Spontaneous REM sleep has a longer onset latency and increased duration (30–60 min) of individual episodes. 8 min) latency to onset of REM sleep and a very short (15 min), and the individual epochs are rarely >12 min in duration. Despite these differences, mPRF levels of endogenous acetylcholine (ACh) significantly increase during both Carb-REM (48) and REM sleep. In addition to functional data suggesting non-M1 muscarinic cholinergic receptor modulation of REM sleep, autoradiographic data have demonstrated a predominant number of M2 muscarinic receptors in the mPRF of both rat and cat (4, 8, 49).

Neurons of the mPRF do not contain the enzyme choline acetyltransferase (ChAT) and thus are unable to synthesize ACh (39). The mPRF does contain ChAT-positive terminals arising from the more rostral and dorsal cell groups comprising the laterodorsal tegmental (LDT) and pedunculopontine tegmental (PPT) nuclei (51, 62). Electrical stimulation of LDT or PPT neurons causes a monotonic increase in ACh release within the mPRF (46) and promotes REM sleep enhancement in unanesthetized animals (68). Electrophysiological studies have shown increased LDT and PPT neuron discharge (26) and depolarization of mPRF neurons (36) during REM sleep. The association of REM sleep with increased cholinergic neuron discharge, increased ACh release in the mPRF, and enhanced excitability of mPRF neurons is consistent with in vitro data showing that most mPRF neurons are depolarized by the cholinergic agonist carbachol (32). Considered together, these facts identify a neuronal network (LDT-PPT-mPRF), a neurotransmitter (ACh), and a receptor system (muscarinic cholinergic) modulating REM sleep.

Muscarinic cholinergic receptors (mACHRs) are linked to guanine nucleotide binding proteins (G proteins), and in the brain stem mACHRs increase during REM sleep (57). Molecular cloning studies have revealed five subtypes of mACHRs in mammalian brain (m1-m5), and four of these subtypes (M1-M4) have been pharmacologically defined using relatively selective muscarinic antagonists (18). Agonist activation of m2, m3, or m5 subtypes via a stimulatory G protein leads to stimulation of phospholipase C (PLC), activation of protein kinase C (PKC), and an increase in intracellular calcium. The m2 and m4 receptor subtypes are linked to a G protein that inhibits the enzyme adenylate cyclase (AC), decreases production of adenosine 3',5'-cyclic monophosphate (cAMP), and inhibits protein kinase A (PKA). Knowledge of these signal transduction cascades (18) and the finding that the M2 receptors are the predominant subtype in the mPRF (4, 8, 49) were consistent with the discovery that a pertussis toxin-sensitive, Gi-like G protein and AC within the mPRF modulate Carb-REM sleep generation (67). The present study was undertaken in an effort to further understand the role of mAChRs in the control of REM sleep.
characterize signal transduction processes by which mACHRs contribute to REM sleep generation. This study tested the hypothesis that cAMP and PKA in the mPRF modulate cholinergic REM sleep generation. Portions of these results have been presented as abstracts (16, 17).

METHODS

Surgical procedure. Five adult male cats (3.5–5 kg) were anesthetized with halothane (1–3% in O2) and implanted with standard electrodes for quantifying states of sleep and wakefulness (69). These polygraphic measures included the electroencephalogram (EEG), electrooculogram (EOG), nasal electromyogram (EMG), and pontogeniculooccipital (PGO) waves recorded from the lateral geniculate body of the thalamus. At the time of surgery, cats also were implanted with bilateral stainless steel guide tubes (24 gauge), stereotaxically placed to permit repeated drug or vehicle administration to the mPRF (posterior = 2.0, lateral = 1.5, horizontal = −5.0; 30° posterior angle) (11). All surgical and experimental procedures strictly adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

mPRF microinjections. Three to four weeks after surgery, cats were acclimated to sleeping in the laboratory in a head-restrained position. When cats displayed normal REM sleep percentages (15–20%) over a 120-min recording, the microinjection experiments were begun. With cats in a state of quiet wakefulness, 0.25 µl of saline (control) or drug was administered into the mPRF over a 30-s period via a 31-gauge microinjector connected to polyethylene tubing (PE-20) and a microdrive-activated Hamilton syringe. All injections were unilateral, and in three of the five animals, both mPRF sites were used. Two animals contained unilateral sites incapable of eliciting the Carb-REM state because of stereotaxic misplacement of the guide tubes; these sites were excluded from the study. Administered compounds, concentration and amount administered, and number of injections (n) included saline (control; n = 30), carbachol (8.8 mM; 0.4 µg; n = 30), the cAMP analog dibutylryl cAMP (DBcAMP) (87 mM; 10.7 µg; n = 9), the cAMP analog 8-bromo-cAMP (8-BrcAMP) (87 mM; 9.4 µg; n = 9), the PKA stimulator Sp-cAMP[S] (87 mM; 1.0 µg; n = 9), the PKA inhibitor Rp-cAMPS (8.7 mM; 1.0 µg; n = 9). The role of the second messenger cAMP in Carb-REM sleep was investigated by administering 8-BrcAMP (n = 9) or DBcAMP (n = 9) into the mPRF 15 min before carbachol. The contribution of PKA as a modulator of cAMP-dependent protein phosphorylation to Carb-REM sleep was similarly investigated by pretreating the mPRF with Sp-cAMP[S] (n = 12) or Rp-cAMPS (n = 10) 15 min before carbachol administration. Sleep/wake states were polygraphically recorded for 120 min immediately after each microinjection and again 24 h later to ensure that administered compounds had no long-term effects on REM sleep. For all experiments, the order of microinjections was random and each microinjection was separated by a minimum of 3 days. The doses of cAMP analogs and PKA stimulator and inhibitor were equimolar to doses of carbachol previously shown to evoke the Carb-REM state (7).

Statistical analysis. Every minute of each 2-h polygraphic recording was scored as either wakefulness, non-REM (NREM) sleep, or REM sleep according to standard criteria (69). Dissociated states were not seen after injections of carbachol or drugs alone. Occasionally, dissociated states were present after a drug pretreatment to carbachol. All dissociated states as well as transition states were scored in a consistent manner. REM sleep was defined by muscle atonia, ≥10 PGO spikes/min and <5 EEG spindles/min. NREM sleep included muscle tone, <10 PGO spikes/min and >5 EEG spindles/min. In addition to polygraphic recordings, real-time video monitoring during every experiment helped standardize behavioral state classification. Drug main effects on percent, temporal organization, and time course of sleep/wake states were statistically evaluated using completely randomized analysis of variance (ANOVA) and ANOVA for repeated measures. When ANOVA revealed significant main effects and interactions, the effect of different compounds on sleep/wake states was evaluated statistically using Dunnett’s and Tukey’s multiple comparison tests. Statistical significance was represented by P values < 0.05.

Histologic analysis. At completion of the study, cats were deeply anesthetized with pentobarbital sodium, and brains were perfused with saline followed by 10% buffered Formalin. After fixation and dehydration in 30% sucrose-Formalin, brain stem blocks were sectioned at 40 µm thickness, mounted onto gelatin-coated glass slides, and stained with cresyl violet. Injection sites were localized using the atlas of Berman (11).

RESULTS

Localizaton of pontine microinjection sites. The sagittal brain stem section illustrated in Fig. 1A shows the location of a typical mPRF microinjection site. Figure 1B illustrates the stereotaxic placement of each of the eight microinjection sites utilized in this study. Carbachol microinjected into each of these sites was effective in producing the Carb-REM state, as confirmed by analysis of polygraphic measures (Fig. 1C) and quantification of percent time spent in REM sleep compared with saline control (Fig. 1D). The following results comprise data from 212 polygraphic recordings.

mPRF administration of cAMP agonists DBcAMP and 8-BrcAMP decreased Carb-REM sleep. The percent of time spent in REM sleep (Fig. 2A) was significantly altered by mPRF drug administration (F = 46.8; df = 5, 65; P < 0.0001). Administration of carbachol caused a significant increase (252.7%) in REM sleep compared with control (saline). When microinjected alone, neither DBcAMP nor 8-BrcAMP altered REM sleep compared with control. Carb-REM was significantly decreased by pretreating the mPRF with either DBcAMP (DB + Carb; −44.0%) or 8-BrcAMP (8Br + Carb; −37.1%). The effects of DBcAMP and 8-BrcAMP were not long lasting, as evidenced by the lack of changes in REM sleep 24 h after injections. Carb-REM occurred at the expense of NREM sleep (Fig. 2B), which was significantly decreased following carbachol, DB + Carb, and 8Br + Carb. The only drug-induced change in the percent of wakefulness was a significant increase following DB + Carb (Fig. 2C).

PKA stimulation, but not inhibition, decreased Carb-REM sleep. To further test the hypothesis that mPRF cAMP plays a role in Carb-REM sleep, the PKA stimulator Sp-cAMP[S] and the inhibitory stereoisomer Rp-cAMP[S] were microinjected into the mPRF. ANOVA revealed a statistically significant (F = 30.3; df = 5, 69; P < 0.0001) drug main effect on REM sleep (Fig. 3A). mPRF administration of carbachol caused a 298.7% increase in REM sleep time, and pretreatment of the mPRF with Sp-cAMP[S] (Sp + Carb) significantly decreased (−41.2%) this Carb-REM state. When the PKA

spikes/min and ≤5 EEG spindles/min. NREM sleep included muscle tone, <10 PGO spikes/min and >5 EEG spindles/min. In addition to polygraphic recordings, real-time video monitoring during every experiment helped standardize behavioral state classification. Drug main effects on percent, temporal organization, and time course of sleep/wake states were statistically evaluated using completely randomized analysis of variance (ANOVA) and ANOVA for repeated measures. When ANOVA revealed significant main effects and interactions, the effect of different compounds on sleep/wake states was evaluated statistically using Dunnett’s and Tukey’s multiple comparison tests. Statistical significance was represented by P values < 0.05.

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inhibitor Rp-cAMP[S] was administered as a pretreatment to Carb (Rp1Carb), there was no significant decrement in the ability of Carb to increase REM sleep. There were no significant differences in REM sleep following mPRF administration of Rp-cAMP[S] or Sp-cAMP[S] alone. Analyses 24 h after each injection showed that Sp-cAMP[S] and Rp-cAMP[S] had no long-term effects on REM sleep generation. Carb-REM came at the expense of NREM sleep. Post hoc Dunnett's tests showed that carbachol alone and carbachol following injection of Sp-cAMP[S] or Rp-cAMP[S] significantly decreased NREM sleep time (Fig. 3B) but did not produce any significant changes in the percent time awake (Fig. 3C).

mPRF administration of cAMP analogs disrupted the temporal organization of Carb-REM sleep. The temporal organization of REM and Carb-REM sleep was analyzed by quantifying the effects of mPRF microinjections on the frequency, duration, and latency to onset of REM sleep. Figure 4 shows the mean ± SD for REM sleep latency, frequency, and duration associated with each mPRF microinjection condition. Latency to onset of REM sleep (Fig. 4, A and D) was significantly decreased by Carb, and this decrease in REM latency was not reversed by any of the signal transduction-altering compounds. The number of REM sleep episodes during a 2-h recording (Fig. 4, B and E) was significantly increased only after 8Br+Carb. REM sleep epoch duration (Fig. 4, C and F) was significantly increased by Carb, and this increase was significantly reduced by pretreatment with DB-cAMP (DB1Carb) and 8Br-cAMP (8Br+Carb).

The ability of signal transduction-altering compounds to disrupt the temporal organization of REM sleep was evaluated by quantifying the mean (±SE) cumulative minutes of REM sleep as a function of the time postinjection. Figure 5A plots the effects of Carb, 8Br-cAMP, and DB-cAMP on the time course of REM sleep. Two-way ANOVA for repeated measures revealed statistically significant main effects of time (F = 195.0; df = 3,359; P < 0.0001) and of drug (F = 312.2; df = 5,359; P < 0.0001) as well as a significant time x drug interaction (F = 31.3; df = 15,359; P < 0.0001) (Fig. 5A). The ability of DB-cAMP and 8Br-cAMP to signifi-

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**Fig. 1.** Sagittal section of cat brain stem (A) showing a typical microinjection site localized to the medial pontine reticular formation (mPRF) (posterior = 1.5, lateral (L) = 1.6, horizontal = -5.5; stereotaxic coordinates according to Berman (11)). Arrow marks injection site. 6N, abducens nerve; 7G, genu of facial nerve; IC, inferior colliculus; IO, inferior olive; PAG, periaqueductal gray; SC, superior colliculus; TB, trapezoid body; TR, tegmental reticular nucleus. **B:** localizations of eight microinjection sites (L = 1.2–1.9) from 5 cats are represented by ● (the 7 symbols result from overlap of an injection site). C: 1-min polygraphic recordings from rapid eye movement (REM) sleep and carbachol-induced REM (Carb-REM) sleep demonstrate the similarity between natural REM sleep and the carbachol-enhanced state. Resp., respiratory airflow trace; EOG, electrooculogram; EEG, electroencephalogram; LGB, presence of pontogeniculooccipital waves from the lateral geniculate body of the thalamus; EMG, electromyogram. Time is shown in seconds and calibration bars are noted. D: each minute of the 2-h polygraphic recording was scored as either waking (W), non-REM sleep (NREM), or REM sleep. Compared with a microinjection of saline (control), carbachol always significantly increased REM sleep duration and decreased the latency to onset of REM sleep.
cantly decrease the carbachol-induced increase in cumulative minutes of REM sleep was present at 30, 60, 90, and 120 min after mPRF drug administration. Figure 5B shows the time course of Carb-REM sleep following mPRF administration of carbachol, the PKA stimulator Sp-cAMP[S], and the PKA inhibitor Rp-cAMP[S]. Two-way ANOVA revealed a statistically significant time main effect ($F = 69.8; df = 3,280; P < 0.0001$), drug main effect ($F = 235.6; df = 5,280; P < 0.0001$), and time × drug interaction ($F = 11.2; df = 15,280; P < 0.0001$) on Carb-REM sleep. The PKA stimulator Sp-cAMP[S] caused a significant decrease in Carb-REM sleep at 60, 90, and 120 min of the recording. The PKA inhibitor Rp-cAMP[S] had no effect on the time course of Carb-REM sleep.

DISCUSSION

Although the cholinergic model of REM sleep has been known for more than 30 years (29), only recently...
has this model been applied to the study of signal transduction pathways modulating EEG and behavioral arousal (44, 67). It is relevant, therefore, to consider the present data in light of the strengths and limitations of the cholinergic model of REM sleep.

Cholinergic REM sleep enhancement as a paradigm for studies of signal transduction pathways modulating arousal states. All experimental models have limited domains of validity, and it is clear that no single neurotransmitter or brain region regulates EEG or behavioral arousal. It is precisely this complexity, however, that makes cholinergic REM sleep enhancement such a powerful investigational tool. Advantages of the cholinergic model include the ability to enhance REM sleep from a discrete site in the pons (Fig. 1, A and B) and to permit inferences regarding the regulatory role of a particular brain region (the mPRF) (9), a specific neurotransmitter (ACh) (44), and distinct receptor subtypes (muscarinic cholinergic) (7, 71).

Even within the reticular formation there is a humbling complexity of neurotransmitters and receptors (38), many of which are coupled to G proteins. Administering signal transduction-altering compounds alone into the mPRF would be expected to affect many G protein-linked receptor systems. Such simultaneous activation of multiple signal transduction systems would make it difficult to suggest a modulatory link between any single receptor/transduction pathway and REM sleep. Therefore, another advantage of the cholinergic model is its ability to provide some insights into the signal transduction processes activated by mAChRs.

The present experiment continued a series of studies (44, 67) seeking to characterize the effects of signal transduction-altering compounds on cholinergic REM sleep enhancement (Fig. 1, C and D). By comparing the REM sleep effects of mPRF cholinomimetics alone to mPRF pretreatment with compounds that alter PKA and cAMP, the present study quantified the sleep/wake effects of these signal transduction-altering compounds (Figs. 2–4).

The use of cAMP-altering compounds in vivo. The feasibility of microinjecting DBcAMP, 8-BrcAMP, Sp-cAMP[S], and Rp-cAMP[S] into the mPRF of intact, unanesthetized cats is supported by many experiments...
that have utilized these compounds with in vivo systems. Intracerebroventricular injections of DBcAMP have been performed in rats to examine effects of cAMP on gene expression (1) and behavior (33, 40). 8-BrcAMP has been microinjected into the brain stem of rats to study the effects of cAMP on respiration (10) and behavior reactivity (24). Successful analyses of antinociception (21) and drug-seeking behavior (61) have been accomplished by brain infusion of Sp-cAMP[S] and Rp-cAMP[S] in intact rats. Thus the ability to use signal transduction-altering compounds in an in vivo system is an important tool for elucidating cellular mechanisms underlying autonomic control.

The notion that cAMP contributes to REM sleep generation is consistent with data from rat showing that in the pons and medulla cAMP levels were lowest during REM sleep compared with NREM sleep or wakefulness (55). Direct measures of cAMP in the hypothalamus of rat also suggest that cAMP is involved in regulating the sleep-wake cycle (2, 56). As described below, the present data support the view that, in the pontine reticular formation, the second messenger cAMP modulates cholinergic REM sleep enhancement.

cAMP modulates cholinergic REM sleep. DBcAMP and 8-BrcAMP are two membrane-permeable analogs of cAMP that mimic the intracellular effects of cAMP (20, 40). When microinjected directly into the pontine reticular formation of cat, DBcAMP and 8-BrcAMP significantly decreased the REM sleep enhancement produced by carbachol (Figs. 2 and 5A). Several lines of evidence suggest that the mechanism(s) by which these cAMP analogs caused a decrease in cholinergic REM sleep involve the signal transduction cascade modulated by m2 and m5 mAChR subtypes (Fig. 6). Previously, it has been shown that mPRF microinjection of pertussis toxin and forskolin inhibited the ability of carbachol to enhance REM sleep (67), suggesting a role for a G-like G protein and AC in cholinergic REM sleep generation. By inhibiting an inhibitory G protein, pertussis toxin can stimulate AC, resulting in cAMP production. The diterpine forskolin enhances cAMP via direct stimulatory actions on AC (60). Thus the present finding that mPRF administration of DBcAMP and 8-BrcAMP significantly blocked cholinergic REM sleep enhancement is consistent with previous data (67) showing decreases in cholinergic REM sleep enhancement by pertussis toxin and forskolin, compounds that increase cAMP levels.

PKA modulates cholinergic REM sleep. Microinjecting Sp-cAMP[S] into the mPRF decreased the ability of carbachol to cause the REM sleep-like state (Figs. 3 and 5B). Sp-cAMP[S], a cAMP analog with increased membrane permeability, greater resistance to phosphodiesterase inhibitors, and higher specificity for PKA than other cAMP analogs (72), facilitates the phosphorylation of intracellular proteins (70). Multiple lines of evidence are consistent with the possibility that protein phosphorylation plays a key role in sleep cycle control. REM sleep can be enhanced for many hours (7) and for days (13, 23) following the administration of microgram quantities of cholinomimetics into the pons or amygdala. The probability that mPRF levels of cAMP and PKA modulate cholinergic REM sleep enhancement also is supported by the finding that microinjection of the PKA inhibitor Rp-cAMP[S] did not alter cholinergic REM sleep generation (Figs. 3 and 5B).

cAMP agonists altered the temporal organization of cholinergic REM sleep. Analysis of the temporal organization of cholinergic REM sleep showed that the time course of REM sleep enhancement by carbachol was suppressed by DBcAMP, 8-BrcAMP, and Sp-cAMP[S] but not by Rp-cAMP[S]. These results parallel the ability of these same compounds to decrease the mean percent of REM sleep averaged across the 120-min recording. As shown in Fig. 4, cAMP analogs, PKA stimulator, and PKA inhibitor had specific effects on the latency, frequency, and duration of REM sleep. These data suggest that cAMP modulates the maintenance but not the initiation of each REM sleep episode. This finding differs from the results obtained following
mPRF administration of pertussis toxin, which decreased the number and duration of REM sleep epochs (67). If these differing effects on REM sleep timing can be related to unique steps in the signal transduction cascade, then the results imply unique modulatory roles for specific receptors, effectors, and second messengers. Pertussis toxin acts specifically at membrane-bound Gi proteins, whereas the cAMP analogs DB-cAMP, 8-BrcAMP, and Sp-cAMP are intracellular agonists of cAMP. Therefore, specific components of the G protein-AC-cAMP-PKA pathway may regulate different temporal components of REM sleep.

Differential effects on REM sleep and cholinergic enhancement of REM sleep. Recent reviews make it clear that natural REM sleep and Carb-REM sleep are remarkably similar in their effects on multiple physiological systems (3). Statistically significant similarities during the two states (Fig. 1C) have been reported for measures of motor atonia (52), breathing (45), blood pressure (66), heart rate (28), and EEG frequency (27). Significant similarities during the two states also have been reported for measures of discharge rate for reticular (65), medullary raphe (73), and pontine respiratory neurons (31); state-dependent changes in membrane excitability (32, 36, 42); brain glucose utilization (47); ACh release in the mPRF (43, 44); and gene expression (63, 64, 75).

Despite the foregoing similarities between REM sleep and the cholinergically induced REM sleep-like state, the amount of REM sleep after mPRF saline administration was not significantly different from REM sleep amounts after DB-cAMP, 8-BrcAMP, Sp-cAMP[S], and Rp-cAMP[S] were microinjected alone into the mPRF (Fig. 5). It is logical to speculate that simultaneous activation of arousal-promoting and REM sleep-promoting transduction pathways accounts for the absence of REM sleep effect (Figs. 2–4) after mPRF administration alone of the cAMP analogs- and PKA-altering compounds. Data supporting this speculation come from the finding that REM sleep is facilitated by G protein-linked mAChRs and inhibited by G protein-linked opioid receptors in the mPRF (22, 41). Thus one might expect a self-canceling outcome from the simultaneous activation of receptors, effectors, or second messengers promoting both wakefulness and REM sleep.

Fig. 6. Schematic model of intracellular signal transduction mechanisms possibly contributing to Carb-REM sleep. The cholinergic agonist carbachol binds to all subtypes of muscarinic receptors (m1-m5). Activation of m1, m3, or m5 subtypes stimulates phospholipase C (PLC) via a guanine nucleotide-binding protein (G). PLC cleaves phosphatidylinositol-4,5-biphosphate (PIP2) into membrane-bound diacylglycerol (DAG) and inositol triphosphate (IP3), which leads to release of intracellular Ca2+ stores and activation of protein kinase C (PKC). The interaction of carbachol with m2 or m4 subtypes inhibits (indicated by X) adenylate cyclase (AC) via a pertussis toxin-sensitive G protein (G). The inhibition of AC blocks production of cAMP, limiting the amount of substrate available to activate protein kinase A (PKA). The phosphorylation of intracellular proteins by PKA elicits the physiological response via numerous possible intermediate processes (some of which are shown boxed). mPRF microinjection of both pertussis toxin (PTX; 1), which inhibits G, and forskolin (FSK; 2), which stimulates AC, significantly reduced the ability of carbachol to induce the REM sleep-like state (67). The present data show that pontine administration of cAMP analogs DB-cAMP (3) and 8-BrcAMP (4) decreased Carb-REM sleep. In addition, mPRF injection of the PKA stimulator Sp-cAMP[S] (5) decreased Carb-REM sleep, whereas the PKA inhibitor Rp-cAMP[S] (6) did not alter Carb-REM sleep. Thus 6 lines of evidence support a role for cAMP and PKA as modulators of cholinergic REM sleep generation.
Future experimental work will be necessary to confirm or refute the foregoing speculation.

Limitations and conclusions. There are several limitations inherent to this study. First, it should be clear that the signal transduction-altering compounds may have altered the excitability of other cell groups in addition to cholinceptive mPRF neurons. Interneurons intrinsic to the mPRF are known to exhibit state-dependent changes in excitability (reviewed in Ref. 52). Furthermore, pontine administration of signal transduction-altering drugs also may alter presynaptic cholinergic terminals within the mPRF recently shown to modulate ACh release (58) and REM sleep generation (15). It is improbable, however, that the present results are an artifact of drug diffusion to regions outside the mPRF. It long has been noted that, "a major advantage of the microinjection technique is its ability to stimulate a relatively circumscribed neuronal pool" (74). This view is supported by biophysical studies of drug diffusion (54) and distribution kinetics (34) within the brain. Diffusion data obtained from radiolabeled drug studies show that 30 min after brain microinjections of 0.5-µl volumes, 72% of the drug remained within a radius of 0.75 mm (74). This diffusion distance is consistent with mapping studies of cholinergic REM sleep generation suggesting a diffusion radius of ~1 mm 4 h after microinjections of 0.25 µl of cholinomimetic into the pons (9). A second limitation of this study is the fact that only one dose of each compound was used. Characterizing the dose-dependent effects of cAMP analogs on REM sleep generation is open to future study. Third, although this study did not address the role of m1, m3 or m5 mAChR-activated signal transduction pathways, such an involvement in REM sleep generation is highly probable. G protein-activated transduction cascades are known to amplify cell-to-cell signaling by promiscuous interactions within a cell (53). Calcium is a potent contributor to cell signaling, and activation of m1, m3, and m5 subtypes is known to stimulate PLC, activate PKC, and increase intracellular calcium (35) (Fig. 6). G protein-activated calcium influx can stimulate nitric oxide synthase (NOS), and stereospecific NOS inhibition in the mPRF decreases mPRF ACh release and inhibits REM sleep (44). A fourth limitation is the fact that the present data permit only indirect inferences concerning intracellular actions of mPRF administered compounds. These results encourage future studies aiming to provide direct measurements of signal transduction molecules within the sleep-related regions of the brain stem.

In conclusion, the present data are consistent with multiple lines of evidence (Table 1) supporting the hypothesis that mPRF levels of cAMP modulate cholinergic REM sleep enhancement. The recent discovery that ACh release in the mPRF is regulated by muscarinic autoreceptors presumed to be localized on presynaptic cholinergic terminals (58) and the finding that cholinergic REM sleep can be inhibited by vesicular ACh transporter blockade within the mPRF (15) suggest that cAMP may contribute to transmembrane signal transduction cascades in both presynaptic cholinergic and postsynaptic cholinceptive neurons within the pontine brain stem.

Table 1. Summary of the actions of signal transduction-altering compounds and the effect on Carb-REM sleep

<table>
<thead>
<tr>
<th>Compound</th>
<th>Gα</th>
<th>Protein</th>
<th>AC</th>
<th>cAMP</th>
<th>PKA</th>
<th>Carb-REM Sleep</th>
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<tr>
<td>Carbachol</td>
<td>+</td>
<td>(18)</td>
<td>(18)</td>
<td>(50)</td>
<td>(50)</td>
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<tr>
<td>Pertussin toxin</td>
<td>-</td>
<td>(18)</td>
<td>(37)</td>
<td>(19)</td>
<td>(19)</td>
<td></td>
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<tr>
<td>Forskolin</td>
<td>+</td>
<td>(60)</td>
<td>(60)</td>
<td>(60)</td>
<td>(60)</td>
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<tr>
<td>Dibutyryl cAMP</td>
<td>+</td>
<td>(40)</td>
<td>(40)</td>
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<tr>
<td>8-Bromo-cAMP</td>
<td>+</td>
<td>(20)</td>
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</tr>
<tr>
<td>Sp-cAMP[S]</td>
<td>+</td>
<td>(72)</td>
<td></td>
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<tr>
<td>Rp-cAMP[S]</td>
<td>-</td>
<td>(72)</td>
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</table>

Perspectives

About 10 years ago, the cloning of multiple mAChRs and the ability to localize mAChR mRNA in brain (12) stimulated efforts to elucidate the functional significance of mAChR subtypes for REM sleep generation. Many studies show that non-M1 mAChRs in the mPRF modulate REM sleep generation. For example, electrophysiological data showed that cholinergic agonists depolarized most mPRF neurons but hyperpolarized about one-fourth of these cells (32). ACh usually increases neuronal excitability via mAChRs, but non-M1 mAChRs have been shown to hyperpolarize some parabrachial neurons (25). The cholinergically induced inhibition in the minority of mPRF neurons is mediated by non-M1 mAChRs via an inwardly rectifying potassium current (30). Microinjection studies of cat mPRF suggested a predominant role for non-M1 mAChRs in REM sleep generation (71). An added complexity is the need to specify mAChR subtype involvement at both presynaptic and postsynaptic sites within the mPRF. Studies quantifying the inhibition of feline REM sleep by mPRF administration of mAChR antagonists noted postsynaptic M2 modulation of REM sleep (5) and presynaptic M2 modulation of ACh release (6). In pontine regions of cat brain rostral and dorsal to the mPRF, microdialysis application of cholinergic antagonists led to the suggestion that M3 mAChRs play a critical role in REM sleep.
generation (59). Autoradiographic data show that the M2 subtype comprises the predominant mACHR binding site in the mPRF of cat (8, 49) and rat (4). Interestingly, in rat pontine reticular formation the density of M2 binding sites was not correlated with regions shown by microinjection to be most efficacious for cholinergic REM sleep enhancement (4). Considered together, the foregoing data demonstrated the importance of non-M1 mAChRs for REM sleep generation and encouraged the present study to focus on the signal transduction pathway linked to m2 and m4 mAChRs. Activation of m2 and m4 receptors stimulates an inhibitory G protein that decreases enzymatic activity of AC, leading to decreased cAMP and inhibition of PKA (18). The present results (Table 1, Fig. 6) show that mPRF administration of compounds that alter receptor, messenger, and effector molecules comprising the m3–m4 signal transduction pathway significantly alter cholinergic REM sleep generation. The suppression that mPRF cholinomimetics activate G proteins is indirect but supported by ongoing autoradiographic [35S]GTPγS binding studies. These data show that carbachol was able to activate G proteins in cholinergic and cholinceptive rat brain stem nuclei. This cholinergically elicited G protein activation was blocked by atropine, consistent with the view that G protein activation was muscarinically mediated (14). Data from many laboratories convincingly demonstrate that pontine cholinergic neurotransmission plays a key role in REM sleep generation (3, 4, 38, 45, 63, 68). The present results further specify that the mPRF transmembrane signal transduction processes subserving m2 and m4 mAChRs are involved in REM sleep regulation.

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