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Contribution of muscarinic M_1 receptors to the cholinergic suppression of synaptic responses in layer II of the entorhinal cortex

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HIGHLIGHTS

- Cholinergic inputs suppress excitatory synaptic transmission in entorhinal cortex.
- Pirenzepine, an M_1-preferring antagonist, blocks the cholinergic suppression.
- Methoctramine, an M_2-preferring antagonist, does not block the suppression.
- A selective M_1 blocker, but not an M_4 blocker, reduces the suppression.
- Results are consistent with a primary role of M_1 receptors in the suppression.

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ABSTRACT

The entorhinal cortex is thought to play roles in sensory and mnemonic function, and the cholinergic suppression of the strength of synaptic inputs is likely to have important impacts on these processes. Field excitatory postsynaptic potentials (fEPSPs) in the medial entorhinal cortex evoked by stimulation of the piriform cortex are suppressed during theta EEG activity in behaving animals, and cholinergic receptor activation suppresses synaptic responses both *in vivo*, and in layer II entorhinal neurons *in vitro*. Here, we have used *in vitro* field potential recordings to investigate the transmitter receptors that mediate the cholinergic suppression of synaptic responses in layer I inputs to layer II of the medial entorhinal cortex. Bath-application of the cholinergic agonist carbachol suppressed the amplitude of fEPSPs with an EC_{50} of 5.3 μM, and enhanced paired-pulse ratio. The M_2/M_4 preferring receptor blocker methoctramine, or the M_4 receptor blocker PD102807, did not prevent the cholinergic suppression. However, the M_1/M_4 receptor blocker pirenzepine and the M_1 receptor blocker VU0255035 reduced the suppression, suggesting that the cholinergic suppression of synaptic responses in the entorhinal cortex is dependent in large part on activation of M_1 receptors.

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Neurons in layer II of the entorhinal cortex receive synaptic inputs from sensory and associational regions, and they also provide the hippocampal formation with its largest cortical sensory input [3]. These strong interconnections suggest that the entorhinal cortex plays important roles in the sensory and mnemonic functions of the medial temporal lobe [3,19]. There has also been a growing interest in the role of the entorhinal cortex in spatial navigation because of the discovery of spatial "grid cells" in the medial entorhinal cortex [7]. During periods of behavioral mobility, the entorhinal cortex and hippocampus generate theta-frequency (4–12 Hz) EEG activity that is promoted by septal cholinergic inputs [8]. Theta activity helps to coordinate firing among entorhinal neurons [1], modulates transmission through the hippocampal formation [22], and is thought to promote learning-related synaptic plasticity [15,27].

In contrast to the excitatory effects of acetylcholine on membrane potential and neuronal excitability [2,11], cholinergic inputs to the entorhinal cortex and hippocampus generally result in a suppression of excitatory synaptic transmission [14,16,21,27]. Synaptic responses in layer II of the entorhinal cortex are suppressed during theta activity *in vivo*, and cholinergic agonists suppress entorhinal EPSPs both *in vivo* and *in vitro* [14]. Similar suppression effects are observed in entorhinal layers III and V [5,27] and in layer V inputs to layer II of medial entorhinal cortex [21]. The cholinergic suppression of synaptic strength may serve to offset hyperexcitability associated with cholinergic depolarization [12], enhance the relative salience of active synaptic inputs, or may affect which synaptic inputs may contribute to learning-related synaptic plasticity [16].

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Although the cholinergic suppression of EPSPs has usually been attributed to M_1 receptors, there have been several reports that support the involvement of M_2 and M_4 receptors. The cholinergic suppression of EPSPs in layer V inputs to layer II of the entorhinal cortex is blocked by the M_1 receptor antagonist pirenzepine [21], and other reports have supported a role for M_1 receptors in the suppression of EPSPs in the CA1 region [24]. However, the cholinergic suppression of CA1 EPSPs is also effectively blocked by gallamine which has a greater affinity for M_2 *versus* M_1 receptors [10], and while the suppression of CA1 EPSPs is markedly reduced in M_1 receptor knock-out mice, a residual suppression suggests that other receptor subtypes also contribute [18]. Further work has also shown that, although there is an attenuation of the carbachol-induced suppression in M_1 knock-out mice, the cholinergic suppression of EPSPs is completely blocked in M_4 knock-outs [6] indicating that M_4 receptors play a major role in the CA1 region.

In the present study, we used field potential recordings from acute brain slices to examine the muscarinic receptors that mediate the carbachol-induced suppression of fEPSPs in layer I inputs to layer II of the medial entorhinal cortex. The cholinergic suppression of synaptic responses in the entorhinal cortex is known to be associated with reduced transmitter release [5,14,27], but the muscarinic subtypes that modulate the suppression of layer I inputs to layer II have not been determined. Muscarinic receptor blockers with differing affinities were used to assess the involvement of different muscarinic receptor subtypes [4].

1. Methods

1.1. In vitro slice preparation

Acute brain slices were prepared in accordance with the guidelines of the Canadian Council on Animal Care. Slices were obtained from 5- to 7-week-old rats anesthetized with halothane. Brains were submerged in ice-cold ACSF containing (in mM) 2 KCl, 1.4 NaH_2PO_4, 2.7 $MgSO_4$, 0.5 $CaCl_2$, 26 $NaHCO_3$, 10 dextrose, and 250 sucrose saturated with 95% O_2 and 5% CO_2. Horizontal slices (400 μM) were cut using a vibratome (WPI, Vibroslice NVSL), and recovered in room temperature ACSF (~22 °C) containing 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 2 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, 10 dextrose, L-ascorbic acid (0.4 mM), uric acid (0.35 mM) and indomethecine (40 μM) for ≥1.5 h. Slices were transferred to a nylon net in a gas–fluid interface chamber (Fine Science Tools) containing a humidified 95%/5% O_2/CO_2 atmosphere, and perfused with oxygenated ACSF (1.5–2.0 ml/min; 32 ± 0.5 °C).

1.2. Stimulation and recording

Field potential recording electrodes were pulled from borosilicate glass (1.0 mm OD) using a horizontal puller (Sutter Instruments, P97), and filled with ACSF (2–6 MΩ). Electrodes were positioned with the aid of a dissecting microscope (Leica, MS5) in layer I near the border of layer II. Synaptic responses were evoked with a concentric bipolar electrode (FHC) placed in layer I, 0.4–0.8 mm rostral to the recording electrode. Cathodal constant current pulses were delivered using a stimulus generator (WPI, Model A300) and isolation unit (Model A360). Evoked fEPSPs were amplified (DC-3 kHz, Axon Instr., Axoclamp 2B) and digitized using pClamp 8.2 software (20 kHz, Digidata 1322A, Axon Instr.). Stimulation intensities were adjusted to evoke fEPSPs with amplitudes of ~65–75% of the maximal.

Synaptic responses were evoked every 20 s to establish a stable baseline of at least 10 min, followed by 10-min constant bath application of the cholinergic agonist carbachol (1–100 μM), and a 20-min washout period in normal ACSF. Responses in a control

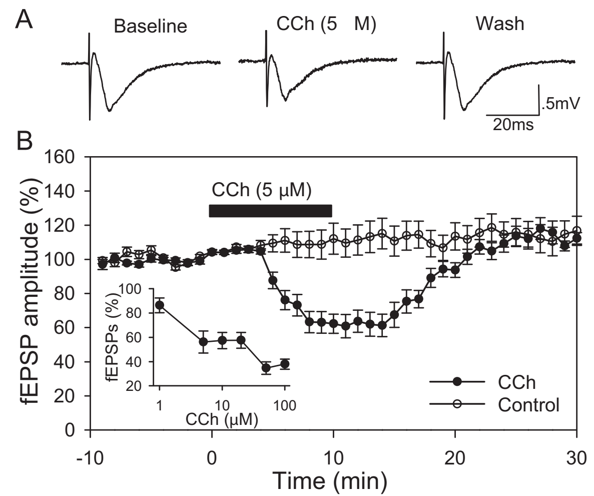

Fig. 1. The cholinergic agonist carbachol (CCh) reduces the amplitude of evoked fEPSPs in the medial entorhinal cortex. (A) Averaged fEPSPs evoked in layer II by layer I stimulation were attenuated by 5 μM carbachol. (B) Carbachol reduced fEPSP amplitude dose-dependently with an EC_{50} of 5.3 μM (inset), and the time-course of changes in fEPSPs induced by 5 μM carbachol (black bar) is shown relative to a control group.

group were recorded without carbachol application. Nonlinear regression analysis using a four-parameter logistic equation determined the EC_{50} of carbachol (SigmaPlot 11.0), and 5 μM carbachol was used in subsequent tests with receptor antagonists. To assess if the cholinergic suppression was due to pre- or post-synaptic mechanisms [14,21], pairs of pulses were administered with an interpulse interval of 30 ms, and paired-pulse facilitation ratio was expressed as the amplitude of the response to the second pulse as a percentage of the amplitude of the response to the first pulse.

Either the M_1 receptor blocker pirenzepine dihydrochloride (1 μM, Ascent Scientific), or the M_2 receptor blocker methoctramine (5 μM, Sigma–Aldrich), was bath applied for 20 min prior to addition of carbachol for 10 min. Pirenzepine can block both M_1 and M_4 receptors at higher doses, and methoctramine blocks both M_2 and M_4 receptors [4,9,26]. Effects of the more selective M_1 receptor antagonist VU0255035 (5 and 10 μM; Eli Lilly and Company) [23] and M_4 receptor antagonist PD102807 (0.5 and 5 μM; Tocris Bioscience) [17] were also determined. Concentrated stock solutions were obtained by dissolving pirenzepine and methoctramine in distilled water, VU0255035 in DMSO (final concentration < 0.1%), and PD102807 in 1% HCl (final concentration 0.003%).

Peak amplitudes of synaptic potentials were measured using pClamp 8.2 software. Effects of carbachol and receptor blockers were assessed using mixed-design ANOVAs and Neuman–Keuls tests based on averaged fEPSPs obtained during the 10 min baseline period, the last 5 min of carbachol application, and the last 5 min of the follow-up period. Averages of ten consecutive evoked responses were obtained for graphical display.

2. Results

2.1. Cholinergic suppression of evoked synaptic responses

Stimulation of layer I resulted in negative synaptic field potentials in layers I–II of the medial entorhinal cortex (*e.g.*, Fig. 1A) and 10-min constant bath application of carbachol suppressed evoked responses reversibly and dose-dependently (1–100 μM; n = 4–7, $F_{5,24}$ = 9.71, p < 0.001; Fig. 1B, inset). One micromolar carbachol suppressed response to 87.2 ± 4.9% of baseline, and 100 μM carbachol suppressed responses to 36.9 ± 3.4% of baseline (−0.26 ± 0.02

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