Somatosensory information, conveyed through the gracilis nucleus (GN), is regulated by descending corticofugal (CF) glutamatergic fibers. In addition, the GN receives cholinergic inputs with still unclear source and functional significance. Using both the in vitro slice and intracellular recording with sharp and patch electrodes and in vivo extracellular single-unit recordings, we analyzed the effects of activation of cholinergic receptors on synaptic, intrinsic, and functional properties of rat GN neurons. The cholinergic agonist carbachol (CCh; 1–10 μM) in vitro (1) induced presynaptic inhibition of EPSPs evoked by both dorsal column and CF stimulation, (2) increased postsynaptic excitability, and (3) amplified the spike output of GN neurons. The inhibition by atropine (1 μM) and pirenzepine (10 μM) of all presynaptic and postsynaptic effects of CCh suggests actions through muscarinic M₁ receptors. The above effects were insensitive to nicotinic antagonists. We searched the anatomical origin of the cholinergic projection to the GN throughout the hindbrain and forebrain, and we found that the cholinergic fibers originated mainly in the pontine reticular nucleus (PRN). Electrical stimulation of the PRN amplified sensory responses in the GN in vivo, an effect prevented by topical application of atropine. Our results demonstrate for the first time that cholinergic agonists induce both presynaptic and postsynaptic effects on GN neurons and suggest an important regulatory action of inputs from cholinergic neuronal groups in the pontine reticular formation in the functional control of somatosensory information flow in the GN.

Key words: gracile nuclei; cuneate nuclei; glutamatergic transmission; presynaptic inhibition; M₁ mAChR; somatosensory information; in vitro; in vivo

Introduction

The gracilis nucleus (GN) of the dorsal column (DC) nuclei receives somatosensory information from the hindlimbs throughafferents running in the dorsal columns (Nyberg and Blomqvist, 1982; Rustioni and Weinberg, 1989; DeBiasi et al., 1994). DC fibers are glutamatergic and contact both projecting neurons and inhibitory interneurons (Rustioni and Weinberg, 1989; Broman, 1994; DeBiasi et al., 1994). Stimulation of the peripheral receptive field or electrical stimulation of the DC evokes EPSPs and IPSPs in dorsal column nucleus neurons in vivo (Andersen et al., 1964; Canedo et al., 1998). In addition, dorsal column nuclei receive corticofugal (CF) descending glutamatergic fibers mainly from the sensorimotor cortex (Jabbur and Towe, 1961; Valverde, 1966). Intracellular recordings in an in vitro preparation have shown that the stimulation of CF evokes EPSPs with both NMDA and non-NMDA components, whereas the DC input elicits EPSPs via activation of non-NMDA receptors (Nuñez and Buño, 1999, 2001).

Dorsal column nuclei show acetylcholinesterase activity and acetylcholinesterase-positive fibers (Simon et al., 1981; Avenida and Dykes, 1996), and choline acetyltransferase (ChAT)-positive terminals have been reported in the neuropil (Henderson and Sherriff, 1991). However, the source and functional significance of the cholinergic input to the dorsal column nuclei remains unclear. Cholinergic interaction with glutamatergic transmission (Fernández de Sevilla et al., 2002; Fernández de Sevilla and Buño, 2003) (for review, see Aigner, 1995) is of key importance in the control of normal and pathological behaviors because it can influence forms of synaptic plasticity that underlie learning and memory (Madison et al., 1991; Hasselmo, 1999), and lesions of the cholinergic system may produce cognitive deficits (Hasselmo, 1999). Cholinergic participation in somatosensory control is less understood, although acetylcholine (ACh) amplifies responses at thalamocortical connections. This effect is reduced by disruption of cortical cholinergic activity and blocked by application of atropine (Rasmusson and Dykes 1988; Baskerville et al., 1997; Maalouf et al., 1998) (for review, see Shulz et al., 2003), suggesting actions via muscarinic acetylcholine receptors (mAChR). Cholinergic inputs could also play an important role in the modulation of somatosensory transmission through the
GN, in which forms of short-term plasticity have been described (Nuñez and Buño, 2001).

The aim of the present study was to both characterize the action of the cholinergic agonist carbamylcholine-chloride [carbachol (CCh)] on the intrinsic and synaptic properties of rat GN neurons using an in vitro slice preparation and determine the physiological role of the cholinergic activity by analyzing in vivo the effects of electrical stimulation of descending cholinergic inputs on somatosensory signals in the GN. We report that CCh reduces synaptic responses and increases GN neuronal excitability and spike responses in vitro via activation of postsynaptic and presynaptic M₃, mAChR, respectively. These actions modify the integrative properties of GN neurons facilitating transmission of sustained sensory inputs. In addition, electrical stimulation of cholinergic inputs amplifies somatosensory responses in vivo via activation of muscarinic receptors. The possible origin of cholinergic projections to the GN was mainly from the pontine reticular formation investigated using double labeling with the neuronal solution was in water-immersion objective. Slices were superfused at a rate of 1 ml/min with a 40 scope (BX50WI; Olympus Optical, Tokyo, Japan) equipped with infra-temperature (20 –22°C). Individual slices were transferred to an immersion glucose. In the Mg²⁺-free solution, MgSO₄ was equimodally replaced with CaCl₂.

Sagittal slices (400 μm), cut with a Vibratome (Pelo, St. Louis, MO), were incubated for at least 1 h in carbogen-bubbled ACSF at room temperature (20–22°C). Individual slices were transferred to an immersion recording chamber (2 ml) placed either on the stage of an inverted mi- croscope (Diaphot-TMD; Nikon, Tokyo, Japan) or an upright micro- scope (BX50WI; Olympus Optical, Tokyo, Japan) equipped with infra- and differential interference contrast images and with a 40× water-immersion objective. Slices were superfused at a rate of 1 ml/min with gassed ACSF at room temperature, and total renewal of the bath solution was in ~3 min.

Intracellular recordings with sharp electrodes. The GN neurons were impaled under visual guidance with 90–120 ΜΩ potassium acetate (3 μl) filled micropipettes (pulled with a P87 puller; Sutter Instruments, No- vato, CA). The microelectrode tip was positioned according to the atlas of Paxinos and Watson (1986). Signals were amplified with an Axoclamp 2B (Molecular Devices, Palo Alto, CA), and the traditional bridge current-clamp method was used. Cells were rejected when, in control ACSF, the membrane potential (V_m) fell below −50 mV during the experiment.

Patch-clamp whole-cell recordings. Patch-clamp recordings in the whole-cell configuration were performed using 3–6 ΜΩ fire-polished pipettes (the same pipette puller was used). The internal patch pipette solution contained the following (in μl): 100 potassium chloride; 5.0 EGTA, 10.0 HEPES, 32.5 KCl, 1.0 MgCl₂, 4.0 Na₂-ATP, and 0.4 Na₂-GTP at pH 7.3 (adjusted with KOH). Pipettes were connected to an Axoclamp 2B amplifier (Molecular Devices) or a PC-ONE amplifier (Da- gan, Minneapolis, MN), and cells were recorded in the continuous single-electrode current or voltage-clamp mode. Under voltage clamp, cells were held at a V_m of −60 mV unless otherwise specified. The pipette was positioned in the GN, and neurons were viewed with the Olympus Optical microscope. Fast and slow capacitances were neutralized, and series resistance was compensated (~80%). Patch recordings were re- jected when the access resistance (7–15 MΩ) increased >20% during the experiment. Leak and the linear capacitance current components were subtracted with the Clampex program (Molecular Devices) method. Current pulses, voltage commands, acquisition, and display of the re- corded signals were generated with the Clampex program in both sharp and patch electrode recordings. No significant differences in the effects of CCh were found between sharp and patch electrode current-clamp records, indicating that the intracellular dialysis that occurs with patch electrodes was not affecting the intracellular signal transduction mecha- nisms involved. Once this was established, patch electrodes were used because they enabled voltage-clamp recordings and prolonged current-clamp recordings with favorable signal-to-noise ratios.

Stimulation. A schematic diagram of the localization of the dorsal column nuclei within the slice and of the position of the stimulating and recording electrodes is shown in Figure 1A (left). Electrical stimulation of the DC and CF was through bipolar nichrome electrodes (60 μm diameter) insulated except at the tips and connected to a Grass Instru- ments (Quincy, MA) stimulator triggered by the Clampex program. Sin- gle pairs or barrages of electrical stimuli (0.3 ms, 0.2–1.0 V) applied at the DC or CF (see Fig. 1A, left) induced DC-EPSPs and CF-EPSPs or DC- EPSPs and CF-EPSPs in GN neurons, respectively.

Pharmacology and data analysis. CCh (1–10 μl), atropine (1 μl), a concentration that does not interfere with nicotinic receptors (Alkondon et al., 2000), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (20 μl), tetrodotoxin (TTX) (1 μl), mecamylamine (MMA) (50 μl), and methyllycaconitine (MLA) (0.1 μl) were added to the ACSF and superfused. Recordings were performed under 50 μl picropiricin to block GABA_A inhibitory postsynaptic responses. All chemicals were from Sigma-Aldrich Quimica (Madrid, Spain).

In vivo experiments. Data were obtained from 19 urethane-anesthetized (1.6 g/kg, i.p.) young adult Wistar rats of either sex, weighing 180–250 g. Animals were placed in a stereotaxic device. The body temperature was maintained at 37°C, and the end-tidal CO₂ concentration was controlled. Experiments were performed in accordance with the European Communities Council Di- rective (86/609/EEC).

Tungsten microelectrodes (5 MΩ) were used to obtain single-unit recordings in the GN [anterior (A), −13.6 to −14.6; lateral (L), 0.2–1.0 mm from the bregma; height (H), 0.0–0.5 mm from the surface of the nu- cleus (according to the atlas of Paxinos and Watson, 1986)]. The recording electrode was introduced at a 60° angle to the surface of the nucleus after opening the cisterna magna. The dura mater was removed, and the nucleus surface was covered with mineral oil to prevent drying. Single-unit recordings were filtered (0.3–3 kHz) and amplified via an AC P15 preamplifier (Grass Instruments). Spike amplitude and shape were con- tinuously monitored on-line in an analog oscilloscope. Continuously recorded data were sampled at 8 kHz and fed into a Macintosh computer (Apple Computers, Cupertino, CA) for off-line analysis with Spike 2 software (Cambridge Electronic Design, Cambridge, UK). Recording were performed in control ACSF and during of topic application of at- ropine sulfate (a muscarinic receptor antagonist; 0.5 μl; 1 μl) on the gracilis nucleus. They were applied with a Hamilton microsyringe placed on the gracilis nucleus, tight to the recording place. Chemicals were purchased from Sigma-Aldrich Quimica.

Tactile stimulation was performed by an electronically gated solenoid with a probe of 1 mm in diameter that induced <0.5 mm skin deflection. The control tactile stimulation consisted in pulses lasting 20 ms and delivered at 0.5 Hz, which were directed to a small area of the hindlimb (receptive field).

Electrical stimulation at the caudal pontine reticular nuclei (PRN) (A, −10 mm; L, 1.0 mm; H, 9 mm) was performed by means of bipolar
electrodes, using rectangular pulses (0.3 ms duration, 10–100 μA) at 0.5 Hz or with trains of pulses at 50 Hz during 500 ms.

Summed peristimulus time histograms (PSTHs) (2 ms bins) were calculated using Spike 2 software. The mean tactile response was measured from the PSTH as the number of spikes evoked at 10–50 μs after stimulus onset divided by the number of stimuli. To measure the time course of tactile response, the mean response to five tactile stimuli were calculated before and after reticulotegmental formation stimulation. All data are shown as mean ± SE. Data were compared using Student’s paired test (*p < 0.05; **p < 0.01; ***p < 0.001).

Cholinergic cell groups projecting to the GN: double-labeling experiments. Under general anesthesia (a mixture of 50% ketamine, 40% atropine, and 10% valium, i.p.), rats were injected with 200 nl of CTb (List Biologic, Campbell, CA) in the GN with a 500 nl blunt-point Hamilton syringe. Under microscopic control, the cisterna magna was opened and the dura reflected to introduce the syringe, attached to a micromanipulator, in the gracile nucleus. To control the volume of product injected, CTb was delivered in four successive injections (each 50 nl) separated several minutes to minimize the washout attributable to the flow of CSF. Animals were housed and cared for according to the European Community Council Directive (86/609/EEC) and allowed to survive 7 d. Animals were then perfused transcardially with a mixture of 3% paraformaldehyde, 13% picric acid, and 0.1% glutaraldehyde in 0.1M phosphate buffer. The brains were postfixed overnight in the same fixative and cryoprotected by storing in 30% sucrose solution for frozen sectioning at 40 μm. Sections were collected in three consecutive series to be processed for CTb revealed with Nissl staining and double-labeling procedure. Series processed for CTb were revealed with Intense BL silver enhancement kit (Amersham Biosciences, Piscataway, NJ) after washing in citrate-acetate buffer, pH 5.5; sections were fixed in 5% sodium tiosulate and stored in 0.1 M phosphate buffer before mounting. Sections devoted to double labeling were first processed for CTb and then incubated with 1:100 goat anti-CHAT antiserum (AB144P; Chemicon, Temecula, CA) in a solution containing 20% normal rabbit serum, 5% bovine serum albumin, and 0.5% Triton X-100 in PBS for 36 h; then, sections were incubated in 1:40 rabbit anti-goat IgG and 1:600 goat peroxidase-antiperoxidase in the previous solution for 1.5 h each and revealed with 0.05% 3’-DAB and 0.003% H2O2. Nissl series was used for delimiting structures. Microscopic analysis of sections was under bright-field illumination (Axioskop; Zeiss, Jena, Germany). Only brain sections from animals in which the injections were restricted to the GN were considered (n = 7).

Results

In vitro experiments

The intracellular current-clamp recordings with sharp electrodes included in this study were from 73 GN neurons that showed a resting Vm of −72.7 ± 1.0 mV, an Rm of 48.6 ± 4.3 MΩ, and fired overshooting action potentials with mean peak amplitude of 75.5 ± 1.2 mV. In addition, whole-cell patch-clamp recordings in either current-clamp or voltage-clamp modes of 78 neurons showing Vm of −61 ± 2.0 mV and Rm of 291.1 ± 18.4 MΩ were also included.

CCh modified excitability and intrinsic membrane properties

In current-clamp sharp electrode recordings, CCh depolarized most GN neurons (38 of 52, or 73%) in a dose-dependent manner, as occurs in other systems (Benardo and Prince, 1982; McCormick, 1992; Nuñez et al., 1997; Yajeya et al., 1999; Borde et al., 2000). At CCh concentrations between 1 and 10 μM, the Vm depolarized between 2 and 10 mV, and the average depolarization induced by 10 μM CCh was 6.2 ± 0.7 mV (Fig. 1A, right). In those cells depolarized by CCh (n = 38), the Rm was also increased from 48.6 ± 4.3 to 56.8 ± 5.1 MΩ (16.9% increase; n = 38; p = 0.025) when using sharp electrodes and from 312.8 ± 10.3 to 408.4 ± 12.1 MΩ (a 30.5% increase; n = 20; p = 0.008) when using patch electrodes. Therefore, the I–V relationships, calculated with data obtained in response to hyperpolarizing pulses under current clamp with patch electrodes, displayed steeper slopes under CCh than in control conditions (n = 20 same cells) (Fig. 1B). The I–V relationships were measured from peak voltage values under current clamp at the onset of the hyperpolarization before the initiation of depolarizing sags (Fig. 1B). The linear slopes of the I–V relationships measured in both the control and CCh-treated cells are consistent with CCh acting on voltage-insensitive conductances. Under current clamp, most cells (20 of 31, or 64%) showed a prominent depolarizing sag during hyperpolarizing pulses (i.e., also termed delayed inward rectification) (Fig. 1B, left) that is caused by the activation of the Na+ and K+–mediated Q-current that tends to drive the Vm to more depolarized values (Nuñez and Buño, 1999). Similar I–V relationships were also calculated with values obtained at the end of the hyperpolarizing pulses. These I–V relationships departed from linearity and suggested the activation of a depolarizing conductance during the hyperpolarization (data not shown).

Under voltage clamp, hyperpolarizing pulses from −60 to −100 mV activated the Q-current, as revealed by the gradually activating inward current that was markedly inhibited by CCh (from 100.2 ± 16.6 to 75.3 ± 15.5 pA under 10 μM CCh; a 25% reduction; n = 9; p = 0.003) (Fig. 1C). Superfusion with TTX (1 μM; n = 4) failed to modify the above described effects of CCh (data not shown). Some neurons (n = 10) that were not depolarized by CCh increased the input resistance 5–10%, whereas a few neurons (n = 4) were not affected by CCh; these four cells were not analyzed further. Although CCh always reduced the Q-current under voltage clamp, the depolarizing sag mediated by the Q-current activation could increase in amplitude with the CCh challenge (Fig. 1B), an effect that was probably caused by the marked membrane conductance drop that paralleled the effects of CCh.

The responses to depolarizing current pulses revealed that CCh reduced the current threshold for spike generation from 0.04 ± 0.03 to 0.02 ± 0.01 nA (a 50% reduction; p = 0.02; n = 15) and increased the number of spikes (from 3.1 ± 0.3 to 6.5 ± 0.6; a 109.6% increase; p < 0.001; n = 15) evoked by identical 0.05 nA depolarizing current pulses (Fig. 1B, left). All neurons showed an increased Rm under CCh. Therefore, the significant increase in spike output is caused by the rise in the input resistance. This effect occurred without evident modifications of the delay to the first action potential evoked by depolarizing pulses, which is mediated by the transient K+–mediated A-current (Nuñez and Buño, 1999). The effects of CCh on the membrane resistance and excitability were similar in neurons that showed or lacked the Q-current.

CCh inhibited both DC and CF inputs

We analyzed under current and voltage clamp the effects of CCh on DC-EPSPs and -EPSCs that display a non-NMDA but lack the NMDA component (Nuñez and Buño, 2001). To minimize changes caused by modifications in the driving force, measurements of EPSP amplitudes were at the initial resting Vm, which was set by injecting a hyperpolarizing current if the cell was depolarized by CCh. As occurs in other systems (Hounsagard, 1978; Valentino and Dingledine, 1981; Fernández de Sevilla et al., 2002), bath-applied CCh (10 μM) decreased the DC-EPSP peak amplitude (27 of 38 neurons, or 71%) from 4.1 ± 0.45 to 2.2 ± 0.37 mV (a 46.3% decrease; p = 0.002) (Fig. 2A, left traces). In voltage-clamp conditions, the DC-EPSC was also reduced by 10 μM CCh (38.5% reduction; p < 0.01; n = 10) (Fig. 2A, middle
traces). Synaptic responses were not modified by the CCh challenge in the rest of the neurons (n = 11).

We had shown previously that CF-EPSC displayed both non-NMDA and NMDA components (Núñez and Buño, 2001). We analyzed the effects of CCh on CF-EPSCs under voltage-clamp in control ACSF and in Mg²⁺-free ACSF with 20 µM CNQX. At a V_m of −60 mV in control ACSF, only the non-NMDA component of CF-EPSC was recorded because of the voltage-dependent block by extracellular Mg²⁺ of NMDA receptor channels. The CCh (10 µM) challenge reduced the mean non-NMDA CF-EPSC peak amplitude from 50.6 ± 4.9 pA in the controls to 28.0 ± 3.0 pA under CCh (a 44.4% reduction; n = 10; p = 0.008) as well as the CF-EPSP peak amplitude (Fig. 2B). In Mg²⁺-free ACSF plus CNQX, the block by extracellular Mg²⁺ of NMDA receptor channels is absent and the non-NMDA EPSC component is blocked; thus, an isolated NMDA CF-EPSC component is recorded. In these conditions, the mean amplitude of the isolated NMDA CF-EPSC was reduced from 30.0 ± 2.5 to 16.8 ± 1.9 pA (a 44.1% reduction; n = 7; p = 0.003) (Fig. 2C). These results together suggest that the effects could be mediated by a presynaptic action of CCh because it inhibits to a similar degree the DC-EPSC and both the NMDA and non-NMDA components of CF-EPSPs. However, these results do not completely confirm presynaptically mediated actions.

Finally, we analyzed the dose–response relationship for the cholinergic inhibition of the EPSPs and the increase in the R_C. EPSPs peak amplitude and R_C at different concentrations of CCh (0.5, 1, 5, 10, 20, and 50 µM) were normalized to the control values in each cell, and the normalized values of different cells were averaged and plotted as a function of drug concentration. The plot was well fitted (r² = 0.99 in all cases) to the formalism y = y₀ + a/1 + (x/c)ⁿ, derived from the Hill equation, where c is the IC₅₀. The estimated IC₅₀ values were 1.5, 1.2, and 3.3 µM for the CF-EPSP, DC-EPSP, and R_C respectively (data not shown).

The EPSP amplitude reduction may be underestimated because of the important membrane conductance drop induced by CCh. Therefore, we plotted the amplitude of the EPSPs as a function of R_C, which revealed a negative correlation (r² = 0.95), suggesting a possible underestimation of the action of CCh on EPSP amplitude.

CCh inhibits DC and CF inputs presynaptically

Paired-pulse modulation is a form of short-term presynaptic plasticity characterized by a change in the peak amplitude of the second EPSP (R₂) when it is elicited shortly after (<100 ms) a preceding (R₁) stimulation (Kamiya and Zucker, 1994; Fernández de Sevilla and Buño, 2002; Martin and Buño, 2003) (for review, see Thomson, 2000). Changes in short-term plasticity during paired-pulse stimulation have been recognized as an indication of a presynaptically mediated effect. Therefore, we analyzed the actions of CCh on paired-pulse responses (50–80 ms interval) of both DC- and CF-EPSPs and EPSCs under current and voltage clamp, respectively.

Paired-pulse plasticity of DC-EPSPs was typified by a reduced R₂ EPSP relative to R₁ or paired-pulse depression (PPD). Superfusion with CCh (10 µM) reduced the PPD and, on average, converted it to paired-pulse facilitation (PPF). Indeed, the mean R₂
peak amplitude was 4.5 ± 0.5 mV in control conditions and was reduced by CCh to 3.2 ± 0.1 mV. However, CCh did not modify the amplitude of R2, which was 3.7 ± 0.2 mV in control solution and 3.64 ± 0.3 mV under CCh (data not shown). Therefore, CCh increased the paired-pulse ratio (defined as R2/R1) from 0.83 ± 0.05 in control conditions to 1.12 ± 0.04 under CCh (a 35% increase; p = 0.001; n = 10), a change from PPD to PPF, suggesting a presynaptically mediated effect.

In contrast, the short-term plasticity of CF-EPSPs was characterized by a PPF. The mean control CF-EPSP peak amplitude was 5.5 ± 0.5 mV and CCh inhibited R1 to 3.6 ± 0.4 mV whereas the mean peak amplitude of R2 remained unaltered (6.0 ± 0.5 mV in control and 6.1 ± 0.4 mV under CCh). Therefore, CCh reduced the R1 more than R2, thus increasing the PPF and the paired-pulse ratio from 1.09 ± 0.07 in control conditions to 1.69 ± 0.1 under CCh (a 55% increase; p = 0.001; n = 8; data not shown). Although in control conditions the R1 and R2 differences were not statistically significant under current clamp, they were significant during the CCh challenge and in both control and under CCh in voltage-clamp conditions. These differences could be caused by the conductance increase and the depolarization during R1 that could shunt and reduce the driving force of R2.

Similar results were obtained under voltage clamp in which the PPD that characterized DC-EPSP in control solution, with a mean paired-pulse ratio of 0.81 ± 0.07, was converted to a PPF by CCh, with a paired-pulse ratio of 1.3 ± 0.09 (a 60% increase; p = 0.005; n = 10) (Fig. 3A,B). In addition, the PPF displayed by CF-EPSP in control conditions, with a paired-pulse ratio of 1.15 ± 0.08, was increased by CCh to 1.54 ± 0.06 (a 34% increase; p = 0.006; n = 10) (Fig. 3A,B).

A modification of the mEPSCs frequency is another universally recognized sign of presynaptically mediated effects. Therefore, we measured the changes in mEPSC frequency in the presence of TTX (1 μM). The CCh (10 μM) challenge decreased the mEPSC frequency from 0.91 ± 0.11 Hz in control to 0.44 ± 0.02 Hz under CCh (a 51.6% decrease; n = 8; p = 0.011; Kolmogorov–Smirnov test), a modification that was not paralleled by amplitude changes (18.1 ± 3.3 pA in control and 17.4 ± 2.3 pA under CCh; p < 0.05) (Fig. 3C,D).

The above results together are consistent with CCh acting presynaptically via activation of cholinergic receptors to inhibit transmitter release by reducing release probability at synaptic terminals of both cortical and dorsal column fibers.

\[ M_1 \text{mAChRs mediate the presynaptic and postsynaptic effects of CCh} \]

Preincubation with the wide-spectrum mAChR antagonist atropine (1 μM) prevented all of the effects of 10 μM CCh. The increase in R1m and the excitability, the slow depolarization, and the presynaptic inhibition of DC and CF inputs were all blocked by atropine, suggesting that CCh acted both presynaptically and postsynaptically via activation of muscarinic receptors (Fig. 4A,B,D).

It has been suggested that atropine can also inhibit nicotinic receptors when applied at high concentrations (Alkondon et al., 2000). To test whether nicotinic receptors were involved, we recorded GN neurons during superfusion with MMA (50 μM), the α3β4 nicotinic acetylcholine receptor (nAChR) antagonist, and the α7 nAChR antagonist MLA (0.1 μM). In those conditions, CCh (10 μM) induced a slow depolarization that reached values of 5.6 ± 1.2 mV and increased both the R1m in voltage-clamp recordings (from 290.1 ± 18.8 MΩ in control to 381.1 ± 21.9 MΩ with CCh; n = 9; p < 0.001) and the excitability, indicating that the postsynaptic effects of CCh on GN neurons persisted after blocking nAChRs (Fig. 4A,B).

Under MMA and MLA, CCh also reduced DC- and CF-EPSP amplitudes (4.3 ± 0.5 mV in control to 2.1 ± 0.2 mV under CCh; n = 9; p = 0.015; and 3.1 ± 0.3 mV in control to 1.17 ± 0.1 mV under CCh; n = 9; p = 0.012, respectively) (Fig. 4C,D), and the paired-pulse ratio of both DC-EPSCs and CF-EPSCs were significantly increased from 0.8 ± 0.06 and 1.01 ± 0.04 to 1.21 ± 0.08 and 1.38 ± 0.07 by CCh, respectively (n = 9; p = 0.015 and p = 0.001, respectively; data not shown). These results indicate that nAChRs are not engaged in the presynaptic actions of CCh.

It has been reported that both M1 and M2 mAChRs are found in the pons medulla oblongata (Ge et al., 1995). Therefore, to determine the type of mAChR that mediated the cholinergic effects, we superfused CCh (10 μM) in the presence of the M1 mAChR antagonist pirenzepine and the M2 mAChR antagonist methoctramine, respectively. The effects of CCh on the Vm, excitability, and spike threshold were prevented when pirenzepine (10 μM) was added to the bath (Fig. 4B). In addition, the DC- and CF-EPSP amplitude reductions induced by CCh were prevented by pirenzepine (3.3 ± 1.0 to 3.9 ± 0.94 and 3.4 ± 1.17 to 3.2 ± 1.56 mV, respectively; p > 0.05 in both cases) (Fig. 4C,D). In contrast, the M2 mAChR antagonist methoctramine (10 μM) had no effect on the postsynaptic and presynaptic actions of CCh (Fig.
The above results indicate that both presynaptic and postsynaptic actions of CCh are mediated by M₁ mAChRs.

**Effects of CCh on the spike output in response to synaptic input**

To investigate the possible functional role of the cholinergic regulation of the integrative properties of GN neurons, we analyzed the effect of CCh on the GN neuron spike output in response to suprathreshold synaptic stimulation. EPSPs were elicited with repeated stimulus barrages (10 Hz, 1.9 s at 0.1/s) applied at the DC and CF fibers. In control conditions, DC stimulation evoked a transient response with an initial pair of spikes, followed by subthreshold EPSPs that decreased gradually in amplitude during the stimulation, a “phasic” spike response consistent with EPSP depression and little summation (Fig. 5A, top left). The 10 μM CCh challenge depolarized the GN neurons (6.5 ± 2.1 mV; n = 6) and increased both EPSP summation and the number of spikes evoked by the barrage, resulting in a sustained, more “tonic,” spike response (Fig. 5A, top right). On average, 3.1 ± 1.1 spikes per stimulus were evoked in control conditions, whereas CCh increased the number of spikes to 9.2 ± 0.4 (a 296.7% increase; p = 0.002; n = 6).

The control response evoked by CF stimulation was characterized by initial subthreshold EPSPs that facilitated and summated to evoke an initial burst (two spikes), followed by three spikes separated by silent periods with EPSPs that failed to evoke spikes (Fig. 5B, bottom left). The CCh challenge reduced the amplitude of the initial EPSPs and increased the facilitation and summation of subsequent EPSPs, resulting in a more sustained response with additional spikes and very few failures of EPSPs (Fig. 5B, bottom right). The number of spikes per stimulus was 3.5 ± 0.7 in control condition and increased to 9.6 ± 0.5 with CCh (a 274.2% increase; p = 0.003; n = 6).

We also analyzed the effects of CCh on DC and CF subthreshold synaptic signals. We applied the same synaptic stimulation at lower intensities at DC and CF fibers, both in control conditions and during superfusion of 10 μM CCh (Fig. 5C). The profiles of the control DC and CF responses were different because the DC input showed marked depression with little summation (Fig. 5C, top, Control), whereas the CF input evoked an initial facilitation and summation followed by depression (Fig. 5C, bottom, Control). The CCh challenge reduced the peak amplitudes of both R₁, DC-EPSP (45.6 ± 5.8%; p = 0.005; n = 6) and CF-EPSP (55.2 ± 4.7%; p = 0.004; n = 6). However, the profiles of the effects of CCh on the subsequent responses were markedly different for the DC and CF stimulations (Fig. 5C, CCh, top and bottom). The initial depression of DC-EPSPs in control conditions was changed to facilitation by CCh, whereas the final depression was not much affected (102.5 ± 10.1%; p = 0.08; n = 6). The initial facilitation of CF-EPSPs was increased by CCh, and the final depression was abolished. In addition, CCh increased the temporal summation of DC-EPSP (208.2 ± 15.2%; p = 0.0008; n = 6).

These results show that CCh is able to modify the time course of the responses evoked by stimulation barrages as a result of reducing the first EPSPs of the response but not the last one. Therefore, the temporal summation of the EPSPs estimated by a summation ratio (defined as R₉₀₀₀₉₀₀₀ₒₐₛ₉ₒₐₛ / Rₒₛ₉ₒₐₛ ) was 0.5 ± 0.1 in control and 0.7 ± 0.3 during CCh (DC input, n = 5, p > 0.5, 0.7 ± 0.2 in control and 3.0 ± 0.4 during CCh; for CF input, n = 5, p < 0.001) (Fig. 5D). These results could be explained by the increase in the membrane time constant (τ) for the DC input (τ = R₉ₒₛ₉ₒₐₛ / Cₚₒₛₑ₃ₒₐₛ) and by the increase in both τ and NMDA receptor component in the CF input attributable to the depolarization induced by CCh.

The above results together indicate that activation of M₁ mAChRs modifies both the transfer of somatosensory information and its descending control by favoring the conduction of sustained versus transient signals in the GN.

**Anatomical identification of cells of origin of the cholinergic input**

The anatomical material revealed that the CTb deposits were restricted to the GN, specifically in the GN in all animals selected.
Discussion

Cholinergic receptors are widely distributed in the CNS in which at least five genetically distinct mACHRs (Levey et al., 1991) and 16 genetically different nAChRs (Lukas et al., 1999) are present. In our regions of interest in which presynaptic cholinergic and postsynaptic GN neurons are present, M1 and M3 mACHRs are found (Ge et al., 1995). Consistent with those reports, present results demonstrate that cholinergic agonists regulate neuronal activity in the GN as revealed in vitro by the effects of bath-applied CCh. The CCh challenge induced three major effects: (1) a presynaptic inhibition of CF- and DC-EPSPs, (2) an increase in postsynaptic excitability, and (3) an amplification of the spike responses evoked both by CF and DC stimulation. These effects were mediated by M1-type mACHRs. In addition, electrical stimulation in the PRN in which presynaptic cholinergic neurons are present induces an atropine-sensitive increase in spontaneous spike activity and amplifies somatosensory-evoked responses. These original findings suggest an important action of cholinergic neurons in the PRN in which presynaptic cholinergic neurons are present.
ergic inputs acting via M₁ mAChRs in the regulation of somatosensory information flow at the level of the first synaptic relay station.

Modification of intrinsic membrane properties
Depolarizing muscarinic responses are caused by reductions of potassium conductances via a G-protein-coupled signaling mechanism in other systems (Benardo and Prince, 1982; McCormick, 1992; Borde et al., 1995, 1999; Nuñez et al., 1997; Yajeya et al., 1999). In GN neurons, the CCh-induced depolarization is inhibited by muscarinic antagonists atropine and pirenzepine, indicating that CCh acted via activation of M₁ mAChRs. The CCh challenge was associated with an increased membrane resistance, probably mediated by the inhibition of several K⁺ channel types normally active at the resting V_m. Present results are consistent with CCh inhibiting both voltage-insensitive and voltage-gated conductances. This conclusion is consistent with the I–V relationships calculated early during voltage deflections evoked by hyperpolarizing pulses that were linear both in control conditions and under CCh, suggesting inhibition of a leak channel by CCh. However, late depolarizing sags that increased with hyperpolarization were present, and I–V relationships clearly departed from a linear model during the sags, consistent with the activation of voltage-gated conductances.

The characteristic time course and voltage dependence of the sag evoked by hyperpolarizing pulses (present results) and its block by low concentrations of Cs⁺ (Nuñez and Buño, 1999) are consistent with the activation of the Na⁺- and K⁺-mediated Q-current (also termed i_Q) (Adams and Halliwell, 1982; Edman and Grampp, 1989; McCormick and Pape, 1990). The Q-current was markedly inhibited by CCh, as revealed under voltage clamp, a reduction that could also contribute to increased R_m (present results). These changes in intrinsic properties were paralleled by an increased excitability, as revealed by both a reduced current threshold to evoke spikes and the enhanced spike frequency evoked by identical depolarizing current pulses.

Regulation of synaptic responses
Immunohistochemical studies in the rat show that DC and CF inputs to dorsal column nuclei are glutamatergic (Rustioni and Weinberg, 1989; Broman, 1994; DeBiasi et al., 1994) and that dorsal column nuclei neurons express both AMPA and NMDA receptors (Watanabe et al., 1994; Kus et al., 1995; Popratiloff et al., 1997). Consistent with these findings, we had reported that responses of GN neurons evoked by both DC and CF inputs were glutamatergic and that non-NMDA receptors mediate DC-EPSPs, whereas CF-EPSPs also display an NMDA component (Nuñez and Buño, 1999, 2001).

Present results suggest that the CCh challenge diminished the peak amplitude of both DC- and CF-EPSPs most likely by reducing transmitter release through activation of presynaptic receptors, as suggested by the changes in paired-pulse responses and the decreased mEPSC frequency that parallel the EPSC inhibition (Kamiya and Zucker, 1994; Fernández de Sevilla et al., 2002; Fernández de Sevilla and Buño, 2003). The presynaptic effects of CCh were inhibited by low concentrations of atropine, indicating that CCh acted through the activation of presynaptic muscarinic receptors at both DC and CF terminals, as occurs in other systems (Hounsgaard, 1978; Qian and Saggu, 1997; Alkondon et al., 2002). Therefore, it is likely that similar presynaptic mechanisms mediate the mACHR antagonist pirenzepine but were not affected by the M₂ mAChR antagonist methoctramine, consistent with a presynaptic action via M₁ mAChR activation. In addition, nAChR antagonists did not modify CCh effects, indicating that nicotinic receptors were not engaged in the effects of CCh.

In other systems, the presynaptic muscarinic inhibition is mediated through the block of N-type voltage-gated Ca²⁺ channels by mAChR that act via a G-protein-coupled signaling pathway (Qian and Saggu, 1997). The resulting reduced Ca²⁺ influx inhibits glutamate release by decreasing transmitter release probability (Valentino and Dingledine, 1981; Fernández de Sevilla et al., 2002). Therefore, it is likely that similar presynaptic mechanisms mediate the muscarinic inhibition in the GN.

Morphological study
ChAT-positive fibers and terminals have been reported in the dorsal column nuclei (Simon et al., 1981; Henderson and Sher-
riff, 1991; Avendaño and Dykes, 1996), suggesting an important role of the cholinergic input to the GN in sensorimotor modulation. Cholinergic projections from the laterodorsal and pedunculopontine tegmental nuclei to the pontine tegmentum have been reported in cats and rats (Mitani et al., 1988; Shiromani et al., 1988; Semba et al., 1990). However, the source of the cholinergic innervation described in the GN in the rat remained unclear. In the present results, the number of CTb-positive neurons found to project to the GN nuclei is low, probably because of the superficial location of the GN nuclei and the difficulty of avoiding the washout produced by the flow of CSF, which probably decreased the CTb concentration at the injection site. However, a consistent number of CTb-positive neurons were located in forebrain and brainstem. Interestingly, CTb-positive neurons and double-labeled cells could not be detected in the neuronal Ch5 and Ch6 cholinergic groups described by Mesulam et al. (1983). These results agree with the description that cholinergic neurons of the laterodorsal and pedunculopontine tegmental nuclei project to prosencephalic structures, whereas non-cholinergic neurons project in a descending pattern (Satoh and Fibiger, 1986; Goldsmith and van der Kooy, 1988; Spann and Grofova, 1989; Semba et al., 1990). Our results indicate that the cholinergic projections to the GN arise at the pontine and bulbar reticular formation in which cholinergic cells have also been described (Armstrong et al., 1983; Cuello and Soffroniew, 1984). Therefore, present morphological results indicate that this cholinergic input may originate in brainstem reticular neurons and could underlie the changes in synaptic transmission and neuronal activity described here, as suggested by the effects of their stimulation in vivo.

Functional implications

Present results suggest that cholinergic activity controls the flow of somatosensory signals in the GN by presynaptically inhibiting both ascending DC and descending CF fibers and by postsynaptically regulating the output by increasing the excitability of GN neurons. Interestingly, activation of descending CF fibers also amplifies DC neuronal responses to DC stimulation in vitro (Núñez and Buño, 2001), suggesting that cholinergic activity acting cooperatively with descending glutamatergic CF fibers may potentiate the flow of somatosensory information in the GN. Results obtained in anesthetized rats showed that electrical stimulation of the caudal pontine reticular formation increased the firing rate of GN cells and facilitated sensory responses through activation of cholinergic receptors. Previous recordings of neurons located in the pontine gigantocellular nucleus in behavioral rats showed that these neurons increased the firing rate during waking, waking with movements, and paradoxical sleep (Vertes, 1977). We found that some of these neurons are cholinergic and project to the GN. Thus, it is possible that this increment of cholinergic neuron
activity may facilitate sensory transmission through the GN during waking and paradoxical sleep.

In addition, a diversity of firing modes and response types characterize in vivo recordings of dorsal column nuclei neurons (Andersen et al., 1964; Canedo et al., 1998; Malmierca and Nuñez, 1998; Panetsos et al., 1998; Nuñez et al., 2000). Most of these firing behaviors are absent in the deafferented slice preparation (Nuñez and Buño, 1999, 2001), although low-threshold membrane oscillations could be observed in cultures of dorsal column nuclei neurons (Reboreda et al., 2003). However, these discharge patterns can be reproduced in vitro under muscarinic activation, probably indicating that the natural functional repertoire of GN neurons is under the complex modulation by cholinergic input.

Humans participating in a task requiring alertness and attention display increased regional blood flow in the midbrain reticular formation (Kinomura et al., 1996), in which cholinergic neurons are located. We can speculate that the change in neuronal activity of cholinergic brainstem reticular formation neurons during these cognitive tasks may favor transmission of the relevant information at the first somatosensory relay station, the GN. This hypothesis may be corroborated by the existence of collateral ascending axons from sensory pathways that make synapses with neurons located in the brainstem reticular formation (Scheibel, 1980).

**References**


