Modulation of GABAergic Transmission by Muscarinic Receptors in the Entorhinal Cortex of Juvenile Rats

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Xiao Z, Deng PY, Yang C, Lei S. Modulation of GABAergic transmission by muscarinic receptors in the entorhinal cortex of juvenile rats. J Neurophysiol 102: 659–669, 2009. First published June 3, 2009; doi:10.1152/jn.00226.2009. Whereas the entorhinal cortex (EC) receives profuse cholinergic innervations from the basal forebrain and activation of cholinergic receptors has been shown to modulate the activities of the principal neurons and promote the intrinsic oscillations in the EC, the effects of cholinergic receptor activation on GABAergic transmission in this brain region have not been determined. We examined the effects of muscarinic receptor activation on GABAergic receptor-mediated synaptic transmission in the superficial layers of the EC. Application of muscarine dose-dependently increased the frequency and amplitude of spontaneous inhibitory postsynaptic currents (IPSCs) recorded from the principal neurons in layer II/III via activation of M₃ muscarinic receptors. Muscarine slightly reduced the frequency but had no effects on the amplitude of miniature IPSCs recorded in the presence of tetrodotoxin. Muscarine reduced the amplitude of IPSCs evoked by extracellular field stimulation and by depolarization of GABAergic interneurons in synaptically connected interneuron and pyramidal neuron pairs. Application of muscarine generated membrane depolarization and increased action potential firing frequency but reduced the amplitude of action potentials in GABAergic interneurons. Muscarine-induced depolarization of GABAergic interneurons was mediated by inhibition of background K⁺ channels and independent of phospholipase C, intracellular Ca²⁺ release, and protein kinase C. Our results demonstrate that activation of muscarinic receptors exerts diverse effects on GABAergic transmission in the EC.

INTRODUCTION

The entorhinal cortex (EC) mediates the majority of connections between the hippocampus and other cortical areas (Witter et al. 1989, 2000a) and thus is regarded as the gateway to the hippocampus. Sensory inputs from olfactory structures, parasubiculum, perirhinal cortex, claustrum, and amygdala converge onto the superficial layers (layers II/III) of the EC (Burwell 2000) that give rise to dense projections to the hippocampus; the axons of the stellate neurons in layer II of the EC form the perforant path that innervates the dentate gyrus (Steward and Scoville 1976), whereas those of the pyramidal neurons in layer III form the temporooroammonic pathway that synapses onto the distal dendrites of pyramidal neurons in CA1 and the subiculum (Steward and Scoville 1976; Witter et al. 2000a,b). Moreover, neurons in the deep layers of the EC (layers V/VI) relay a large portion of hippocampal output projections back to the superficial layers of the EC (Dolorfo and Amaral 1998a,b; Kohler 1986; van der Linden and Lopes da Silva 1998) and to other cortical areas (Witter et al. 1989). The EC is part of a network that aids in the consolidation and recall of memories (Dolcos et al. 2005; Hyman et al. 1984; Kotzbauer et al. 2001) and schizophrenia (Arnold et al. 1991; Falkai et al. 1988; Joyal et al. 2002; Prasad et al. 2004). Furthermore, the EC participates in the induction and maintenance of temporal lobe epilepsy (Avoli et al. 2002; Spencer and Spencer 1994).

The cholinergic system plays a fundamental role in cortical function with respect to attention, learning, and memory (Hasselmo 2006). Cholinergic innervation of the cerebral cortex originates mainly from the basal forebrain (Amaral and Kurz 1985; Rye et al. 1984; Shute and Lewis 1967); the EC receives a diffuse cholinergic innervation from the nucleus basalis of Meynert in the basal forebrain, the septum, and the nucleus pallidus, which terminates primarily in layers II and V (Alonso and Köhler 1984; Gaykema et al. 1990; Lewis and Shute 1967; Lysakowski et al. 1989; Mellgren and Srebro 1973; Milner et al. 1983), two layers that gate hippocampal input and output, respectively. Accordingly, activation of cholinergic receptors especially the muscarinic receptors promotes intrinsic oscillation (Dickson and Alonso 1997; Golombiewski et al. 1994; Klink and Alonso 1997b; Konopacki and Golombiewski 1992) and inhibits synaptic transmission (Hamam et al. 2007; Richter et al. 1999). Furthermore, muscarinic receptor activation depolarizes layer II EC neurons (Klink and Alonso 1997a) and generates graded persistent activity (Egorov et al. 2002) by both activating a nonspecific cationic conductance and inhibiting a K⁺ conductance (Shalinsky et al. 2002). However, the effects of cholinergic receptors on GABAergic inhibitory transmission in the EC have not been determined, although the principal neurons in the EC receive extensive inhibitory innervations from local GABAergic interneurons. Here we examined the roles of muscarinic receptors in GABAergic transmission onto the principal neurons in the superficial layers of the EC, and our results demonstrate that activation of muscarinic receptors exerts diverse effects on GABAergic transmission.

METHODS

Slice preparation

Horizontal brain slices (400 μm) including the EC, subiculum, and hippocampus were cut using a vibrating blade microtome (VT1000S; Leica, Wetzlar, Germany) from 13- to 20-day-old Sprague Dawley juvenile rats. J Neurophysiol 102: 659–669, 2009. First published June 3, 2009; doi:10.1152/jn.00226.2009.
that they were mediated by GABA A receptors. Miniature IPSCs evoked at 0.2 Hz by low-intensity stimulation (80–100 μA intensity) via a constant-current isolation unit (A360; World Precision Instrument, Sarasota, FL) connected to a patch electrode filled with oxygenated extracellular solution. Series resistance was rigorously monitored by the delivery of a test pulse at 50 ms to establish a stable resistance. The recorded IPSCs were plotted using a computer program (Mini Analysis 6.0.1, Synaptosoft, Decatur, GA) and the amplitude was determined by fitting the waveform to a Lorentzian function with a 90% confidence interval. The rise time of IPSCs was determined as the time between half-maximal amplitude and half-maximal rise time. The decay time of IPSCs was determined as the time between half-maximal amplitude and half-maximal decay time. The half-maximal amplitude of IPSCs was determined as the amplitude at which the IPSCs were reduced to 50% of the peak amplitude. The half-maximal decay time of IPSCs was determined as the time at which the IPSCs were reduced to 50% of the peak amplitude.

Recordings from the superficial layers of the EC

Whole cell patch-clamp recordings using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) in voltage-clamp mode were made from the principal neurons in layer II/III of the EC visually identified with infrared video microscopy (BX51WI; Olympus, Tokyo, Japan) and differential interference contrast optics (Deng et al. 2006, 2007; Lei et al. 2007; Xiao et al. 2009). The recording electrodes were filled with the following solution (in mM): 100 NaCl, 2 NaHCO3, 3.5 KCl, 1.25 MgCl2, 0.5 CaCl2, 1.25 CsCl, 5.0 MgCl2, and 10 glucose, saturated with 95% O2-5% CO2 (pH 7.4). The extracellular solution contained (in mM) 130 NaCl, 24 NaHCO3, 3.5 KCl, 1.25 NaH2PO4, 0.5 CaCl2, 5.0 MgCl2, and 10 glucose, saturated with 95% O2-5% CO2 (pH 7.4). To record GABA A receptor-mediated spontaneous IPSCs from the principal neurons in the EC using the same internal and external solution at a holding potential of +30 mV (Deng and Lei 2006, 2008; Deng et al. 2006). Under these conditions, the recorded currents were completely blocked by application of 50 μM bicuculline methobromide (10 μM), confirming that they were mediated by GABA A receptors. Miniature IPSCs (mIPSCs) were recorded by including TTX (1 μM) in the preceding external solution to block action potential (AP)-dependent responses. Evoked IPSCs (eIPSCs) were recorded from stellate and pyramidal neurons in the EC using the same internal and external solution at a holding potential of +30 mV by placing a stimulation electrode 100 μm from the recorded cell in layer III. Synaptic responses were evoked at 0.2 Hz by low-intensity stimulation (80–100 μs duration; 10–40 μA intensity) via a constant-current isolation unit (A360; World Precision Instrument, Sarasota, FL) connected to a patch electrode filled with oxygenated extracellular solution. Series resistance was rigorously monitored by the delivery of a test pulse at 50 ms to establish a stable resistance. The recorded IPSCs were plotted using a computer program (Mini Analysis 6.0.1, Synaptosoft, Decatur, GA) and the amplitude was determined by fitting the waveform to a Lorentzian function with a 90% confidence interval. The rise time of IPSCs was determined as the time between half-maximal amplitude and half-maximal rise time. The decay time of IPSCs was determined as the time between half-maximal amplitude and half-maximal decay time. The half-maximal amplitude of IPSCs was determined as the amplitude at which the IPSCs were reduced to 50% of the peak amplitude. The half-maximal decay time of IPSCs was determined as the time at which the IPSCs were reduced to 50% of the peak amplitude.

Dual electrodes recordings from synaptically connected interneuron and pyramidal neuron pairs in the EC

The electrode sealed to interneurons contained the above K+-glutamate intracellular solution, and the electrode sealed to pyramidal neurons was filled with the preceding Cs+-containing intracellular solution except that Cs+-glutamate was replaced with the same concentration of CsCl. Interneuron was held in current-clamp mode and stimulated at a frequency of 0.3 Hz by brief current pulses (duration: 10 ms, amplitude: 0.2–0.25 nA) to initiate APs. Pyramidal neuron was held in voltage-clamp mode (holding potential: −60 mV). The recorded currents were completely blocked by application of biccuculline (10 μM), indicating that they were mediated by GABA A receptors.

Data analysis

Data are presented as the means ± SE. Concentration-response curve of muscarine was fit by Hill equation: \( I = I_{\text{max}} \times \left\{1/[1 + (EC_{50}/[\text{ligand}])]\right\}, \) where \( I_{\text{max}} \) is the maximum response, \( EC_{50} \) is the concentration of ligand producing a half-maximal response, and \( n \) is the Hill coefficient. Student’s paired or unpaired t-test or ANOVA was used for statistical analysis as appropriate; P values are reported throughout the text and significance was set as \( P < 0.05. \) For sIPSC or mIPSC cumulative probability plots, events recorded 5 min prior to and 5 min after reaching the maximal effect of muscarine were selected. Same bin size (25 ms for frequency and 2 pA for amplitude) was used to analyze data from control and muscarine treatment. Kolmogorov-Smirnov test was used to assess the significance of the cumulative probability plots. N, number in the text, represents the numbers of cells examined.

Chemicals

Pirenzepine, AF-DX 116, PD 102807, SKF96365, tertiapin, l-norpinidine, and U73122 were purchased from TOCRIS (Ellisville, MO). Ro318220, GDP-β-S, and GTP-γ-S were from BIOMOL (Plymouth Meeting, PA). 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphorylcholine (edelfosine) was purchased from Calbiochem (Darmstadt, Germany). Muscarine, atropine, McN-A-343, 1,1-dimethyl-4-diphenylacetoxypiperidinium iodide (4-DAMP), bupivacaine, and other chemicals were products of Sigma-Aldrich (St. Louis, MO).
RESULTS

Muscarine increases the frequency and amplitude of sIPSCs recorded from the principal neurons in the EC

Stellate and pyramidal neurons are the two types of principal cells in the superficial layers of the EC. Here we identified these two types of neurons by their morphology and location because previous studies demonstrate that these neurons can be reliably differentiated by these two criteria (Deng and Lei 2007; Lei et al. 2007). Stellate neurons are usually located in layer II or the border of layer II and III, and they have larger and polygonal soma with variable number of main dendrites radiating out from the cell body but are devoid of a clearly dominant dendrite. Pyramidal neurons have a pyramidal or elongated soma with dendrites orientated in a bidirectional way; an apical dendrite running to the surface of the cortex and basal dendrites extending toward the deeper layers. We recorded sIPSCs from both stellate and pyramidal neurons in layer II/III of the EC. Bath application of muscarine (10 μM) significantly increased the frequency (258 ± 39% of control, P = 0.005, Fig. 1A, 1–3) and amplitude (141 ± 11% of control, P = 0.009, Fig. 1A4) of sIPSCs in eight of eight stellate neurons examined. Similarly, application of muscarine (10 μM) significantly increased the frequency (230 ± 34% of control, P = 0.008, Fig. 1B, 1–3) and amplitude (136 ± 9% of control, P = 0.009, Fig. 1B4) of sIPSCs in seven of seven pyramidal neurons examined. Because there were indistinguishable differences for muscarine-induced increases in sIPSC frequency (P = 0.59, unpaired t-test) and amplitude (P = 0.72, unpaired t-test) recorded from stellate neurons and pyramidal neurons (Fig. 1C), suggesting that the muscarinic effects are not cell-specific, we performed the rest of the experiments on both stellate and pyramidal neurons. The EC50 value was measured to be 0.46 μM (Fig. 1D).

Involvement of M3 receptors

We next examined whether the muscarinic effects on sIPSCs were mediated by activation of muscarinic receptors. Application of atropine (5 μM), a broad-spectrum muscarinic receptor antagonist, did not significantly alter sIPSC frequency (109 ± 9% of control, n = 6, P = 0.38, Fig. 2A) and amplitude (99 ± 4% of control, n = 6, P = 0.89) by itself and completely blocked muscarine-induced increases in sIPSC frequency (98 ± 5% of control, n = 6, P = 0.68, A) and amplitude (99 ± 3% of control, n = 6, P = 0.8), suggesting that muscarine increases sIPSCs via activation of muscarinic receptors. We further probed the roles of muscarinic receptor subtypes in muscarine-induced increases in sIPSCs. Muscarinic receptors comprise the M1-like (M1, M3, M5) and M2-like (M2, M4) receptors (Hammer et al. 1980; Lucas-Meunier et al. 2003). The M1-like receptors are coupled to Gαq/11 and stimulate phospholipase C (PLC) resulting in an increase in intracellular Ca2+ release and activation of protein kinase C (PKC), whereas the M2-like receptors are coupled to Gαi proteins leading to an inhibition of protein kinase A and a reduction in cyclic AMP (Caulfield and Birdsell 1998; Lucas-Meunier et al. 2003). Pirenzepine was used to differentiate these two families of muscarinic receptors because this compound selectively blocks M1-like receptors (Caulfield and Birdsell 1998; Lucas-Meunier et al. 2003). Pretreatment of slices with and continu-
Muscarine slightly depresses mIPSC frequency and markedly inhibits the amplitudes of eIPSCs

We next examined the effects of muscarine on mIPSCs recorded in the presence of TTX (1 μM). Application of muscarine (10 μM) did not increase but slightly reduced mIPSC frequency (85 ± 5% of control, n = 7, P = 0.02, Fig. 3, A–C). The amplitude of mIPSCs was not significantly changed after application of muscarine (95 ± 3% of control, n = 7, P = 0.1, Fig. 3D), suggesting that muscarine has no effects on postsynaptic GABAergic inputs. Application of muscarine (10 μM) did not increase but instead remarkably reduced the amplitude of eIPSCs (17 ± 5% of control, n = 5, P < 0.001, Fig. 3E), consistent with previous results (Salgado et al. 2007). Muscarine-induced depression of eIPSC amplitude was blocked by co-application of 4-DAMP (2 μM, 101 ± 5% of control, n = 6, P = 0.81, Fig. 3F), suggesting that the effect of muscarine on eIPSCs is mediated via activation of M3 receptors. This phenomenon (increases in sIPSCs but reduction in eIPSCs) has also been observed for norepinephrine in the hippocampus (Madison and Nicoll 1988), nicotine in striatum (Liu et al. 2007), and 5-HT in the EC (Deng and Lei 2008).

We then used dual electrodes and recorded simultaneously APs from an interneuron and eIPSCs from a pyramidal neuron in synaptically connected interneuron and pyramidal neuron pairs in layer III of the EC. The electrode sealed to interneuron (n = 30) showed pronounced afterhyperpolarization. As demonstrated previously (Deng and Lei 2008), there are two types of interneurons in layer III of the EC. Type I interneurons showed typical rebound burst firing, whereas type II interneurons displayed prominent voltage sag in response to hyperpolarizing current injection with no rebound burst firing. The electrode sealed to pyramidals showed marked sag in response to hyperpolarizing current injection with no rebound burst firing.
injection with rebound burst firing (Deng and Lei 2008). Under these conditions, bath application of muscarine (10 μM) significantly reduced the amplitudes of eIPSCs (49 ± 6% of control, n = 4 pairs, P = 0.004, Fig. 3, G and H). Examination of the APs simultaneously recorded from interneurons showed that muscarine significantly depolarized the resting membrane potentials (RMPs; control: −64.8 ± 1.3 mV, muscarine: −61.2 ± 1.5 mV, n = 4, P = 0.02, paired t-test, Fig. 3G) and reduced the amplitudes of APs (control: 116.3 ± 10.3 mV, muscarine: 106.4 ± 12.4 mV, n = 4, P = 0.0006, paired t-test, Fig. 3, G and I). Together, these results suggest that a reduction of presynaptic AP amplitudes may contribute to muscarine-mediated depression of eIPSCs in the EC.

Muscarine increases the excitability of GABAergic interneurons in the EC

The preceding results suggest that muscarine exerts distinct effects on sIPSCs, mIPSCs, and eIPSCs. Both sIPSCs and eIPSCs are AP-dependent, whereas mIPSCs are not. We next determined the effects of muscarine on the firing frequency of APs recorded from identified GABAergic interneurons in layer III of the EC. Layer III interneurons did not show spontaneous firing. We therefore persistently injected minimal positive current to induce firing. Under these circumstances, the firing frequency (103 ± 15% of control, n = 5, P = 0.83) and amplitude (98 ± 2% of control, n = 5, P = 0.36, data not shown) of the APs were not significantly changed after continuous recordings for 35 min. However, bath application of muscarine (10 μM) significantly increased the frequency (104 ± 349% of control, n = 7, P = 0.04, Fig. 4, A and B) but decreased the amplitudes (91 ± 12% of control, n = 7, P = 0.02.). Among the seven interneurons recorded, there were four type I and three type II interneurons. Because each interneuron responded to muscarine, we pooled the data obtained from the types I and II interneurons for the rest of the experiments.

We then recorded muscarine-induced changes in RMPs in current-clamp mode from layer III interneurons in the extracellular solution containing TTX (0.5 μM) to block potential indirect effects from synaptic transmission. A negative current (−50 pA for 500 ms) was injected every 5 s to assess the changes of input resistance induced by muscarine (Fig. 4C).

Under these circumstances, application of muscarine (10 μM) generated membrane depolarization (control: −65.1 ± 1.5 mV, muscarine: −61.6 ± 1.0 mV, n = 5, P = 0.008, Fig. 4, C and D) and increased the input resistance (control: 341 ± 52 MΩ, muscarine: 421 ± 60 MΩ, n = 5, P = 0.006, Fig. 4, C and D), suggesting that muscarine reduces membrane conductance.

We also examined muscarine-induced depolarization by recording the HCs at −60 mV in voltage-clamp mode in the extracellular solution containing TTX (1 μM). Application of muscarine (10 μM) induced an inward HC (−18.9 ± 2.9 pA, n = 9, P < 0.001, Fig. 4E).

Muscarine-induced increases in inward HCs were blocked when 4-DAMP (2 μM) was applied (−1.4 ± 2.3 pA, n = 6, P = 0.57, Fig. 4E), indicating that

**Fig. 3.** Muscarine slightly depresses miniature IPSC (mIPSC) frequency and markedly inhibits the amplitudes of evoked IPSCs (eIPSCs) by extracellular stimulation and depolarization of GABAergic interneurons in synaptically connected interneuron and pyramidal neuron pairs. A: mIPSCs recorded from a neuron before and during the application of muscarine (10 μM) in the presence of TTX (1 μM). B: pooled time course of mIPSC frequency (n = 7). Note that muscarine slightly inhibited mIPSC frequency. C: cumulative probability of mIPSC frequency before and during the application of muscarine (n = 7). D: cumulative probability of mIPSC amplitude before and during the application of muscarine (n = 7). E: application of muscarine (10 μM) decreased eIPSC amplitude (n = 5). Inset: the current averaged from 10 traces before, during, and after application of muscarine. Note that muscarine reversibly depressed eIPSC amplitude. F: application of 4-DAMP (2 μM) blocked muscarine-induced depression of eIPSC amplitude (n = 6). G: application of muscarine (10 μM) reduced the amplitudes of AP recorded from interneurons and eIPSCs recorded from pyramidal neurons in interneuron-pyramidal neuron pairs. Top: averaged trace from 20 individual action potentials (APs) prior to (left) and during (right) the application of muscarine (10 μM) recorded from an interneuron. Inset: the enlarged overlay of the 2 APs. Note that application of muscarine induced depolarization and slightly reduced the amplitude of AP. Middle panel: Superimposed IPSCs evoked by individual APs before (left) and during (right) the application of muscarine. Note that application of muscarine reduced the amplitude of the IPSCs and increased the frequency of spontaneous IPSCs. Bottom: averaged IPSC before (left) and during (right) the application of muscarine. H: summary time course of muscarine-mediated inhibition of IPSC amplitudes from 4 interneuron-pyramidal neuron pairs. I: summarized AP amplitude recorded from interneurons before and during the application of muscarine (n = 4 pairs).
muscarine depolarizes entorhinal interneurons via activation of M₃ receptors.

**Muscarnine induces membrane depolarization by inhibiting background K⁺ channels in interneurons**

We then tested whether muscarine depolarizes GABAergic interneurons by opening a cationic conductance. If this is the case, the extracellular Na⁺ and Ca²⁺ should be the major cations to mediate membrane depolarization. We initially replaced the extracellular NaCl with the same concentration of NMDG-Cl. In this condition, application of muscarine (10 μM) still induced a comparable inward HC (control: −18.9 ± 2.9 pA, n = 9; NMDG: −18.0 ± 2.1 pA, n = 8, P = 0.81, unpaired t-test, Fig. 4F). Furthermore, omission of extracellular Ca²⁺ failed to change significantly muscarine-induced increases in inward HCs (control: −18.9 ± 2.9 pA, n = 9; 0 Ca²⁺: −19.4 ± 4.0 pA, n = 6, P = 0.92, unpaired t-test, Fig. 4G). Finally, muscarine-induced increases in inward HCs were insignificantly changed (versus muscarine alone, Fig. 4H) when the extracellular solution contained other cation channel blockers: Gd³⁺ (10 μM, −20.8 ± 2.3 pA, n = 5, P = 0.66), La³⁺ (10 μM, −18.8 ± 2.5 pA, n = 6, P = 0.98), and SKF96365 (50 μM, −18.6 ± 0.8 pA, n = 6, P = 0.93). These results together suggest that muscarine-induced depolarization is not mediated via activation of a cationic conductance.

We then tested the hypothesis that muscarine inhibits a resting K⁺ conductance to generate membrane depolarization. If so, the muscarine-induced currents should have a reversal potential close to the K⁺ reversal potential. We used a ramp protocol (from −120 to −60 mV, at a speed of 0.1 mV/ms) to construct the voltage-current curve before and during the application of muscarine (10 μM). The extracellular solution was supplemented with (in μM) 1 TTX, 100 Cd²⁺, 100 Ni²⁺, 10 DNQX, 50 D-APV, and 10 bicuculline to block synaptic current and other voltage-gated ion channels. Under these conditions, muscarine induced a current which had a reversal potential (−88.5 ± 2.9 mV, n = 5) close to the calculated K⁺ reversal potential (−92.2 mV) in our recording conditions (Fig. 5A), suggesting that muscarine induces membrane depolarization by inhibiting resting membrane K⁺ channels. This result was also consistent with the previous data showing that muscarine increased the input resistance (Fig. 4, C and D).

We next tried to characterize the properties of the involved K⁺ channels. Muscarine-induced increase in inward HCs recorded from interneurons in the EC was not significantly changed (vs. muscarine alone, Fig. 5B) by application of TEA (10 mM, −17.4 ± 3.2 pA, n = 9, P = 0.73), Cs⁺ (3 mM, −19.5 ± 1.9 pA, n = 9, P = 0.89), or 4-aminopyridine (4-AP, 2 mM, −21.8 ± 3.8 pA, n = 6, P = 0.55), suggesting that muscarine-mediated inhibition of K⁺ channels is insensitive to the classic K⁺ channel blockers. Moreover, application of tertapain (50 mM), an inward rectifier K⁺ channel inhibitor, failed to change significantly muscarine-induced increases in inward HCs (−19.0 ± 2.8 pA, n = 7, P = 0.98 versus muscarine alone, Fig. 5B) suggesting that muscarine-induced membrane depolarization is unlikely to be mediated by the inward rectifier K⁺ channels although this type of K⁺ channels is involved in the modulation of RMPs. The muscarinic effect was unlikely to be mediated by M-type K⁺ channels because application of linopirdine (10 μM), a
selective M-channel blocker, failed to change muscarine-induced increases in inward HCs significantly (−19.7 ± 2.9 pA, n = 6, P = 0.86 vs. muscarine alone, Fig. 5B) and the voltage-threshold for M-channel activation is positive to −60 mV rendering this type of channels less likely to be involved in controlling RMPs.

Because the two pore-domain K+ channels (K_{2p}) are involved in controlling RMPs and they are insensitive to the classic K+ channel blockers (TEA, 4-AP, CsCl), we next examined the roles of K_{2p} in muscarine-induced membrane depolarization in GABAergic interneurons. The family of K_{2p} channels includes TWIK, THIK, TREK, TASK, TALK, and TRESK (Bayliss et al. 2003; Lesage 2003), some of which are sensitive to Ba^{2+} and bupivacaine. We therefore tested the roles of Ba^{2+} and bupivacaine in muscarine-induced membrane depolarization. Application of Ba^{2+} (3 mM) alone induced an inward HC (−28.2 ± 5.2 pA, n = 9, P < 0.001, Fig. 5C) by itself, suggesting that Ba^{2+}-sensitive K+ channels have a significant role in controlling RMPs. However, muscarine-induced increases in inward HCs were not significantly changed in the presence of Ba^{2+} (−13.7 ± 3.3 pA, n = 9, P = 0.25 vs. muscarine alone, unpaired t-test, Fig. 5C). Likewise, bath application of bupivacaine (200 μM) induced an inward HC (−11.1 ± 1.7 pA, n = 10, P < 0.001, Fig. 5D) and failed to alter muscarine-induced increases in inward HCs (−14.0 ± 3.7 pA, n = 10, P = 0.32 vs. muscarine alone, unpaired t-test, Fig. 5D).

Signal transduction mechanism

Our results demonstrated that muscarine modulates GABAergic transmission by activating M₃ receptors on GABAergic interneurons. Activation of M₃ receptors is coupled to Go_{α/11} resulting in activation of PLC that further generates two intracellular messengers, IP₃ to increase intracellular Ca^{2+} release and diacylglycerol to activate PKC. We therefore examined the role of this pathway in muscarine-induced depolarization in interneurons. We first tested whether G-proteins were involved in muscarine-induced increases in inward HCs. We replaced GTP in the intracellular solution with GDP-β-S (4 mM), a G protein inactivator, and recorded HCs from interneurons in layer III. Application of muscarine (10 μM) in the presence of GDP-β-S significantly reduced muscarine-induced inward HCs (−4.7 ± 0.9 pA, n = 8, P < 0.001 vs. muscarine alone, unpaired t-test, Fig. 6A). Furthermore, intracellular application of GTP-γ-S (0.5 mM), a nonhydrolysable GTP analogue, induced an inward HC by itself (−59.0 ± 21.3 pA, n = 6, P = 0.04, Fig. 6B) and significantly reduced muscarine-induced increases in inward HCs (n = 10).

FIG. 5. Muscarine generates membrane depolarization in entorhinal interneurons via inhibition of background K⁺ channels. A: voltage-current relationship recorded by a ramp protocol (from −120 to −60 mV, at a speed of 0.1 mV/ms) before and during the application of muscarine (10 μM) when extracellular K⁺ concentration was 3.5 mM. Traces in the figure were averaged traces from 5 cells. Muscarine-induced net current had a reversal potential of −88.5 ± 2.9 mV (n = 5), close to the calculated K⁺ reversal potential (−92.2 mV). Inset: the enlarged net current induced by muscarine. B: muscarine-induced increases in HCs were insensitive to TEA (n = 9), CsCl (n = 9), 4-AP (n = 6), tertiapin (n = 7), and linopirdine (n = 6). C: bath application of Ba^{2+} (3 mM) induced an inward HC but did not significantly change muscarine-induced increases in inward HCs (n = 9). D: bath application of bupivacaine (200 μM) induced an inward HC but failed to change significantly muscarine-induced increases in inward HCs (n = 10).

FIG. 6. Muscarine-induced depolarization of entorhinal interneurons is G protein dependent but independent of PLC, intracellular Ca^{2+} release, and PKC activity. A: intracellular application of GDP-β-S (4 mM) via the recording pipettes significantly reduced muscarine-induced increases in inward HCs (n = 8). B: intracellular application of GTP-γ-S (0.5 mM) induced an inward HC by itself and significantly reduced muscarine-induced increases in inward HCs (n = 6). C: pretreatment of slices with and co-application of edelfosine (20 μM) for 2 h failed to block muscarine-induced enhancement of inward HC (n = 5). D: pretreatment of slices with and continuous bath application of Ro318220 (1 μM) did not significantly change muscarine-induced increases in inward HCs (n = 5).
increases in inward HCs (−3.7 ± 0.9 pA, n = 6, P = 0.001 vs. muscarine alone, unpaired t-test, Fig. 6B). Together these results suggest that G-proteins are involved in muscarine-induced increases in inward HCs.

Because activation of M3 receptors increases PLC activity, we next tested whether PLC was required for muscarine-induced membrane depolarization. Slices were pretreated for >2 h with U73122 (20 μM), a PLC inhibitor, and the bath was continuously perfused with the same concentration of U73122. However, application of U73122 failed to change significantly muscarine-induced increases in inward HCs (−19.3 ± 2.7 pA, n = 5, P = 0.94 vs. muscarine alone, unpaired t-test, Fig. 6C).

Similar treatment of slices with edelfosine (20 μM), another PLC inhibitor, did not alter muscarine-induced increases in HCs (−25.4 ± 4.6 pA, n = 8, P = 0.24 versus muscarine alone, unpaired t-test, Fig. 6D). We also examined the requirement of intracellular Ca2+ release and PKC in muscarine-mediated depolarization of interneurons. Intracellular dialysis of BAPTA (20 mM) via the recording pipettes failed to change significantly muscarine-induced increases in inward HCs (−17.0 ± 4.3 pA, n = 5, P = 0.72 vs. muscarine alone, Fig. 6E). Pretreatment of slices with and continuous bath application of Ro318220 (1 μM), a selective PKC inhibitor, did not significantly alter muscarine-induced increases in inward HCs (−19.1 ± 4.2 pA, n = 5, P = 0.96 vs. muscarine alone, Fig. 6F). These results suggest that muscarine-mediated depolarization in entorhinal interneurons is independent of intracellular Ca2+ release and PKC activity.

**Discussion**

Whereas the effects of muscarine on principal neurons and neural network activity in the EC are under extensive investigations, its potential actions on GABAergic transmission remain unexplored. Our results demonstrate for the first time that activation of muscarinic receptors modulates GABAergic transmission in the EC in distinct modes; activation of M3 muscarinic receptors increases sIPSC frequency and amplitude but inhibits the amplitude of eIPSCs and the frequency of mlPSCs with no effects on the amplitude of mlPSCs. Muscarine increases the firing frequency but reduces the amplitude of APs. Muscarine depolarizes GABAergic interneurons by inhibiting background K+ channels that are insensitive to the classic K+ channel blockers (TEA, Cs+, 4-AP, tertiapin), Ba2+, and bupivacaine. Muscarine-induced modulation of GABAergic transmission requires the function of G proteins but is independent of PLC, intracellular Ca2+ release and PKC activity.

We have shown that bath application of muscarine profoundly increases sIPSC frequency and amplitude but inhibits the amplitudes of IPSCs evoked by extracellular stimulation or depolarization of GABAergic interneurons in synaptically connected interneuron and pyramidal neuron pairs. Muscarine slightly inhibits the frequency of mlPSCs recorded in the presence of TTX with no effects on mlPSC amplitude. The result that muscarine does not modulate mlPSC amplitude indicates that activation of muscarinic receptors does not modulate postsynaptic GABA_A receptors. Muscarine has been shown to reduce GABA release in rat auditory cortex (Salgado et al. 2007), lateral amygdala, nucleus accumbens, and striatum (Sugita et al. 1991). However, these studies were conducted by record-
GABA release, demonstrated by increased sIPSC frequency and amplitude, via activation of cation channels although Ca\(^{2+}\) influx via either cation channels per se or subsequent activation of voltage-gated Ca\(^{2+}\) channels due to the membrane depolarization induced by cation channel openings could facilitate GABA release. First, if the effect of muscarine on GABA release is mediated via activation of cation channels, application of muscarine should increase mIPSCs recorded in the presence of TTX because under this circumstance Ca\(^{2+}\) influx via the activated cation channels or voltage-gated Ca\(^{2+}\) channels should increase GABA release. We clearly showed that muscarine slightly depressed mIPSC frequency with no effects on mIPSC amplitude. Second, replacement of extracellular Na\(^{+}\) with NMDG or omission of extracellular Ca\(^{2+}\) failed to change muscarine-induced increases in inward HCs recorded from GABAergic interneurons. Third, application of the selective M-type K\(^{+}\)/H11001 channel inhibitor, failed to change muscarine-induced depolarization in interneurons, or Ca\(^{2+}\) influx resulting in activation of PLC. We have shown that the muscarine-induced membrane depolarization in interneurons is dependent on G proteins but independent of PLC, intracellular Ca\(^{2+}\) release, and PKC. Our results support an action mode in which activation of muscarinic receptors depolarizes entorhinal interneurons via intracellular signals other than PLC or a direct interaction of G proteins with K\(_{\text{ip}}\) channels. Consistent with our results, G\(_{\text{q}}\)-mediated inhibition of K\(_{\text{ip}}\) channels is mediated via either intracellular signaling molecules (Chemin et al. 2003; Kang et al. 2006; Mathie 2007; Veale et al. 2007) or direct G protein coupling (Chen et al. 2006; Deng et al. 2006) depending on the involved types of K\(_{\text{ip}}\) channels. Specifically, in line with our results, application of U73122, a PLC inhibitor, failed to block muscarine-induced depolarization in neonatal rat cerebellar granule cells (Boyd et al. 2000).

GABAergic interneurons synchronize neural network activities and serve as the precision clockwork for gamma and theta oscillations. Neural oscillatory events are thought to be crucially involved in different sleep states, in various cognitive processes including selective attention, and in the encoding and binding of information for associating features into unified perceived objects at the cortical level (Freund 2003). Furthermore, oscillation and synchronization of neural activity is also important for epileptogenesis (Tolner et al. 2005). Muscarinic receptor activation in the EC promotes intrinsic oscillations both in vivo (Alonso and Garcia-Austt 1987a,b; Dickson et al. 1995; Mitchell and Ranck 1980) and in vitro (Klink and Alonso 1997a,b). Muscarinic receptor-induced synchronization of neural activity in the EC requires concurrent glutamatergic and GABAergic synaptic inputs (Dickson and Alonso 1997). However, the roles of muscarinic receptor activation on GABAergic function in the EC have not been explored previously. Our results clearly filled this gap and suggest that muscarinic modulation of GABAergic transmission in the EC may contribute to the synchronous events induced by muscarinic receptor activation. Moreover, synchronized synaptic potentials observed in epileptic human mesial temporal lobe tissue were also found to be dependent on fast GABAergic transmission (Schwartzkroin and Haglund 1986) suggesting that the epileptogenic synaptic events are probably the compound action of both glutamatergic-mediated excitatory postsynaptic potentials and GABAergic-mediated inhibitory postsynaptic potentials (Schwartzkroin and Knowles 1984). Similar data have been reported during perfusion of hippocampal slices with 4-AP (Rutecki et al. 1987), and simultaneous firing of interneurons and principal cells has been described in the high potassium model of epilepsy in the hippocampus in vitro (McBain 1994), as well as during paroxysmal events in vivo (Bragin et al. 1995; Bragin et al. 1997; Steriade and Contreras 1995; Steriade et al. 1994). Our results therefore provide a cellular and molecular mechanism that could explain the roles of muscarinic receptors in epilepsy as well.