivo studies are faced with additional limitations not encountered under in vitro binding conditions. This section identifies some of those problems, and interprets the present results in light of those limitations.

One problem for any study using the technique of intracerebral microinjection is that the actual brain concentration of the injected drug is unknown. The amount of antagonist microinjected into the mPRF was calculated precisely for the present study (Table 2). However, the brain concentration can only be estimated because the volume of distribution and the rate of antagonist diffusion away from the injection site are unknown. The concentration of a drug that inhibits a response by 50% is referred to as the IC\textsubscript{50}, and IC\textsubscript{50} values commonly are used to compare relative drug potencies. The term ID\textsubscript{50} (50% inhibitory dose) is used here rather than IC\textsubscript{50} (50% inhibitory concentration) to emphasize the point that the present experiments were conducted in vivo, thus brain concentrations of the microinjected drugs are unknown. Because methoctramine and 4-DAMP are small molecules of approximately the same size, it can be assumed that their rate of diffusion in the brain is similar (discussed in detail by Billard et al.). Thus, although the exact brain concentrations are unknown, comparisons of relative potencies can be made following injections of equal volumes and concentrations.

Another challenge for in vivo studies is that the affinities of muscarinic antagonists are determined using cloned mAChR subtypes and in vitro binding assays in which conditions such as pH, temperature, and ionic composition are controlled. The affinities of mAChR antagonists under physiological conditions are, in fact, unknown. The approach used in the present study was to compare potencies determined in vivo with affinities determined in vitro in order to make conclusions about the role of a particular mAChR subtype in mediating REM sleep. One unproved assumption underlying this approach is that the assay conditions used in vitro provide an environment that is similar to physiological conditions. This approach is encouraged, however, by two findings. First, affinities for mAChR determined from radioligand binding studies correspond well to affinities determined in functional assays (reviewed in Caulfield). Second, affinities for cloned mAChR subtypes expressed in cell lines are quite similar to affinities determined using natively expressed receptors. These findings suggest that antagonist affinities for mAChR subtypes determined in vitro do, in fact, provide a realistic estimate of in vivo affinities.

In addition to determining ID\textsubscript{50} values for methoc-
Modulation of REM Sleep by mAChR Subtypes

In vitro receptor autoradiography has demonstrated that the predominant mAChR subtype in the mPRF of cat is M2 and the homologous PhO of rat is M2. Recent electron microscopic studies show the presence of postsynaptic M2 receptors in the same regions of rat pontine reticular formation where microinjection of cholinergic agonists increases REM sleep. In vivo microinjection studies revealed that manipulating M2- and M4-coupled signal transduction pathways in the mPRF alters REM sleep. Through the use of in vivo microdialysis and high pressure liquid chromatography, muscarinic autoreceptors of the M2 subtype have been shown to modulate mPRF acetylcholine (ACh) release. The present data show by ID$_{50}$ comparison that in the mPRF methoctramine and 4-DAMP are approximately equipotent for blocking the amount of muscarinic evoked REM sleep, and in vitro equilibrium binding studies show that methoctramine and 4-DAMP have similar affinities for the M2 mAChR subtype. Taken together with the above mentioned results, the present data suggest that M2 mAChRs in the mPRF play a role in modulating the amount of cholinergically generated REM sleep. The endogenous neurotransmitter, ACh, increases in the feline mPRF during both spontaneous and cholinergically induced REM sleep. Thus, the present results also imply that the amount of spontaneous REM sleep is modulated by M2 mAChRs in the mPRF.

The foregoing conclusion is in agreement with previous microinjection studies performed using cat and rat. Interestingly, when muscarinic antagonists were delivered to cat brain stem by microdialysis, 4-DAMP was found to be more potent than methoctramine or pirenzepine for blocking cholinergically evoked REM sleep. This finding was interpreted by the authors to suggest that the M3 subtype is important for REM sleep generation (Table 4). Two important differences between the microdialysis study and the present study must be taken into account when attempting to reconcile the data from these two laboratories: site of antagonist administration and amount of antagonists administered. Sakai and Onoe used microdialysis to deliver muscarinic agonists and antagonists into the peri-locus coeruleus alpha (peri-LC$_{\alpha}$), which is located more dorsally and rostrally than the mPRF region microinjected in the present study (shown in Fig. 2). Although the M2 mAChR subtype is predominate throughout cat pontine reticular formation, mAChR subtypes in the peri-LC$_{\alpha}$ region of cat have not yet been identified. Regarding the amounts of muscarinic antagonists delivered, Table 4 summarizes the amounts (moles) and concentrations (M) of muscarinic antagonists microinjected or dialyzed into the pons in an attempt to determine which mAChR subtype(s) modulate REM sleep. Comparing the relative potencies of these muscarinic antagonists across microdialysis and microinjection studies is precluded by the fact that with microdialysis it is not possible to know the absolute amount of drug delivered. Within studies, however, it is clear that in the mPRF methoctramine and 4-DAMP are equipotent for blocking cholinergically evoked REM sleep (Figs. 5, 6) whereas in the peri-LC$_{\alpha}$ 4-DAMP is more potent than methoctramine or pirenzepine.

Cholinergically Induced Respiratory Rate Depression

Previous data have shown that mPRF microinjection of bethanechol causes a REM sleep-dependent decrease in minute ventilation and in the ventilatory response to hypercapnia. The present study demonstrates, for the first time, that mPRF bethanechol causes a sleep-dependent decrease in respiratory rate that mimicked the naturally occurring sleep-dependent decrease in breathing rate (Fig.
The decrease in respiratory rate during the bethanechol-induced REM sleep-like state was similar to the decrease in respiratory rate observed during the REM sleep-like state evoked by mPRF administration of carbachol. The finding that bethanechol decreased respiratory rate below control levels (Fig. 7) may be accounted for by the dose of bethanechol used, and further studies are required to determine if a lower dose of bethanechol would decrease breathing rate with a magnitude similar to the decrease observed during natural sleep.

Neither methoctramine nor 4-DAMP blocked the bethanechol-induced decrease in respiratory rate. This finding is consistent with previous data showing that mPRF administration of atropine did not block the bethanechol-induced decrease in minute ventilation. Thus, activation of mAChRs in the mPRF initiates respiratory rate depression, and this depression cannot be reversed by mPRF application of muscarinic antagonists. Taken together, these data support the concept that the respiratory rate depression caused by mPRF bethanechol results from mPRF influences on respiratory rhythm generating networks. It is not known whether these influences are direct or indirect, but REM sleep generating regions of the feline mPRF do receive monosynaptic projections from pontine, dorsal medullary, and ventral medullary respiratory groups.

### CONCLUSION

Within the constraints and limitations imposed by current mAChR pharmacology, the present data support the conclusion that ACh acts via M2 mAChRs in the mPRF to modulate the amount of REM sleep. This conclusion is further supported by anatomical studies demonstrating the prevalence of M2 mAChRs in cat and rat pontine...
reticular formation. Future development of subtype selective muscarinic agonists will provide tools for direct identification of the mAChR subtypes mediating REM sleep generation.

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