A Novel Role of Brainstem Pedunculopontine Tegmental Adenylyl Cyclase in the 
Regulation of Spontaneous REM Sleep in the Freely Moving Rat

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Running head: PPT cAMP pathway in REM sleep regulation

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ABSTRACT

Physiological activation of kainate receptors and GABA-B receptors within the pedunculopontine tegmentum (PPT) is involved in regulation of rapid eye movement (REM) sleep. Since these two types of receptors may also directly and/or indirectly activate the intracellular cyclic adenosine monophosphate (cAMP) signaling pathway, we hypothesized that this signaling pathway may be involved in the PPT to regulate spontaneous REM sleep. To test this hypothesis, four different doses (0.25, 0.50, 0.75, and 1.0 nmol) of a specific adenylyl cyclase (AC) inhibitor, SQ22536, were microinjected bilaterally (100 nl/site) into the PPT and the effects on REM sleep in freely moving chronically instrumented rats were quantified. By comparing alterations in the patterns of REM sleep following control injections of vehicle or one of the four different doses of SQ22536, the contributions made by each dose of SQ22536 to REM sleep were evaluated. The results demonstrated that the local microinjection of AC inhibitor SQ22536 into the PPT decreased the total amount of REM sleep for three hours and increased slow-wave sleep (SWS) for two hours in a dose-dependent manner. This reduction in REM sleep was due to increased latency and decreased frequency of REM sleep episodes. These results provide evidence that inhibition of AC within the PPT can successfully reduce REM sleep. These findings suggest that activation of the cAMP-signaling pathway within the cholinergic cell compartment of the PPT is an intracellular biochemical/molecular step for generating REM sleep in the freely moving rat.
INTRODUCTION

Rapid eye movement (REM) sleep is a distinctive sleep stage that alternates with episodes of slow-wave sleep (SWS). Over the last 25 years, considerable progress has been made in identifying the neuroanatomical, neurochemical, and neurophysiological events underlying the generation and modulation of REM sleep, which involves the activation of brainstem pedunculopontine tegmentum (PPT) cholinergic cells (Datta, 1995; Datta and Siwek, 2002). The PPT is situated in the dorsolateral mesopontine tegmentum and contains a prominent group of cholinergic neurons which project widely throughout the brain stem and forebrain (for review, see Datta, 1995). Single-cell recordings from the PPT in behaving cats and rats have identified several different classes of cells whose firing rates correlate with both wakefulness and REM sleep (Datta et al., 1989; El-Mansari et al., 1989; Steriade et al., 1990; Datta, 1995; Datta and Siwek, 2002).

Neurotransmitter-mediated excitation and inhibition of PPT cells are important processes for the regulation of REM sleep (Datta, 1995). Local microinjection studies in the cat and rat have suggested that the excitatory neurotransmitter, glutamate, may be involved in the direct and/or indirect activation of PPT cholinergic cells which trigger and maintain REM sleep (Datta and Siwek, 1997; Datta et al., 2001). Subsequent pharmacological and single-cell recording studies have shown that the activation of PPT kainate type glutamate receptors induces REM sleep (Datta, 2002; Datta et al., 2002; Datta and Siwek, 2002). On the other hand, the inhibitory neurotransmitter GABA activates GABA-B receptors which inhibit PPT cholinergic cells and suppresses REM sleep (Datta et al., 2003; Ulloor et al., 2004). Despite tremendous progresses in the identification of specific neurotransmitters and receptors involved in the regulation of the PPT cells neuronal activity and REM sleep, no other attempt has been made to study the intracellular signal transduction mechanisms of PPT cells that may be involved in the regulation of REM sleep.

A number of recent studies in behaving cats and rats have looked at the involvement of signal transduction pathways in the pontine reticular formation (PRF), a site known to be involved in the regulation of REM sleep, (Shuman et al. 1995; Capece and Lydic, 1997; Marks and Birabil, 2000). These studies have indicated that the signal transduction pathway activated by muscarinic cholinergic receptors
involves a pertussis toxin-sensitive G protein, adenylate cyclase (AC), cyclic adenosine monophosphate (cAMP), and protein kinase A (PKA). In the rat, this signal transduction pathway in the PRF is also shown to be involved in spontaneous REM sleep (Marks and Birabil, 2000). One recent study has demonstrated that the activation of GABA-B receptors within the PPT suppresses REM sleep in the freely moving rat (Ulloor et al., 2004). It is known that the GABA-B receptors couple to Gi/Go G-proteins (Takahashi et al. 1998; Sivilotti and Nistri, 1991; Mody et al., 1994; Thompson, 1994; Kerr and Ong, 1995; Couve et al., 2000; Robbins et al., 2001). It is also known that Gi/Go G-proteins inhibit AC and inhibition of AC prevents activation of the cAMP-PKA signal transduction pathway (Gilman, 1987; Marinissen and Gutkind, 2001). Therefore, it is reasonable to suggest that the suppression of REM sleep initiated by active GABA-B-receptors in the PPT may be due to inhibition of the cAMP-PKA signal transduction pathway. Recent pharmacological and physiological studies have also shown that the activation of PPT kainate type glutamate receptors induces REM sleep (Datta, 2002; Datta et al., 2002; Datta and Siwek, 2002). It is well known that the activation of kainate receptors increases the cytoplasmic free calcium concentration (Haak et al. 1997; Bleakman and Lodge, 1998; Michaelis, 1998; Bernard et al. 1999; Kovacs et al. 2000). In neurons, calcium ions can stimulate the production of cAMP and the activation of PKA by activating AC (Ginty et al. 1991; Cali et al. 1994; Xia et al. 1996; Waltereit et al. 2001). Thus, it is possible that the activation of kainate receptors in the PPT could also activate the cAMP-PKA signal transduction pathway to induce REM sleep.

Since the activation of PPT GABA-B and kainate receptors is involved in the regulation of REM sleep, and activation of these two types of receptors can also directly and/or indirectly modulate the intracellular cAMP signal transduction pathway (which is known to be involved in the PRF), in the present study we hypothesized that the activation and/or inactivation of PPT intracellular cAMP signaling may also be involved in the regulation of REM sleep. To test the role of the cAMP-signaling pathway in the regulation of spontaneous REM sleep, we examined polygraphic wake-sleep signs following local microinjections of control vehicle or AC-dependent cAMP inhibitor (SQ22536) into the PPT of freely moving rats. By comparing the alterations in the patterns of REM sleep following
injections of control vehicle or different doses of SQ22536, the contribution made by each dose of
SQ22536 in spontaneous REM sleep was evaluated.

METHODS

Subjects and housing

Experiments were performed on 52 adult male Sprague-Dawley rats (Charles River, Wilmington, MA) weighing between 250 and 350 g. The rats were housed individually at 24 °C with food and water provided ad libitum with lights on from 07:00 to 19:00 (light cycle) and off from 19:00 to 07:00 (dark cycle). The principles for the care and use of laboratory animals in research, as outlined by the National Institutes of Health Publication No. 85-23 (1985) were strictly followed.

Drug and vehicle for microinjections

The drug used in this study was a membrane permeable inhibitor of adenylyl cyclase (AC), 9-(Tetrahydro-2-furanyl)-9H-purin-6-amine (SQ22536; Mol. Wt. 205.22), purchased from RBI/Sigma Chemicals Co. (St. Louise, MO, USA). The SQ22536 was dissolved in 0.9% saline to make four different doses (0.25, 0.50, 0.75, and 1.0 nmol/100 nl). This 0.9% saline (100 nl) was also used for the vehicle control microinjection. Control saline and drug solutions were freshly prepared under sterile conditions before each use. The selection of this drug was based on the selective inhibitory effect on intracellular AC and cAMP (Fabbri et al., 1991; Shi and Bunney, 1992; Johnson et al., 1997; Natsume and Kometani, 1999). This commercially available SQ22536 is suitable for our local microinjection studies because it is water soluble, cell-permeable, has reversible effects, and has been used successfully for microinjection studies in behaving animals (Marks and Birabil, 2000).

Surgical Procedures and Implantation of Electrodes
Treatment of the animals and surgical procedures were in accordance with an approved institutional animal welfare protocol (AN-14084). Efforts were made to minimize the number of animals used and their suffering. Rats were anesthetized with pentobarbital (40 mg/kg, i.p.), placed in the stereotaxic apparatus, and secured using blunt rodent ear bars (Paxinos and Watson, 1997). With the use of sterile procedures, cortical electroencephalogram (EEG), dorsal neck muscle electromyogram (EMG), electrooculogram (EOG), hippocampal EEG (to record theta waves), and pontine EEG (to record P-waves) recording electrodes were chronically implanted, as described elsewhere (Datta, 2000). In addition, stainless steel guide tubes (26 gauge) with an equal length stylett inside were stereotaxically implanted bilaterally 2 mm above the PPT (A: 1.0; L: 1.8; H: 3.0) as described previously (Datta et al., 2001).

*Intracerebral microinjections and experimental design*

After the adaptation recording sessions, microinjection sessions began. During experimental sessions, animals were connected to the polygraphic recording system 15-minutes before a microinjection into the PPT. The microinjection system consisted of a 32-gauge stainless steel injector cannula with a 26-gauge collar that extended 2.0 mm beyond the implanted guide tube. The collar was connected to a 1.0 µl motor-driven Hamilton microsyringe with PE 20 tubing. While the animal was connected to the recording system, the stylettes were removed and an injector filled with either control vehicle (100 nl volume of 0.9% saline) or one of the four doses of SQ22536 (0.25, 0.50, 0.75, and 1.0 nmol in 100 nl) was introduced through one of the guide tubes for the injection. This procedure was repeated in the other guide tube. One minute after the insertion of the injector cannulae, 100 nl of control saline or one of the four doses of SQ22536 was bilaterally microinjected over a 60 sec period (Pump II Pico Plus, Harvard Apparatus, Holliston, MA). The injector cannulae were gently withdrawn 2 minutes after the injections, and the stylettes were reinserted into the guide tubes. During the microinjections, animals were free to move around the cage with the cannulae in place. Immediately after completion of the microinjection procedure, polygraphic variables were recorded continuously for a session of six-hours (between 10:00
and 16:00), when rats would normally be sleeping (Datta, 2000). Each of these rats received a total of 2 microinjections (100 nl each, one in the right and one in the left PPT) in a single experimental recording session. None of the rats were used for more than one microinjection recording session. At the end of all experimental sessions and before perfusion, 100 nl of black ink was microinjected 1 mm dorsal to each injection site for localizing the injection sites as described earlier (Datta et al., 2001).

**Determination of behavioral states and data analysis**

For the purpose of determining possible effects on sleep and wakefulness, three behavioral states were distinguished based on the visual scoring of polygraphic records as described earlier (Datta, 2002). In this study, microinjections of SQ22536 or saline into the PPT did not produce any dissociated sleep/wake states. The behavioral states of wakefulness (W), slow-wave sleep (SWS), and REM sleep were scored in successive 10 sec epochs. The polygraphic measures provided the following dependent variables, which were quantified for each recording session: (1) percentage of recording time spent in W, SWS, and REM sleep; (2) latency to onset of the first episode of REM sleep after the onset of recordings; (3) total number of REM sleep episodes; and (4) mean duration of REM sleep episodes. The effects of the five different treatments (1. Control saline; 2. 0.25 nmol SQ22536; 3. 0.50 nmol SQ22536; 4. 0.75 nmol SQ22536; 5. 1.0 nmol nmol SQ22536) on the percentages of W, SWS, and REM sleep were statistically analyzed using a two-way ANOVA with time as a repeated measure variable (six levels corresponding to six one hour epochs following injections) and treatment as a between-subject variable (five levels corresponding to the five different treatments). Following a two-way ANOVA, post-hoc Scheffe-F tests were done to determine the individual levels of significant difference between the control (saline) and the four different doses of SQ22536 treatment protocols at six individual data points. The latency, number, and duration of REM sleep episodes were analyzed using a one-way ANOVA followed by post-hoc Scheffe-F tests. Statistical analyses (two-way ANOVA, one-way ANOVA, and Scheffe F-test) were performed with the use of StatView statistical software (Abacus Concepts, Berkeley, CA).
Histological localization of injection site

At the conclusion of the microinjection experiments, rats were deeply reanesthetized with pentobarbital (60 mg/kg, i.p.) and perfused transcardially with heparinized cold phosphate buffer (0.1 M, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed and processed for NADPH-diaphorase staining and histological localization of injection sites as described earlier (Datta et al., 1997, 2001). This NADPH-diaphorase staining was done to identify the cholinergic cell compartment of the PPT (Vincent et al., 1983; Bredt et al., 1991; Hope et al., 1991; Bredt and Snyder, 1992).

RESULTS

(Insert Figure 1)

A total of 52 rats were bilaterally microinjected with control saline or one of the four different doses of SQ22536 into the PPT. Histological identification showed that in 41 rats, both injections were placed within the cholinergic cell compartment of the PPT (Fig. 1). In the remaining 11 rats, microinjection sites were away from the PPT. The analysis below quantifies the effects of control saline (n=10 rats), 0.25 nmol SQ22536 (n=7 rats), 0.50 nmol SQ22536 (n=7 rats), 0.75 nmol SQ22536 (n=10 rats), and 1.0 nmol SQ22536 (n=7 rats) microinjected into the PPT cholinergic cell compartment on the W, SWS, and REM sleep states.

(Insert Figure 2)

Effects of SQ22536 microinjection into the PPT on sleep-wake architecture

Figure 2 illustrates representative sleep-wake architectures for the 6-h post-injection recording sessions (10 AM to 4 PM) starting immediately after each of five different bilateral microinjections. The figure shows that the latency between the end of bilateral microinjections of saline and the first episode of REM sleep was much shorter than after bilateral microinjections of any one of the four different doses of SQ22536. In contrast, the latencies between the end of bilateral microinjections of SQ22536 and the
first episode of SWS were shorter compared to after bilateral microinjections of control saline. For about 3 h after microinjections of SQ22536, the numbers of REM sleep episodes were fewer compared with after bilateral microinjections of control saline. During the 5th and 6th hours after bilateral microinjections of SQ22536, the numbers of REM sleep episodes were similar to the number of REM sleep episodes in the 5th and 6th hours after bilateral microinjections of control saline. These results demonstrate that bilateral microinjections of SQ22536, into the cholinergic cell compartment of the PPT change the sleep-wake architecture of the rat.

(Insert Figure 3)

Effects of SQ22536 microinjection into the PPT on W

The changes in the percentage of time spent in W after bilateral microinjections of saline control and the different SQ22536 doses are summarized in Fig. 3. A Two-way ANOVA indicated a significant main effect of treatment \([F(4,36) = 3.98, p < 0.01]\), time \([F(5,180) = 47.32, p < 0.001]\), and a significant treatment X time interaction \([F(20,180) = 3.35, p < 0.001]\) for total percentage of time spent in W. The results of post hoc analysis (Scheffe F-test) on the total percentage of time spent in W are presented in Fig. 3. Compared with saline control microinjections, three of the four different doses of SQ22536 (0.50, 0.75, and 1.0 nmol) caused a significant reduction of the total percentage of W. This SQ22536-induced reduction in the total percentage of W was dose dependent. The reduction in W after 0.50 and 0.75 nmol doses lasted for the first hour of recordings. The total percentage of W after the lowest dose of SQ22536 (0.25 nmol) was lower compared to after the saline control, but this reduction did not reach significance. The W reducing effect of SQ22536 microinjection at the higher dose (1.0 nmol) lasted for the first 2 hours of recordings. These results show that the application of SQ22536 into the cholinergic cell compartment of the PPT causes a short lasting reduction in the total percentage of W.

(Insert Figure 4)

Effects of SQ22536 microinjection into the PPT on SWS

The changes in the percentage of time spent in SWS after microinjection of saline control and different SQ22536 doses are summarized in Fig. 4. A two-way ANOVA indicated a significant main effect of treatment \([F(4,36) = 6.44, p < 0.001]\), time \([F(5,180) = 21.0, p < 0.001]\), and a significant
treatment X time interaction [F(20,180) = 3.61, p < 0.001] for total percentage of time spent in SWS. The results of post hoc analysis (Scheffe F-test) for the total percentage of time spent in SWS are presented in Fig. 4. After microinjections of SQ22536, there was a dose-dependent increase in the total percentage of time spent in SWS. After the second lowest dose (0.50 nmol) of SQ22536, the rats spent more time in SWS than the saline injected rats during the first hour of recording. As the doses of SQ22536 increased to 0.75 and 1.0 nmol, SWS remained significantly higher for the first 2 hours of recording. This SQ22536 microinjection-induced increase in SWS was relatively short-lasting compared to receptor-mediated action in the PPT.

(Insert Figure 5)

Effects of SQ22536 microinjection into the PPT on REM sleep

The changes in the percentage of time spent in REM sleep after microinjection of the saline control and the different SQ22536 doses are summarized in Fig. 5. A two-way ANOVA indicated a significant main effect of treatment [F(4,36) = 7.53, p < 0.001], time [F(5,180) = 94.16, p < 0.001], and a significant treatment X time interaction [F(20,180) = 5.34, p < 0.001] on total percentage of time spent in REM sleep. The results of post hoc analysis (Scheffe F-test) on total percentage of time spent in REM sleep are presented in Fig. 5. Compared with post-control-injection recordings, after microinjection of SQ22536, there was a dose-dependent decrease in the total percentage of time spent in REM sleep. Post hoc analysis indicated that the total percentage of REM sleep after microinjection of 0.50 nmol dose of SQ22536 was significantly lower in the second and third hours post injection compared with after microinjection of saline. The lowest dose of (0.25 nmol) SQ22536 also reduced the total percentage of REM sleep in the second hour but this reduction did not reach the level of significance. Microinjections of 0.75 and 1.0 nmol doses of SQ22536 suppressed REM sleep completely for the first two hours post injection. Post hoc analysis indicated that the total percentage of REM sleep after these two higher doses (0.75 and 1.0 nmol) was significantly decreased even in the third and fourth hours post injection compared with after microinjection of saline.
The effects of different doses of SQ22536 on latency, number, and duration of REM sleep episodes are illustrated in Fig. 6. One-factor ANOVAs indicated a significant effect on the latency \([F(4,36) = 59.47, p < 0.001]\) and number \([F(4,36) = 61.65, p < 0.001]\), but not in the duration \([F(4,36) = 2.43, p = 0.09]\) of the REM sleep episodes. Post hoc analyses indicated that the latency to the first episode of REM sleep was significantly delayed after microinjections of 0.50, 0.75, and 1.0 nmol doses of SQ22536 compared with the saline control. The total number of REM sleep episodes was significantly decreased after microinjections of 0.50, 0.75, and 1.0 nmol doses of SQ22536 compared with the saline control. Similar post hoc analyses did not reveal any significant difference in the mean duration of REM sleep episodes after microinjection of any one of the doses of SQ22536 compared with the saline control. These results demonstrate that the changes in the total percentage of time spent in REM sleep after microinjection of the different doses of SQ22536 are due to changes in the latency and number of REM sleep episodes.

As we have mentioned above, histological examination revealed that in 11 rats guide tubes were placed between 0.6 and 0.9 mm away from the cholinergic cell compartment of the PPT. These guide tubes were in the caudal part of the midbrain. Of those 11 rats, five received bilateral microinjections of SQ22536 and six received saline control. Two of the SQ22536 microinjected rats received 0.75 nmol and three received 1.0 nmol doses of SQ22536. In these rats injected with SQ22536, the total percentages of W \((39.4 \pm 6.2)\), SWS \((52.4 \pm 7.4)\), and REM sleep \((8.2 \pm 1.3)\) were comparable to the total percentages of W \((36.2 \pm 7.8)\), SWS \((56.7 \pm 6.5)\), and REM sleep \((7.1 \pm 1.1)\) in the saline injected rats. These results show that the microinjection of SQ22536 into these sites did not cause any changes in the sleep-wake parameters.

**DISCUSSION**

The principal findings of this study are that 1) microinjection of the selective adenylyl cyclase inhibitor SQ22536 into the PPT decreased spontaneous REM sleep in a dose-dependent manner, 2)
SQ22536 microinjection into the PPT reduced the total amount of wakefulness and increased slow-wave sleep in a dose-dependent manner, 3) SQ22536 microinjections 0.6 to 0.9 mm anterior to the PPT did not produce any change in REM sleep. These results show for the first time that the direct inhibition of adenyl cyclase within the cholinergic cell compartment of the PPT suppresses physiological REM sleep. These results also suggest that activation of cAMP in the cholinergic cell compartment of the PPT in rats is an intracellular physiological mechanism involved in spontaneous REM sleep.

We chose to study chronically implanted freely moving rats because this preparation provides the most physiological approach to a longitudinal analysis of the behavioral and electrographic events related to the wake-sleep cycle (Marks and Birabil, 2000; Datta et al., 2002; Ulloor et al., 2004). After the accommodation period of each experimental condition, the rats demonstrated regular values of wake and sleep stages. Thus, the microinjections of a pharmacologically active selective signal transduction-altering inhibitor that affected electrographic signs of wake-sleep patterns could be evaluated accurately (Capece and Lydic, 1997; Marks and Birabil, 2000). Moreover, understanding the role of the intracellular signal transduction pathway in a behaviorally active living organism is a vital step towards creating ways to converge functional biochemistry and integrative physiology. In this particular study, in order to identify the involvement of the intracellular signal transduction pathway in the induction of REM sleep, we chose a specific inhibitor, SQ22536, to block AC-dependent cAMP (Fabbri et al., 1991; Shi and Bunney, 1992; Johnson et al., 1997; Natsume and Kometani, 1999; Froehlich and Wand, 1997; Marks and Birabil, 2000). The local microinjection of SQ22536 and other signal transduction-altering compounds into the brain of freely moving animals has already been shown to be an important technique when studying the involvement of signal transduction pathways modulating behaviors (Kantak et al., 1981; Barraco et al., 1988; De Lima and Davis, 1995; Cook et al., 1995; Capece and Lydic, 1997; Marks and Birabil, 2000). However, a major limitation of the microinjection method relates to the diversity in the neurochemical nature of the neuronal population affected by the drug application. In this study, a selective inhibitor of AC-dependent cAMP was injected into the part of the PPT (pars compacta) where most cells are known to be cholinergic (Mesulum et al., 1983; Rye et al., 1987). Nonetheless, we acknowledge that if there are non-cholinergic cells located within the targeted cholinergic cell groups
they will also be affected by the application of this drug. We also acknowledge that we have no way of knowing whether SQ22536 acted on pre- or post-synaptic elements of the neurons. Another interpretive limitation of this study is that, while we suggest that our application of SQ22536 into the PPT revealed an endogenous mechanism of REM sleep regulation, future study is needed to be certain. Additional interpretative limitation comes from the fact that the PPT has also been implicated in the modulation of respiration. Thus, it is possible that the observed reduction in REM sleep may partially be due to impaired respiration (Radulovacki et al., 2004). Since in this study, we did not record respiration, it is impossible to rule out that possibility.

In recent years, a number of studies have attempted to identify endogenous neurotransmitters’ and their specific receptors’ involvement in the cholinergic cell compartment of the PPT in regulating single cell activity of the PPT and regulating REM sleep (Datta, 2002; Datta et al., 1997, 2001, 2002, 2003; Datta and Siwek, 1997, 2002; Garcia-Rill et al., 2003; Kobayashi et al., 2004; Ulloor et al., 2004). However, other than the results of this study, there is no direct evidence to demonstrate that the intracellular signal transduction pathways in the cholinergic cell compartment of the PPT is involved in the regulation of REM sleep. The results of this study, for the first time, provide evidence that the direct inhibition of AC by microinjecting SQ22536 suppresses REM sleep by increasing the latency and decreasing the frequency of REM sleep episodes. Since SQ22536 inhibits the production of the intracellular second-messenger cAMP by inhibiting AC (Gilman, 1987; Johnson et al., 1997; Marks and Birabil, 2000), these results suggest that the production of intracellular cAMP in the PPT is involved in the generation of REM sleep. This finding is interesting because in another area of the rat’s brainstem, the medial pontine reticular formation (mPRF), microinjection of carbachol induces a REM sleep-like state and microinjection of SQ22536 increases REM sleep (Marks and Birabil, 1998, 2000). This finding indicates that inhibition of cAMP production in the mPRF is an important step for the induction of REM sleep. This site-specific role of cAMP in the regulation of REM sleep is also supported by the result from the present study as microinjection of SQ22536 into the caudal midbrain in sites adjacent to the PPT was not effective in causing any changes in REM sleep. Other studies in the cat have demonstrated that pretreatment of the mPRF with agents that activate AC, cell-permeable analogs of cAMP, or agonists of
protein kinase A (PKA) all antagonize the carbachol or neostigmine microinjection-induced REM sleep-like state (Shuman et al., 1995; Capece and Lydic, 1997). These findings also indicate that inhibition of the cAMP signal transduction pathway in the mPRF might be involved in the carbachol and neostigmine microinjection-induced REM sleep-like state in the cat. Yet in another study, microinjection of pituitary adenyl cyclase-activating polypeptide (PACAP) into the mPRF of rats increased REM sleep (Ahnaou et al., 1999). This result indicates that the activation of the cAMP-dependent signal transduction pathway in the mPRF is involved in the induction of REM sleep. Thus, based on the results of these earlier studies and the present study, it is reasonable to suggest that the intracellular cAMP signal transduction pathway in the brainstem is critically involved in the regulation of REM sleep. Since the activation of the cAMP signal transduction pathway causes protein phosphorylation by activating protein kinase A (PKA) that ultimately causes activation of ion channels, receptor up/down regulation, neurotransmitter synthesis/release, and gene expression (Gilman, 1987; Elazar and Fuchs, 1991; Ginty et al., 1991; Wang et al., 1991, 1993), it is logical to speculate that part of this cAMP activation-mediated REM sleep generating mechanism in the cholinergic cell compartment of the PPT involves activation of the cAMP-dependent PKA signal transduction pathway. However, we acknowledge that future experimental work will be necessary to confirm or refute this pathway’s involvement.

In light of the present study, it is interesting to compare the effects of microinjection of the GABA-B receptor-selective agonist, baclofen, into the PPT (Ulloor et al., 2004). The results of that study demonstrated that the activation of GABA-B receptors in the cholinergic cell compartment of the PPT suppresses PPT REM-on cells and decreases REM sleep by increasing REM sleep latency and decreasing the number of REM sleep episodes. It has also been demonstrated that the activation of PPT GABA-B receptors decreases wakefulness and increases slow-wave sleep (Ulloor et al., 2004). In the present study, we have demonstrated that the microinjections of the selective AC inhibitor into the PPT also caused decreased REM sleep by increasing latency and decreasing number of episodes of REM sleep. This AC inhibitor also caused a reduction in wakefulness and an increase slow-wave sleep. Another interesting common characteristic between activation of GABA-B receptors and inhibition of AC in the PPT is neither processes changed the episode durations of REM sleep. The similarity in action on REM sleep by
GABA-B receptor activation and AC inhibition in the PPT and the fact that the activation of GABA-B receptors could inhibit AC suggests that the PPT GABA-B receptor mediated REM sleep regulation mechanism also involves inhibition of AC (Gilman, 1987; Takahashi et al., 1998; Marinissen and Gutkind, 2001). To explain the mechanism behind the fact that the inhibition of AC in the PPT did not change REM sleep episode duration may require future study.

Since the results of the present study demonstrated that inhibition of AC in the PPT suppresses REM sleep, it is reasonable to suggest the possibility that the activation of AC may increase REM sleep. Earlier studies have demonstrated that the activation of kainate type glutamate receptors in the PPT increases REM sleep (Datta, 2002; Datta and Siwek, 1997; Datta et al., 2001, 2002). Since it is known that the activation of kainate receptors can activate AC by increasing intracellular calcium ions (Ginty et al., 1991; Wang et al., 1991, 1993; Cali et al., 1994; Xia et al., 1996; Haak et al., 1997; Bleakman and Lodge, 1998; Michaelis, 1998; Bernard et al., 1999, Kovacs et al., 2000; Waltereit et al., 2001), it is possible that the PPT kainate receptor activation-mediated induction of REM sleep may also have operated through the activation of AC. Since AC-dependent cAMP pathway inhibition in the PPT suppressed REM sleep, and other studies have suggested that dysregulation of the normative developmental changes in the PPT could lead to a number of disorders including schizophrenia, panic attacks, bipolar disorder, and obsessive-compulsive disorder (Garcia-Rill et al., 2003; Kobayashi et al., 2004), it would be interesting to design future studies to understand the role of PPT cAMP signal transduction mechanisms in the pathophysiology of these neuropsychiatric disorders. However, at the present time this remains an unexplored area of research.

In conclusion, the present study shows for the first time that the modulation of AC in the cholinergic cell compartment of the PPT may be an important step for the regulation of REM sleep. The data provide a novel perspective on the regulatory aspect of PPT cells’ activity in the regulation of REM sleep. The results also suggest that the activation of the cAMP-signaling pathway in the cholinergic cell compartment of the PPT may be an important step in a series of biochemical and molecular events for the induction of REM sleep.
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LEGENDS

Figure 1. Histological localization of SQ22536 microinjection sites in the pedunculopontine tegmentum (PPT) of the rat that produced changes in REM sleep. A: Coronal histological section of the rat brainstem at stereotaxic antero-posterior level A:1.0 showing SQ22536 microinjection sites and ink microinjection sites. To localize SQ22536 microinjection sites without interfering with NADPH labeling of cholinergic cells, using same injector 100 nl of black ink was microinjected 1 mm dorsal to the SQ22536 microinjection sites (arrows). B: Outline drawing of A showing anatomical location of SQ22536 (arrow heads) and black ink (arrows) microinjection sites and surrounding structures. Note that the SQ22536 microinjection sites showing a cluster of NADPH-diaphorase-positive cholinergic cells in and around the microinjection sites. Abbreviations of anatomical terms: Aq, aqueduct; CG, central gray; DR, dorsal raphe nucleus; LDT, laterodorsal tegmentum; LL, lateral lemniscus; Me5, mesencephalic trigeminal tract/nucleus; mlf, medial longitudinal fasciculus; MnR, median raphe nucleus; PPT, pedunculo pontine tegmental nucleus; SCP, superior cerebellar peduncle;. Scale bar in lower right corner = 1 mm.

Figure 2. Examples to show the changes in sleep-wake architecture after bilateral microinjections of different doses of SQ22536 into the PPT. These 5 hypnograms from 5 different rats plotted as step histograms plot the occurrence and duration of polygraphically defined wakefulness (W), slow-wave sleep (S), and REM sleep (R) after control vehicle and four different doses of SQ22536. All microinjections were made at 10:00 AM (0 min) and followed by 6 h of continuous recording (720 min).
Figure 3. Wakefulness after bilateral microinjections of 4 different doses of SQ22536 and control saline into the PPT. Bars represent percentages (means ± SE) of wakefulness during each of the 6-h periods after injections of control saline and 0.25, 0.50, 0.75, and 1.0 nmol SQ22536. Note dose-dependent decrease of wakefulness after SQ22536 microinjections. Asterisks represent the levels of statistical significance (Scheffe F test) of the differences relative to control saline: *p<0.05; **p<0.01; ***p<0.001.

Figure 4. Slow-wave sleep after bilateral microinjections of 4 different doses of SQ22536 and control saline into the PPT. Bars represent percentages (means ± SE) of slow-wave sleep during each of the 6-h periods after injections of control saline and 0.25, 0.50, 0.75, and 1.0 nmol SQ22536. Note the dose-dependent increase of slow-wave sleep after SQ22536 microinjections. Asterisks represent the levels of statistical significance (Scheffe F test) of the differences relative to control saline: *p<0.05; **p<0.01.

Figure 5. REM sleep after bilateral microinjections of 4 different doses of SQ22536 and control saline into the PPT. Bars represent percentages (means ± SE) of REM sleep during each of the 6-h periods after injections of control saline and 0.25, 0.50, 0.75, and 1.0 nmol SQ22536. Note the dose-dependent decrease of REM sleep after SQ22536 microinjections. Asterisks represent the levels of statistical significance (Scheffe F test) of the differences relative to control saline: *p<0.05; ***p<0.001.

Figure 6. Effects on the latency, the total number, and mean duration of REM sleep episodes observed after microinjection of 4 different doses of SQ22536 (0.25, 0.50, 0.75, and 1.0 nmol) into the PPT. Data presented as mean ± SE. Note that compared to the control saline, microinjections of SQ22536 increased latency for the first episode of REM sleep and decreased the number of REM sleep episodes in a dose-dependent manner. Asterisks represent the levels of statistical significance (Scheffe F test) of the differences relative to control saline: *p<0.05; 0.25, 0.50, 0.75, and 1.0 nmol ***p<0.001.
Fig. 1 (Datta et al.)
Fig. 2 (Datta et al.)

- Control Saline
- SQ22536 (0.25 nmol)
- SQ22536 (0.50 nmol)
- SQ22536 (0.75 nmol)
- SQ22536 (1.0 nmol)

Time (Min)
Fig. 3 (Datta et al.)
Fig. 4 (Datta et al.)
Fig. 5 (Datta et al.)
Fig. 6 (Datta et al.)

**Graph 1:** REM Sleep Latency (Min)
- Saline Control
- SQ (0.25 nmol)
- SQ (0.50 nmol)
- SQ (0.75 nmol)
- SQ (1.0 nmol)

**Graph 2:** Number of REM Sleep Episodes
- Saline Control
- SQ (0.25 nmol)
- SQ (0.50 nmol)
- SQ (0.75 nmol)
- SQ (1.0 nmol)

**Graph 3:** Duration of REM Sleep (Sec)
- Saline Control
- SQ (0.25 nmol)
- SQ (0.50 nmol)
- SQ (0.75 nmol)
- SQ (1.0 nmol)