

Acetylcholine Excites GABAergic Neurons of the Ferret Perigeniculate Nucleus Through Nicotinic Receptors

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SUMMARY AND CONCLUSIONS

1. The actions of acetylcholine (ACh) on the GABAergic neurons of the perigeniculate nucleus (PGN) were investigated with the use of extra- and intracellular recording techniques in spontaneously spindling ferret thalamic slices maintained *in vitro*.

2. Local application of ACh to PGN neurons resulted in rapid depolarization followed by a longer lasting hyperpolarization. Neither of these responses were abolished by blockade of synaptic transmission with tetrodotoxin (TTX) nor with low Ca^{2+} and elevated Mg^{2+} solution, indicating that they are direct postsynaptic actions of ACh on PGN cells. Functionally, the rapid depolarizing response could activate both single spike activity, as well as low-threshold Ca^{2+} spike-mediated bursts.

3. The fast depolarizing response to ACh was selectively blocked by application of the nicotinic antagonist hexamethonium, whereas the slow hyperpolarizing response to ACh was selectively blocked by application of the muscarinic antagonist (–)scopolamine. Application of both hexamethonium and (–)scopolamine blocked the modulation of PGN action-potential firing by ACh.

4. Local application of the nicotinic agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP) resulted in a depolarizing response and an increase in membrane conductance, whereas application of the muscarinic agonist DL-muscarine chloride resulted in a hyperpolarizing response and an increase in membrane conductance. When applied to spontaneously spindling PGN cells, both DMPP and DL-muscarine blocked the occurrence of spindle oscillations. However, only DMPP was associated with depolarization and the generation of single spike activity.

5. These results indicate that the GABAergic cells of the PGN possess postsynaptic nicotinic as well as muscarinic receptors. Activation of the nicotinic receptors results in rapid depolarization of these neurons and can activate both single spike and burst activity. These responses may be important in the generation of pontogeniculo-occipital (PGO) waves and membrane depolarizations during rapid-eye-movement (REM) sleep and arousal.

INTRODUCTION

The perigeniculate nucleus (PGN), an extension of the nucleus reticularis thalami (nRt), is a collection of GABAergic neurons intricately interconnected with the dorsal lateral geniculate nucleus (LGNd) (see Jones 1985). The PGN is bilaterally innervated by cholinergic fibers from the peribrachial (PB) area of the pedunculopontine tegmental (PPT) and laterodorsal tegmental (LDT) nuclei (Smith et al. 1988), suggesting that release of acetylcholine (ACh) by these systems may control the state of activity in the PGN. Autoradiographic localization of putative cholinergic receptors demonstrate that the PGN contains a high density of both nicotinic (Clarke et al. 1985) as well as muscarinic receptors (Wamsley et al. 1984). These results suggest that

postsynaptic action of ACh on PGN neurons may be mediated by both nicotinic and muscarinic receptors. Previous studies have reported a dominant inhibitory effect of ACh on neurons of the nRt/PGN (Ben-Ari et al. 1976; Dingledine and Kelly 1977); although one study suggested that ACh may also excite nRt cells through muscarinic receptors (Kiyama et al. 1986). None of these studies demonstrated nicotinic receptor-mediated responses in PGN or nRt cells, even though activation of these receptors in LGNd relay cells causes fast excitation through depolarization (McCormick and Prince 1987a).

In this study we describe a rapid nicotinic excitation followed by slow muscarinic inhibition sequence in PGN cells to ACh and demonstrate that these responses may control the pattern of activity generated in thalamic circuits.

METHODS

For the preparation of slices, male or female ferrets, 3–4 mo old, were deeply anesthetized with pentobarbital sodium (30–40 mg/kg) and killed by decapitation. The forebrain was rapidly removed, and the hemispheres were separated with a midline incision. Slices (400 μm thick) were cut with the use of a vibratome (Ted Pella) in the sagittal plane. A modification of the technique developed by Aghajanian and Rasmussen (1989) was used to increase tissue viability. During preparation of slices, the tissue was placed in a solution (5°C) in which NaCl was replaced with sucrose while maintaining an osmolarity of 307 mosM. After preparation, slices were placed in an interface style recording chamber (Fine Sciences Tools), maintained between 33.5 and 34.5°C, and allowed at least 2 h to recover. The bathing medium contained (in mM) 126 NaCl, 2.5 KCl, 1.2 MgSO_4 , 1.25 NaH_2PO_4 , 2 CaCl_2 , 26 NaHCO_3 , and 10 dextrose and was aerated with 95% O_2 -5% CO_2 to a final pH of 7.4. For the 1st 20 min that the thalamic slices were in the recording chamber, the bathing medium contained an equal mixture of the normal NaCl and the sucrose-substituted solutions.

Intracellular recording electrodes were formed on a Sutter Instruments P-80 micropipette puller from medium-walled glass (WPI, 1B100F) and beveled on a Sutter Instruments beveler. Micropipettes were filled with 2 M K acetate and 2% biocytin for intracellular labeling of recorded neurons. Biocytin-filled neurons were visualized through standard avidin-biotin-horseradish peroxidase reaction with diaminobenzidine (Horikawa and Armstrong 1988). In addition, the location of the PGN was confirmed through immunocytochemical staining for γ -aminobutyric acid (GABA) (Schwartz and Meineke 1992). Only those neurons exhibiting a stable resting membrane potential of at least -60 mV and electrophysiological properties as reported previously (Bal and McCormick 1993) were included for analysis. Electrical stimulation of the afferent fibers was achieved through the placement of a concentric stimulating electrode in the optic radiation and delivering 1–10 stimuli (100- μs duration; 10- to 500- μA amplitude; 100-Hz frequency).

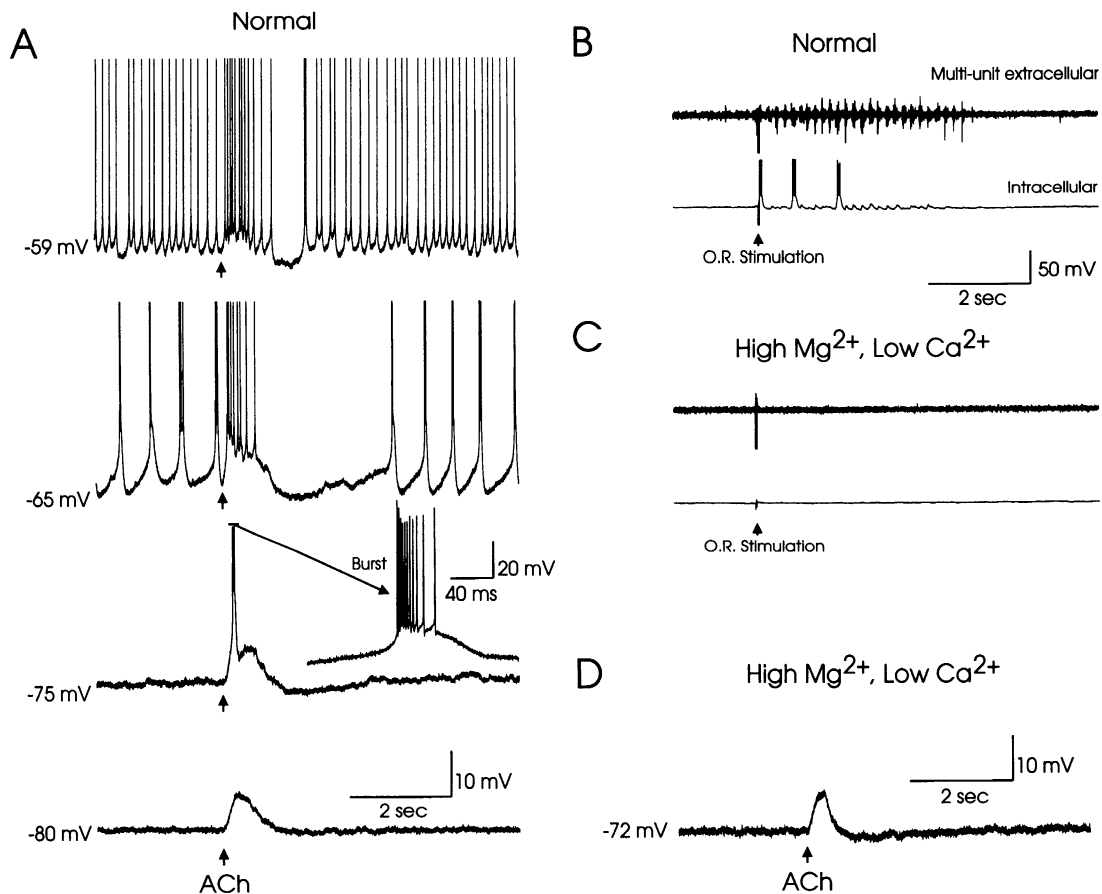


FIG. 1. Intracellular recordings of responses to acetylcholine (ACh) in perigeniculate nucleus (PGN) neurons. *A*: application of ACh causes a rapid depolarization followed by a late hyperpolarization at various membrane potentials. When the cell is in the tonic firing mode of action-potential generation (-59 mV), ACh application results in an increase in firing frequency followed by a decrease in firing frequency. At -65 mV, the cell spontaneously generated rhythmic bursts of action potentials at ~ 1 Hz. At this membrane potential, the application of ACh blocked the rhythmic bursts, caused a depolarization with single spike tonic firing, followed by a period of hyperpolarization, and finally the reinstatement of the rhythmic bursting. At -75 mV, ACh application results in a burst of action potentials. At -80 mV, a subthreshold depolarizing response followed by a small hyperpolarization is seen. *B*: electrical stimulation of the optic radiation (O.R.) in normal solution initiates a spindle oscillation in both multiunit extracellular and intracellular recordings of PGN neurons. Extracellular electrode was placed within $50 \mu\text{m}$ of the intracellular electrode in the PGN. *C*: in high- Mg^{2+} and low- Ca^{2+} solution, electrical stimulation of O.R. does not activate action potentials or postsynaptic potentials, indicating that synaptic transmission is blocked. *D*: in high- Mg^{2+} and low- Ca^{2+} solution, the neuron still responds to ACh with an excitation-inhibition sequence. All intracellular data were collected from the same ferret PGN cell.

Drugs were either applied by the pressure-pulse technique in that a brief (10–20 ms; 207–345 kPa; 10–30 psi) pulse of nitrogen was applied to a broken microelectrode (tip diameter, 2–5 μm) containing the drug dissolved in bathing medium or through addition to the bathing medium. With the pressure-pulse technique, the volume of the resulting application was between 5 and 15 pl as estimated from the diameter of the ejected droplet. For agonists and antagonists, application to the exposed surface of the slice was usually sufficient to elicit its pharmacological effect. However, in the case of ACh, it was necessary to lower the drug-applying pipette into the slice to obtain robust responses. Typically, the entry point of the drug-applying electrode was within $50 \mu\text{m}$ of the recording electrode. Hexamethonium dichloride was obtained from RBI. All other drugs were obtained from Sigma.

The data were analyzed with the use of Axotape (Axon Instruments) on a PC-AT style computer.

RESULTS

Intracellular recordings were obtained from 39 PGN neurons. Intracellular injection of hyperpolarizing and depolar-

izing current pulses into PGN cells revealed two distinct modes of action-potential generation as reported previously (Avanzini et al. 1989; Bal and McCormick 1993; Contreras et al. 1993; McCormick and Wang 1991). At potentials negative to -65 mV, these cells generated a high-frequency (250–400 Hz) burst of action potentials in response to intracellular injection of a short depolarizing current pulse. At potentials positive to -60 mV, these cells generated tonic, single spike activity with the frequency of firing being related to the intensity of depolarization (see Bal and McCormick 1993).

Single brief (~ 10 ms) pressure pulse applications of ACh (5 mM in micropipette) to the region surrounding an intracellularly recorded PGN neuron resulted in a rapid depolarization followed by a slow hyperpolarization (see Fig. 1A; $n = 23$). The onset latency of the depolarization was as brief as the delay imposed by the application system (i.e., ~ 10 ms). The response of PGN neurons to ACh was tested at various membrane potentials by intracellular injection of

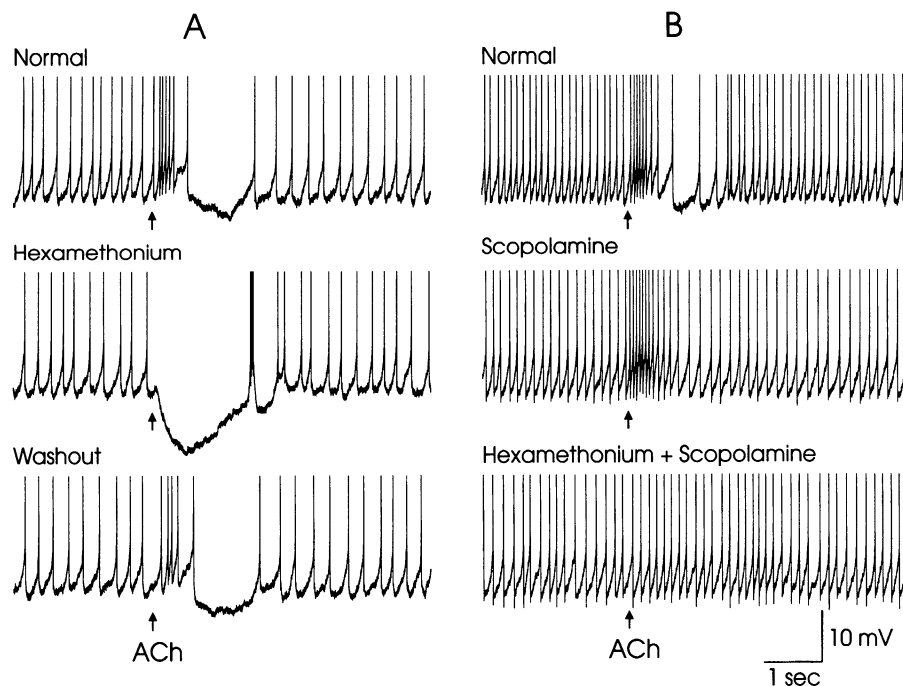


FIG. 2. Effect of nicotinic and muscarinic antagonists on the response of PGN neurons to ACh. *A*: application of ACh to this PGN neuron in normal solution results in the typical excitation-inhibition sequence. Local application of the nicotinic antagonist hexamethonium (2.5 mM in micropipette) completely blocked the early excitatory response to ACh while leaving intact the late inhibitory response. Washing out of the hexamethonium reinstates the excitation-inhibition response. *B*: local application of the muscarinic antagonist scopolamine blocked the late inhibitory response to ACh while leaving intact the early excitatory response. Application of both hexamethonium and scopolamine completely block all modulation of single spike activity by ACh on this PGN neuron.

DC (Fig. 1A). When the membrane potential of the cell was above the threshold for single spike activity, ACh application resulted in an increase followed by a decrease in firing frequency associated with a depolarization-hyperpolarization sequence. Often, the hyperpolarization was followed by the occurrence of a rebound burst. At -65 mV, the cell of Fig. 1A generated rhythmic bursts of action potentials at ~ 1 Hz, presumably through intrinsic membrane mechanisms (see Bal and McCormick 1993). Interestingly, at this membrane potential, the application of ACh blocked the rhythmic bursts and caused a depolarization with single spike tonic firing, followed by a period of hyperpolarization and finally the reinstatement of the rhythmic bursting. At -75 mV, the ACh-induced depolarization activated a low-threshold Ca^{2+} spike and a burst of high-frequency action potentials (Fig. 1A, -75 mV). Hyperpolarization to more negative membrane potentials (e.g., -80 mV) revealed a subthreshold depolarizing response to ACh with no action potentials generated and only a small hyperpolarization, presumably due to the membrane potential being closer to the reversal potential for the muscarinic receptor-mediated increase in K^+ conductance (Fig. 1A) (McCormick and Prince 1986).

To test whether the depolarization-hyperpolarization responses to ACh were direct effects on the PGN cells recorded or were mediated through the release of another neurotransmitter, synaptic transmission was blocked by either application of the highly selective Na^+ channel blocker tetrodotoxin (TTX; $10 \mu\text{M}$ in micropipette) or through bath application of high Mg^{2+} (8 mM) and low Ca^{2+} (0.5 mM). Before the block of synaptic transmission, spontaneous or evoked (electrical stimulation of the optic radiation) spindle oscillations were observed (Fig. 1B) (von Krosigk et al. 1993), and application of ACh caused the typical short-latency depolarization-hyperpolarization sequence (Fig. 1A). After infusing in high- Mg^{2+} , low- Ca^{2+} solution, both the spontaneous spindle oscillations as well as responses to electrical

stimulation of the optic radiation were blocked (Fig. 1C), but the depolarization-hyperpolarization sequence to ACh remained (Fig. 1D; $n = 3$). Likewise, application of TTX to the surface of the slice resulted in the blockage of action potentials, spindle oscillations, and response to electrical stimulation but did not alter the depolarization-hyperpolarization response of the PGN neurons to ACh (not shown). These results indicate that both the depolarization-hyperpolarization responses of PGN cells to ACh are a consequence of direct action on the neuron studied and not mediated through a polysynaptic mechanism.

Both excitatory and inhibitory responses to ACh have been associated with both nicotinic as well as muscarinic receptors (Curtis and Ryall 1966; Krnjevic 1974; McCormick and Prince 1985, 1987a,b; Takagi 1984; Wong and Gallagher 1991). We investigated the pharmacological profile of the receptors mediating the responses of PGN neurons to ACh, using typical nicotinic and muscarinic agonists and antagonists. Local application of the ganglionic nicotinic receptor channel blocker hexamethonium (2.5 mM in micropipette) to the surface of the slice completely abolished the early excitatory response to ACh while leaving intact the late inhibitory response (Fig. 2A; $n = 7$). Interestingly, following the block of the early excitatory response with hexamethonium, the hyperpolarizing response to ACh was enhanced and was now large enough to activate a low-threshold Ca^{2+} spike and associated burst discharge (Fig. 2A, Hexamethonium). The excitatory-inhibitory sequence to ACh was reinstated upon recovery from hexamethonium (Fig. 2A, Washout).

In contrast, local ($10 \mu\text{M}$ to 1.0 mM in micropipette) or bath ($1 \mu\text{M}$) application of the muscarinic receptor antagonist ($-$)scopolamine completely abolished the late inhibitory response to ACh, as reported previously (McCormick and Prince 1986), while leaving intact the early excitatory response (see Fig. 2B; $n = 3$). With concomitant application

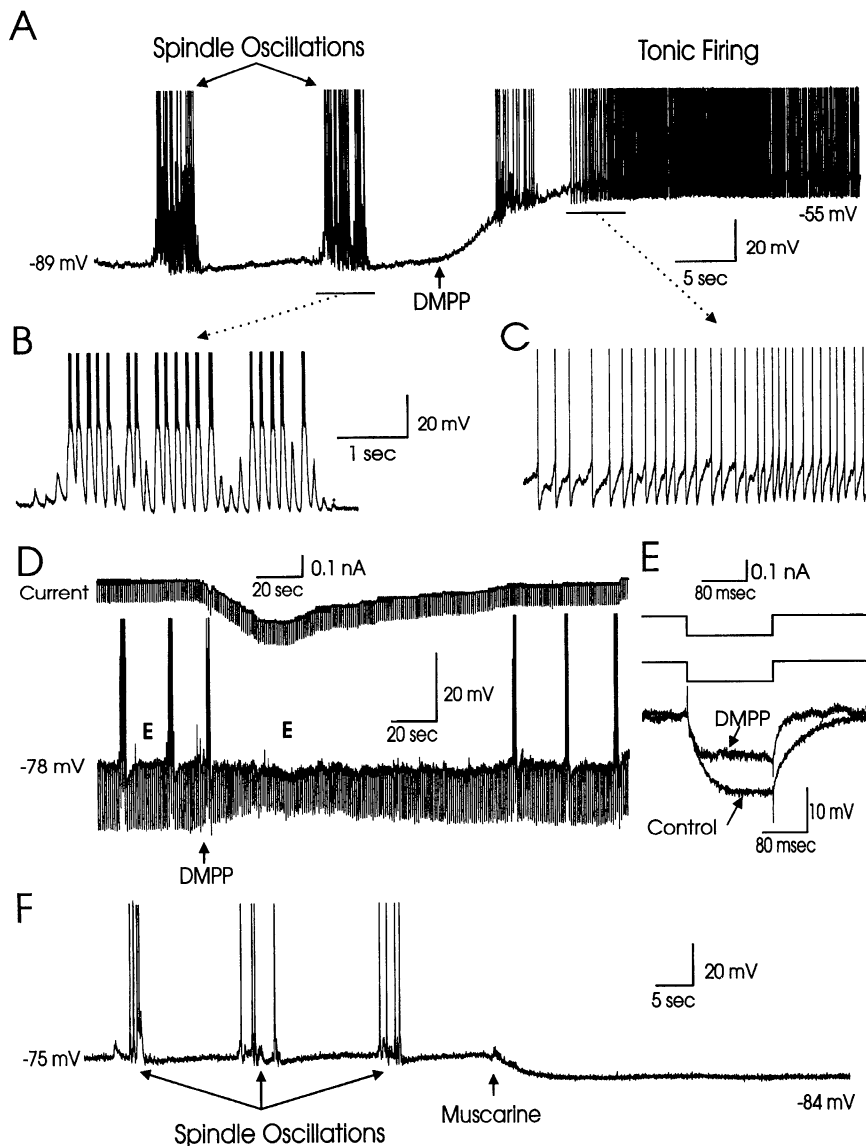


FIG. 3. Pharmacological characterization of receptors mediating responses to ACh in a spontaneously spindling PGN neuron. *A*: local application of the nicotinic receptor agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP) depolarized the PGN neuron, resulting in tonic firing. *B*: expansion of 1 spontaneous spindle oscillation before the application of DMPP. *C*: after the application of DMPP, spindle oscillations are blocked, and the occurrence of tonic activity is seen. *D*: manual voltage clamp of the membrane potential at -78 mV during the local application of DMPP. Examination of the electrotonic membrane response to 150-ms duration, 0.1-nA hyperpolarizing current pulses (expanded in *E*) revealed that DMPP application resulted in an increase in input conductance. As in *A*, DMPP application also caused the cessation of spontaneous spindling. *F*: local application of the muscarinic receptor agonist muscarine chloride hyperpolarized the PGN neuron and stopped spontaneous spindle oscillations.

of both hexamethonium and ($-$)scopolamine, all response to ACh was abolished (Fig. 2*B*). These results indicate that the ACh-induced depolarization is due to the activation of nicotinic receptors, whereas the ACh-induced hyperpolarization is due to muscarinic receptor activation.

To confirm this hypothesis, we applied locally the specific nicotinic receptor agonist 1,1-dimethyl-4-phenylpiperazinium (DMPP; 1.0 mM in micropipette) and the muscarinic receptor agonist DL-muscarine chloride (500 μ M in micropipette) to intracellularly recorded and spontaneously spindling PGN cells. As reported previously, in vitro spindle waves were recorded in the PGN, where they recurred with a regularity of one every 3–30 s (Fig. 3, *A* and *B*) (von Krosigk et al. 1993). Application of nicotinic receptor agonist DMPP to the local region of intracellularly recorded PGN neurons that were spontaneously spindling resulted in a slow membrane depolarization, cessation of spontaneous spindling, and the generation of tonic firing (Fig. 3*A*; $n = 9$). Examination of the electrotonic response to short duration hyperpolarizing current pulses during manual voltage clamp

of the membrane potential with DC following the local application of DMPP revealed an increase in apparent input conductance of 1.9–6.6 nS (Fig. 3, *D* and *E*, $n = 4$). Application of DL-muscarine chloride to spindling PGN cells, on the other hand, resulted in membrane hyperpolarization, cessation of spindle activity (Fig. 3*F*; $n = 4$), and an increase in apparent input conductance of 2.3–10 nS (not shown). The membrane potential, the spontaneous spindles, and the apparent input conductance of the PGN neurons returned to pretreatment levels upon washout of the agonist drugs (e.g., Fig. 3*D*).

These findings support the conclusion that ACh-induced fast excitation is mediated through activation of nicotinic receptors, and ACh-induced slow inhibition is mediated through activation of muscarinic receptors.

DISCUSSION

The results of the present study indicate that the GABAergic neurons of the PGN possess nicotinic receptors that,

when activated, cause a rapid and potent excitation through an increase in membrane conductance similar to nicotinic responses found in selected neurons in the central and peripheral nervous systems and at the neuromuscular junction (e.g., Dennis et al. 1971; Gallagher et al. 1982; McCormick and Prince 1987a,b; Takeuchi and Takeuchi 1960). The presence of strong nicotinic responses as well as the slow muscarinic hyperpolarization following ACh applications to PGN is consistent with receptor autoradiographic techniques that demonstrate this nucleus to possess a high density of both nicotinic (Clarke et al. 1985) as well as muscarinic receptors (Wamsley et al. 1984). The block of ACh depolarizing responses by hexamethonium indicates that the nicotinic receptor in the PGN may be of the ganglionic (vs. neuromuscular) type (Paton and Zaimis 1951), although this feature requires more extensive investigation.

An excitatory response to ACh on nRt neurons was previously suggested by Kayama et al. (1986), where the ionophoretic application of ACh or repetitive stimulation of the LDT was shown to suppress burst discharge and induce tonic firing. However, the cholinergic excitation was suggested to be mediated through muscarinic receptors because the response was blocked by ionophoretic application of scopolamine. We have failed to detect any excitatory response to ACh in nRt/PGN neurons mediated through muscarinic receptors. At present, it is unclear whether the excitatory response studied by Kayama et al. (1986) was due to the activation of nicotinic receptors on nRt neurons.

The cells of the nRt/PGN fire with a bursting pattern of activity during slow-wave sleep, whereas desynchronization of the electroencephalogram (EEG) by waking or by passing into rapid-eye-movement (REM) sleep is associated with tonic discharge (Hirsch et al. 1982; Steriade et al. 1986). In addition, the cholinergic neurons of the ascending reticular activating system increase their rate of spontaneous discharge during wakefulness or REM sleep (Kayama et al. 1992; Steriade et al. 1990). Our present findings suggest that nicotinic activation of PGN neurons may contribute, at least in part, to the cessation of spindle oscillations and the appearance of single spike discharge in nRt/PGN cells. However, the counterbalancing influence of the muscarinic increase in K^+ conductance by ACh (McCormick and Prince 1986) suggests that a burst of activity in ascending or descending cholinergic fibers (Jourdain et al. 1989; Steriade et al. 1987) may be associated with phasic excitation followed by longer periods of decreased excitability.

Electrical stimulation in the brain stem region of the cholinergic input to the PGN in reserpine pretreated and anesthetized cats produces a short-latency depolarization followed by a long-lasting period of hyperpolarization in intracellularly recorded perigeniculate neurons (Hu et al. 1989a). Whereas both in vitro (McCormick and Prince 1986) and in vivo (Hu et al. 1989a) studies have shown that ACh-induced hyperpolarization in PGN is due to a muscarinic receptor-mediated mechanism, the synaptic mechanism responsible for the depolarizing effect of brain stem stimulation remained to be elucidated, although the possibility that it is mediated by nicotinic receptors has been proposed (Steriade et al. 1989). We provide evidence here that the short-latency depolarization observed in PGN cells during brain stem stimulation may indeed be due, at least in part, to the

activation of nicotinic receptors. However, our results do not exclude the possible involvement of glutamate receptors, because glutamate-like immunoreactivity is present within the cholinergic neurons of the laterodorsal tegmental and pedunculopontine nuclei (Clements and Grant 1990).

The mechanism of ponto-geniculo-occipital (PGO) wave generation is critically dependent on the cholinergic input from the brain stem (Hu et al. 1989b) and the activation of nicotinic receptors (Hu et al. 1988). During a PGO wave (Hu et al. 1989b) or in response to electrical stimulation in the peribrachial cholinergic brain stem region (Hu et al. 1989c), LGNd relay neurons are transiently depolarized, and this depolarization is often interrupted by a fast inhibitory postsynaptic potential. Given that the PGN densely and selectively innervate LGNd relay neurons (Bal et al. 1995a,b; Cucchiaro et al. 1991) and the results of the current paper, it seems likely that this inhibitory postsynaptic potential in LGNd relay neurons is generated by nicotinic activation of the PGN. Through these mechanisms the ascending cholinergic system may influence, in both a phasic (e.g., PGO waves) and a tonic (e.g., arousal, REM sleep) manner, the pattern of activity generated in thalamocortical networks.

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