Muscarinic modulation of GABAergic transmission to neurons in the rat subfornical organ

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Xu, Sheng-Hong, Eiko Honda, Kentaro Ono, and Kiyotoshi Inenaga. Muscarinic modulation of GABAergic transmission to neurons in the rat subfornical organ. Am J Physiol Regulatory Integrative Comp Physiol 280: R1657–R1664, 2001.—Cholinergic actions on subfornical organ (SFO) neurons in rat slice preparations were studied by using whole cell voltage- and current-clamp recordings. In the voltage-clamp recordings, carbachol and muscarine decreased the frequency of GABAergic inhibitory postsynaptic currents (IPSCs) in a dose-dependent manner, with no effect on the amplitudes or the time constants of miniature IPSCs. Meanwhile, carbachol did not influence the amplitude of the outward currents induced by GABA. Furthermore, carbachol and muscarine also elicited inward currents in a TTX-containing solution. From the current-voltage relationship, the reversal potential was estimated to be −7.1 mV. These carbachol-induced responses were antagonized by atropine. In the current-clamp recordings, carbachol depolarized the membrane with increased frequency of action potentials. These observations suggest that acetylcholine suppresses GABA release through muscarinic receptors located on the presynaptic terminals. Acetylcholine also directly affects the postsynaptic membrane through muscarinic receptors, by opening nonselective cation channels. A combination of these presynaptic and postsynaptic actions may enhance activation of SFO neurons by acetylcholine.

inhibitory postsynaptic current; synaptic input; acetylcholine; patch clamp

THE SUBFORNICAL ORGAN (SFO), one of the circumventricular organs, plays an important role in cardiovascular regulation, body water balance, and induction of drinking behavior. The SFO receives a cholinergic afferent projection from other brain regions (14). Cholinergic stimulation of the SFO by acetylcholine and carbachol induces water drinking and pressor responses (17, 18). The pressor and drinking responses to acetylcholine and carbachol were blocked by pretreatment with the muscarinic receptor antagonist atropine, whereas nicotinic antagonists had no effect (18). An early in vitro electrophysiological study showed that carbachol increased the firing rates of SFO neurons and that the responses were blocked by atropine (4). Topical application of acetylcholine to the SFO increased the firing rates of these neurons in in vivo studies of rats (26) and cats (7). The effect of acetylcholine was also suppressed by atropine (26). Intracerebroventricular injection of carbachol induced c-Fos expression in the SFO (23). These observations suggest that there are muscarinic acetylcholine receptors in the SFO.

Anatomical (28) and electrophysiological (12, 21) studies indicate that the SFO receives GABAergic synaptic inputs. Several reports suggest that GABA and its analogs influence drinking and cardiovascular responses through the SFO (1, 15, 27). Recent studies have shown the presence of muscarinic subtype receptors in the presynaptic terminals of GABAergic fibers in several regions of the central nervous system (8, 22) and their modulatory action on GABA release (2). The present study examined the effects of acetylcholine action on neurons in the SFO, by the use of brain slice preparations and whole cell patch-clamp recordings.

MATERIALS AND METHODS

The techniques used in making slice preparations and whole cell patch-clamp recordings were similar to those reported previously (13, 29). The present study was carried out according to the guidelines of the Animal Experiment Committee, Kyushu Dental College.

Slice preparation. Male Wistar rats weighing 150–250 g were deeply anesthetized with ketamine (250 mg/kg sc) and decapitated after being stunned. Slices of 300-μm thickness were prepared in a cold bathing medium. The brain was cut midway (−45°) between the coronal and horizontal planes (12). The slices were dissected between the hippocampal commissure and corpus callosum. They were preincubated in the bathing medium for at least 1 h before recording. For recording, one slice was submerged in a recording chamber with a volume of 0.2 ml at a constant temperature of 32°C and a perfusion flow rate of 1.6 ml/min. Chemicals were applied to the slices by perfusion from separate storage bottles containing medium to which they had been added.

Solutions and drugs. The normal bathing and perfusion media used in this experiment contained (in mM) 124 NaCl, 5 KCl, 1.24 KH2PO4, 1.3 MgSO4, 2.1 CaCl2, 26 NaHCO3, and 10 glucose at pH 7.4. All of the bathing and perfusion media used in this study were saturated with a mixture of 95% O2 and 5% CO2. Drugs were purchased from the following pharmaceutical companies: atropine sulfate, (+)-bicuculline methiodide, carbamylcholine chloride (carbachol), hexamethonium chloride, kynurenic acid, (+)-muscarine chloride, and...
aptic currents (EPSCs) and to potentials were clamped to configuration was less than 50 M for further analysis. Series resistance in the whole cell configuration was less than 50 MΩ and compensated. Membrane potentials were clamped to −81 mV for excitatory postsynaptic currents (EPSCs) and to −51 or −56 mV for inhibitory PSCs (IPSCs). To get a current-voltage relationship, the negative going ramp commands of 100 mV were applied at a rate of 50 mVs. Potential values were corrected for the junction potential (11 mV) in analysis. Data were analyzed off-line by using pCLAMP and AxoGraph (Axon Instruments) and an analog-digital converter (MacLab/8, ADI). To analyze synaptic currents quantitatively, the alternating current components were used for analysis with two software packages (AxoGraph v.3 and MacLab v.3.5.1 Spike Histogram). The analysis of synaptic currents was done at a time point when the responses were maximal. Use of an event-detection threshold, which was set at the beginning of the analysis and thereafter held constant, allowed events to be detected with recording segments of 120 s in duration. The Kolmogorov-Smirnov test was used to determine if two distributions were different, with a criterion of P < 0.05. The Student’s paired t-test was performed for analytical comparison. EC50 and IC50 values were obtained by the Prism curve-fitting program (GraphPad Software) based on Michaelis-Menten’s equation. The numerical data are given as means ± SE, and n represents the number of neurons tested.

RESULTS

Glutamatergic and GABAergic synaptic currents. The effects of glutamate and GABA receptor antagonists on the postsynaptic currents were studied. The spontaneous EPSCs (sEPSCs) were almost completely suppressed by the glutamate receptor antagonist kynurenic acid (1 mM, n = 6, 0.92 ± 0.19 to 0.22 ± 0.05 Hz, 76% decrease) and the non-N-methyl-D-aspartate receptor antagonist CNQX (3 mM, n = 8, 0.91 ± 0.12 to 0.20 ± 0.07 Hz, 78% decrease) (29). The sIPSCs were also almost completely suppressed by the GABA receptor antagonist bicuculline (10 μM, n = 6, 6.7 ± 2.4 to 0.33 ± 0.22 Hz, 95% decrease). These results suggest that glutamate and GABA influenced SFO neurons as excitatory and inhibitory neurotransmitters, respectively, under our experimental conditions.

Carbachol-induced suppression of spontaneous and miniature inhibitory postsynaptic inputs. The effects of carbachol on sIPSCs were tested. Of 87 neurons, sIPSCs were decreased in 80 neurons by application of carbachol at 10 μM but increased in one neuron (data not shown). Six neurons showed no response. The effects of carbachol at 10 μM on miniature IPSCs (mIPSCs) remained in nine of 10 neurons in the presence of 0.3 μM TTX (Fig. 1). The mean frequency of the mIPSCs was decreased by carbachol at 10 μM from 5.73 ± 1.83 to 2.94 ± 1.64 Hz (49% decrease). Changes
of amplitudes and time constants were further analyzed for the nine neurons. In all nine neurons, carbachol changed neither the amplitudes (Fig. 1C) nor the time constants (Fig. 2), implying that the action of carbachol occurs in the presynaptic terminals.

The decrement ratio of sIPSC frequency by carbachol was concentration dependent (Fig. 3). The EC50 value for the carbachol effects was 7.2 μM. The effects of the muscarinic receptor agonist muscarine on the IPSCs were also investigated (Fig. 4). Application of muscarine induced a similar inhibitory response to that of carbachol. The EC50 value for the muscarine effects was 25 μM. These values of EC50 were similar to 29.5 μM for carbachol, the value of which was obtained from the septum (9) in studies using slice preparations.

Effects of the muscarinic receptor antagonist atropine on carbachol-induced inhibitory responses. To clarify further the type of receptors involved in the carbachol response, we investigated the effects of the muscarinic receptor antagonist atropine on carbachol-induced inhibitory responses of sIPSCs (Fig. 5). As shown in Fig. 5B, atropine antagonized the inhibitory responses induced by carbachol at 10 μM in a dose-dependent manner. The effects of the nicotinic receptor antagonist hexamethonium on carbachol-induced responses of sIPSCs were also investigated. Hexamethonium at 100 μM did not influence the inhibitory responses induced by carbachol at 10 μM (n = 6, data not shown).

Carbachol does not affect GABA-induced outward currents. To study further the presynaptic action of carbachol in the SFO, we examined its effects on GABA-induced outward currents. Application of GABA at 1 mM induced outward currents (n = 5, 95.4 ± 15.1 pA) at the holding potential of −51 mV. The GABA-induced outward currents were not influenced by carbachol at 10 μM in any of the five neurons tested (97.0 ± 2.7% vs. control, Fig. 6). The perfusion medium contained 0.3 μM TTX.

Selective suppression of carbachol to sIPSCs. The effects of carbachol on sEPSCs were investigated. Figure 5Aa shows that carbachol at 10 μM did not influence EPSCs although it did reduce the frequency of sIPSCs in the same SFO neuron. In 11 of 13 neurons tested, carbachol at 10–100 μM had no effect on sEPSCs. Because carbachol at 10 μM decreased the frequency of sIPSCs in 80 of 87 neurons, these findings suggest that carbachol selectively influences IPSCs.

![Fig. 2. No effects of CCh on time constant of mIPSCs. At left, a current trace of mIPSC. The dotted line shows that the falling phase of the mIPSC fits a single exponential function. We estimated the time constant by curve-fitting procedure with the software of Axograph. The curve-fitting analysis was done for 20 events of mIPSCs in the control and in the presence of CCh in a neuron in Fig. 1. At right, the averaged time constants were 12.8 ± 0.6 ms in the control and 13.4 ± 0.9 ms in the presence of carbachol at 10 μM. Note that CCh produces no significant change in the time constants of mIPSCs.](http://ajpregu.physiology.org/DownloadedFrom/10.22033.3)

![Fig. 3. Dose-dependent inhibition of spontaneous IPSCs (sIPSCs) in frequency by CCh. A: change of frequency of sIPSCs in ratemeter records from 2 SFO neurons, in application of CCh at 0.1–100 μM. The ratemeter record at 0.1 μM was obtained from a SFO neuron. Other ratemeter records (1–100 μM) were obtained from another neuron. Application of CCh was performed at the times indicated by the horizontal bars. The holding potential was −56 mV. B: the dose-response relationship for CCh- and muscarine (Mus)-induced inhibition of the frequency. Changes of mean frequency (Freq) and amplitude (Amp) during CCh application to those in the control are expressed as percentages. Note that CCh and muscarine produce no significant changes in the distribution of sIPSCs amplitudes. Each plot shows a mean value obtained from 9–12 SFO neurons.](http://ajpregu.physiology.org/DownloadedFrom/10.22033.3)
Carbachol induced inward currents. Application of carbachol induced inward currents at the holding potentials of $-51$ mV (Fig. 7A). The mean carbachol-induced inward currents were $29.9 \pm 3.4$ pA in 41 of 69 neurons at $10 \mu$M carbachol and $36.5 \pm 7.8$ pA in 14 of 32 neurons at $100 \mu$M. Application of muscarine at $100 \mu$M also induced inward currents in seven of 12 neurons tested. The mean muscarine-induced inward current was $50.7 \pm 19.3$ pA. The carbachol-induced responses remained in TTX ($0.3 \mu$M)-containing perfusion medium. The mean carbachol-induced inward currents were $37.5 \pm 1.0$ pA in six of 23 neurons at $10 \mu$M carbachol and $44.0 \pm 7.2$ pA in 20 of

![Fig. 4. Muscarine suppresses frequency of sIPSCs in an SFO neuron. A: a representative example of inhibition of sIPSCs by muscarine at 100 \mu M. Top, a current trace; bottom, a ratemeter record of IPSC frequency. B: expanded current records in control (a), during application of muscarine (b), and in washout (c) in A. C: cumulative probability plots of sIPSCs amplitude (a) and interevent intervals (b) in control and during application of muscarine. The mean amplitude and number of sIPSCs were $15.9 \pm 0.2$ pA and 619 events in control (a) and $16.3 \pm 0.2$ pA and 392 events in the presence of CCh during a 120-s recording period. Note that muscarine produces no significant change in the distribution of sIPSCs amplitudes but that the distribution of interevent intervals is shifted toward longer intervals. The holding potential was $-56$ mV.

![Fig. 5. Selective response of CCh to IPSCs and effects of the muscarinic receptor antagonist atropine on CCh-induced suppression of IPSC frequency. A: atropine at 1 \mu M antagonized the CCh (10 \mu M)-induced suppression of sIPSC frequency in an SFO neuron. Top, current traces for control (a), in application of atropine at 1 \mu M (b), and washout (c). Middle, a ratemeter record of IPSC frequency. Bottom, a ratemeter record of EPSC frequency. In a and c, CCh reduced the frequency of sIPSCs but not spontaneous excitatory postsynaptic currents (sEPSCs). B: the dose-response relationship for atropine on the CCh-induced responses. IC$_{50}$ was estimated as 75 nM. n, number of neurons. The holding potentials were $-51$ mV.]
38 neurons at 100 µM in the presence of TTX. The responses were almost completely blocked by atropine at 1 µM (n = 6, 97% decrease) (Fig. 7A). We examined the effects of the M₂/M₃ muscarinic receptor antagonist 4-DAMP on the carbachol (10 µM)-induced currents. The drug at 0.1 µM almost completely suppressed the carbachol-induced currents (n = 6, 95% decrease, data not shown).

To understand the mechanisms involved in the responses, we applied ramp stimulation to SFO neurons in the presence and absence of carbachol. Ten SFO neurons that were sensitive to carbachol were analyzed. In seven of the 10 neurons, membrane conductance was increased with a reversal potential in the presence of carbachol, as shown in Fig. 7B. The mean reversal potential was estimated to be −7.1 ± 5.1 mV. In the three remaining neurons, no marked change of membrane conductance was observed within the range tested. These results suggest that inward currents are induced through muscarinic receptors in the postsynaptic membrane of SFO neurons by activation of non-selective cation channels.

Effects of carbachol on membrane potentials in the current-clamp experiment. In this experiment, the effects of carbachol at 10 and 100 µM on 16 SFO neurons were investigated. Carbachol depolarized the membrane 12.6 ± 4.8 mV at 10 µM (n = 3) and 15.0 ± 3.3 mV at 100 µM (n = 13) in all neurons tested. The mean value of the resting membrane potentials was −61.6 ± 0.6 mV. In seven of 13 neurons, 100 µM carbachol also increased frequency of action potentials. The carbachol-induced depolarization still remained in the TTX-containing medium (Fig. 8, A and B). Carbachol at 100 µM depolarized the membrane 17.0 ± 4.8 mV in all neurons tested (n = 9). The depolarization increased in a dose-related manner (Fig. 8, B and C). The responses
were reversible and repeatable (n = 3, Fig. 8B). These results suggest that carbachol directly depolarizes the membrane of SFO neurons.

**DISCUSSION**

**Suppression of GABA release by activation of muscarinic receptors.** The present observation that IPSCs were almost completely suppressed by the GABA receptor antagonists implies that GABA is a main inhibitory neurotransmitter in the SFO. This is consistent with our previous studies of intracellular recordings (12). Recent studies have demonstrated the presence of muscarinic subtype receptors in the presynaptic terminals of GABAergic fibers in several regions of the central nervous system (8, 22) and their modulatory action on GABA release (2). Baba et al. (2) showed that muscarinic receptor stimulation facilitates GABA release in the rat spinal dorsal horn. Several lines of evidence in the present study demonstrate that acetylcholine modulates neuronal activity in the nerve terminals through muscarinic receptors and inhibits GABA release. Carbachol decreased the frequency of IPSCs without changes of amplitude and time constants. Carbachol did not influence GABA-induced outward currents. These responses still remained in the presence of TTX, implying that the action is not due to action potential-dependent synaptic events. The muscarinic receptor antagonist atropine suppressed the carbachol-induced inhibition of IPSCs, whereas the nicotinic receptor antagonist hexamethonium did not influence it.

Carbachol actions were observed only in IPSCs, not in EPSCs. Our recent study demonstrated that EPSCs are due to glutamatergic synaptic inputs (29). Several studies of synaptic transmissions with whole cell clamp recordings from neurons in the reticular formation (3), the hippocampus (25), and the magnocellular basal forebrain (25) demonstrated that glutamatergic excitatory synaptic inputs are suppressed presynaptically by acetylcholine. Unlike in the other regions of the brain, acetylcholine may not suppress glutamate release but may selectively suppress GABA release in the SFO of rats.

In this study, both GABAergic mIPSCs and whole cell GABA currents were examined. Although the mIPSCs were often of amplitudes >30 pA, the largest peak currents resulting from exogenous application of GABA were only in the order of 140 pA (the mean peak current was 95.4 pA). As suggested by various studies (for example, Ref. 19), it is likely that the desensitization of GABA at high concentrations is very rapid so that it reduces the peak current. In the present study, 1 mM GABA was applied to the bath. The concentration of GABA in the bath gradually changed, and it took 30 s to reach the maximal concentration. As shown in Fig. 6, the GABA-induced outward currents were reduced before the concentration reached the maximum, suggesting that the SFO neurons were also rapidly desensitized by GABA.

**Carbachol induced inward currents.** Carbachol induced inward currents in half the SFO neurons. In most SFO neurons showing carbachol-induced inward currents, an increased membrane conductance was observed and the mean reversal potential was ~7.1 mV. A study in rat septal nucleus neurons in slice preparations (9) found that muscarine induces inward currents with increased membrane conductance and that the mean reversal potential is ~17 mV, suggesting that
the currents are mediated through opening of nonselective cation channels. Although the reversal potential in the study is more negatively shifted than that in the present study, these findings are almost consistent with ours. Thus the present results suggest the involvement of nonselective cation channels in the carbachol-induced responses. However, the net currents induced by carbachol in this study were not always linear, as shown in Fig. 7. This may imply partial involvement of the other ion channels except nonselective cation channels in the carbachol responses. A study in pyramidal neurons dissociated from the rat cerebral cortex (20) revealed that acetylcholine induced an inward current with a membrane conductance decrease due to the suppression of the voltage- and time-dependent $K^+$ current (M current). Shen and North (24) noted that muscarine elicited the inward current through both opening of voltage-independent cation channels and closing of potassium channels in rat locus coeruleus neurons.

Hasuo et al. (9) demonstrated that muscarine activates a nonselective cation channel through an $M_3$ receptor subtype in dorsolateral neurons of rats. The present study showed that the $M_3/M_1$ receptor antagonist 4-DAMP almost completely suppressed the carbachol-induced currents. Although this suggests the involvement of the $M_3$ receptor subtype in the action of the carbachol-induced inward currents of SFO neurons, further systematic pharmacological experiments are necessary to confirm this hypothesis.

**Function of acetylcholine.** GABA-gated ion channels in the SFO have an equilibrium potential more negative than the resting membrane potential (12). Thus the GABAergic synaptic inputs produce inhibitory responses in SFO neurons. Our previous findings (12) by conventional intracellular recordings demonstrated that application of the GABA$_A$ receptor antagonists bicuculline and picrotoxin depolarizes membranes and suggested that GABA neurons tonically inhibit activity of SFO neurons. This study demonstrates that carbachol suppresses GABA release from the presynaptic terminals through the muscarinic receptors. This implies disinhibition of SFO neurons by carbachol. Our present study also demonstrates induction of inward currents and membrane depolarization through the muscarinic receptors by carbachol, indicating a direct postsynaptic action of acetylcholine on SFO neurons. Together, the combination of presynaptic and postsynaptic actions by acetylcholine may facilitate neuronal activity of SFO neurons.

**Perspectives**

The SFO is a brain region that is innervated by a cholinergic pathway that mediates information on body fluid balance. This study demonstrated modulatory action of the cholinergic inputs on activity of SFO neurons. The origin of the cholinergic fibers still remains to be clarified. Lind et al. (16) showed that horseradish peroxidase injection into the SFO labeled neurons in the medial septum. A histochemical study (10) detected expression and immunoreactivity of choline acetyltransferase and vesicular acetylcholine transporter in neurons of the same regions. These findings suggest that cholinergic fibers in the SFO originate from the medial septum.

To demonstrate pharmacologically the subtypes of muscarinic receptors, several antagonists are available (see Ref. 5 for review). In this study, we used a muscarinic receptor antagonist, 4-DAMP, only, and the results suggest $M_3$ receptor involvement in the postsynaptic responses. Further experiments are necessary to identify subtypes of muscarinic receptors involved in presynaptic and postsynaptic responses.

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