Cholinergic Modulation of Neuronal Excitability in the Rat Suprachiasmatic Nucleus

Jyh-Jeen Yang,1 Yu-Ting Wang,1 Pi-Cheng Cheng,1 Yeh-Jung Kuo,2 and Rong-Chi Huang1
1Departments of Physiology and Pharmacology and 2Chinese Medicine, Chang Gung University School of Medicine, Kwei-San, Tao-Yuan, Taiwan

Submitted 28 September 2009; accepted in final form 12 January 2010

Yang JJ, Wang YT, Cheng PC, Kuo YJ, Huang RC. Cholinergic modulation of neuronal excitability in the rat suprachiasmatic nucleus. J Neurophysiol 103: 1397–1409, 2010. First published January 3, 2010; doi:10.1152/jn.00877.2009. The central cholinergic system regulates both the circadian clock and sleep-wake cycle and may participate in the feedback control of vigilance states on neural excitability in the suprachiasmatic nucleus (SCN) that houses the circadian clock. Here we investigate the mechanisms for cholinergic modulation of SCN neuron excitability. Cell-attached recordings indicate that the nonspecific cholinergic agonist carbachol (CCh) inhibited 55% and excited 21% SCN neurons, leaving 24% nonresponsive. Similar response proportions were produced by two muscarinic receptor [muscarinic acetylcholine receptor (mAChR)] agonists, muscarine and McN-A-343 (M1/4 agonist), but not by two nicotinic receptor (nAChR) agonists, nicotine and choline (α7-nAChR-agonist), which, however, produced similar response proportions. Whole cell and perforated-patch recordings indicate that CCh inhibition of firing was mediated by membrane hyperpolarization due to activation of background K+ currents, which were sensitive to submillimolar concentrations of Ba2+ and to millimolar concentrations of TEA. RT-PCR analysis demonstrated the presence of mRNA for M1 to M5 mAChRs in SCN. The CCh-induced hyperpolarization and activation of background K+ currents were blocked by M4 antagonists and to a lesser degree by M1 antagonists but were insensitive to the antagonists for M2 or M3, suggesting the involvement of M4 and M1 mAChRs in mediating CCh inhibition of firing. CCh enhancement of firing was mediated by membrane depolarization, as a result of postsynaptic inhibition of background K+ currents. The multiple actions of cholinergic modulation via multiple receptors and ion channels may allow acetylcholine to finely control SCN neuron excitability in different physiological settings.

INTRODUCTION

The circadian clock in the hypothalamic suprachiasmatic nucleus (SCN) tightly controls the daily patterning of sleep and wakefulness states, which in turn feedback to regulate neural activity in the SCN (Deboer et al. 2003). As the central cholinergic system regulates both the circadian systems and sleep-wake cycle, cholinergic modulation of SCN neurons, via direct cholinergic innervation of SCN from the brain stem and basal forebrain (Bina et al. 1993) or via extra-SCN cholinergic circuit, has been suggested to mediate such a feedback control of vigilance states on neural excitability. All three recordings indicated a consistent picture of cholinergic actions on SCN neuron excitability and neuronal firing rate in SCN neurons. We then used whole cell recordings to investigate cholinergic modulation of membrane potentials and perforated-patch recordings to determine the receptor and ionic mechanisms. Our results yielded a consistent picture of cholinergic actions on SCN neuron excitability. All three recordings indicated ~20% SCN neurons being refractory to carbachol (CCh) stimulation, irrespective of the presence of GABAergic neurotransmission. While all five types of mAChR mRNAs were expressed in SCN, the majority of SCN neurons were inhibited by cholinergic activation of postsynaptic M4 and M1 mAChRs leading to enhancement of background K+ currents. A subset of SCN neurons was excited by CCh most likely mediated via postsynaptic inhibition of background K+ currents. The results suggest that acetylcholine may act on multiple receptors and ion channels to differentially control SCN neuron excitability.

METHODS

Animals

All experiments were carried out according to the guidelines of the Institutional Animal Care and Use Committee of Chang Gung University School of Medicine. Sprague-Dawley rats (17–26 day) were

Address for reprint requests and other correspondence: R.-C. Huang, 259 Wen-Hwa 1st Road, Dept. of Physiology and Pharmacology, Chang Gung University School of Medicine, Kwei-San, Tao-Yuan, Taiwan (E-mail: rongchi@mail.cgu.edu.tw).
kept in a temperature-controlled room under a 12:12 light:dark cycle (light on 0700–1900 h). Lights-on was designated Zeitgeber time (ZT) 0. For day- and nighttime recordings, the animal was killed at ZT 2 and ZT 10, respectively. A total of 235 rats were used in this study. An animal was carefully restrained by hand to reduce stress and killed by decapitation using a small rodent guillotine without anesthesia, and the brain was put in an ice-cold artificial cerebrospinal fluid (ACSF) prebubbled with 95% O2-5% CO2. The ACSF contained (in mM) 125 NaCl, 3.5 KCl, 2 CaCl2, 1.5 MgCl2, 26 NaHCO3, 1.2 NaH2PO4, and 10 glucose.

Reduced SCN preparations

A coronal slice (200–300 μm) containing the SCN and the optic chiasm was cut with a Vibroslice (Campden Instruments, Lafayette, IN) and was then incubated at room temperature (22–25°C) in the incubation solution, which contained (in mM) 140 NaCl, 3.5 KCl, 2 CaCl2, 1.5 MgCl2, 10 glucose, and 10 HEPES, pH 7.4, bubbled with 100% O2. A reduced preparation was obtained by excising a small piece of tissue (circa one-ninth the size of SCN) from the SCN using a fine needle (No. 26002-10, Fine Science Tools, Foster City, CA), followed by further trimming down to two or three smaller pieces with a short strip of razor blade. The reduced preparation (containing tens to hundreds of cells) was then transferred to a recording chamber for recording. The SCN neurons of the reduced preparation could be identified visually with an inverted microscope (Olympus IX70, Japan). The preparation thus obtained allows rapid application of drugs (Chen et al. 2009) and has been used to demonstrate diurnal rhythms in both spontaneous firing and sodium pump activity (Wang and Huang 2004).

Electrophysiology

The reduced SCN preparation was perfused with bath solution containing (in mM) 140 NaCl, 3.5 KCl, 2 CaCl2, 1.5 MgCl2, 10 glucose, and 10 HEPES, pH adjusted to 7.4 with NaOH. All recordings were made with Axopatch 200B amplifier (Axon Instruments, Foster City, CA) at room temperature (22–25°C). The spontaneous firing was recorded in the cell-attached and whole cell current-clamped configurations. For the cell-attached recording, the patch electrode was filled with the bath solution or with the patch solution as described in the following text. The spike counts, in 6-s epochs, always began only after stable recordings were made. At least 1 or 2 min of spontaneous firing rate were counted before the application of drugs. The whole cell recording technique was used for measurement of membrane potentials, whereas the perforated-patch recording technique was used for both membrane potentials and membrane currents. Because no day-night difference was observed in the response proportions to cholinergic agonists, whole cell and perforated-patch recordings were performed during the day. The patch solution contained (in mM) 20 NaCl, 1 CaCl2, 2 MgCl2, 110 K-gluconate, 11 EGTA, 10 HEPES, 3 Na-ATP, and 0.3 Na-GTP, pH adjusted to 7.3 with KOH. The measured liquid junction potential was ~12 mV (Neher 1992) and was corrected for in the presentation of data. Pipette resistance was 4–6 MΩ. For perforated-patch recordings, the patch pipette also included nystatin (Sigma-Aldrich, St Louis, MO) at a final concentration of 250 μg/ml prepared from a stock solution (25 mg/ml DMSO). The signal was low-pass filtered at 1–5 kHz and digitized on-line at 2–10 kHz via a 12-bit A/D digitizing board (DT2821F-DI, Data Translation, Marlboro, MA) with a custom-made program written in the C Language. Data were analyzed and plotted with custom-made programs written in Visual Basic 6.0 and the commercial software GraphPad PRISM (GraphPad Software, San Diego, CA). Data are given as means ± SE.

Data analysis

For cell-attached recordings, the firing rate in control and in drug was determined by averaging a 60-s period (10 6-s epochs) right before and during the application of each drug, respectively. Inhibition of firing by a drug was defined as a decrease in firing rate in drug compared with control using paired t-test ($P < 0.05$). Excitation and no response were defined similarly. Dose-dependent effects of CCh and muscarine on spontaneous firing rate were analyzed with ANOVA, followed by Bonferroni’s test for selected pairs comparison (Fig. 1C). To assess the difference in the response proportions produced by two drugs or the day-night difference by the same drug, $χ^2$ test was performed with PRISM.

Drugs

CCh, choline, nicotine, muscarine, McN-A-343, atropine, mecamylamine, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), galamine trimethylide, himbacine, pirenzepine, and picrotoxin were obtained from Sigma-Aldrich. Tetrodotoxin, AF-DX 384, and J104129 fumarate were purchased from Tocris Cookson (Ellisville, MO), and muscarinic toxin 3 (MT3) was from Peptide Institute (Minoh-shi, Osaka, Japan).

RT-PCR analysis of mAChRs expression

Total RNA of SCN was extracted using the Absolutely RNA Nanoprep kit (Stratagene, La Jolla, CA) according to the manufacturer’s guide; total RNA of brain was extracted using the NeuNleoSpin RNA II kit (Macherey-Nagel, Duren, Germany). The quality and quantity of RNA was determined by measuring A260:A280 ratio. RNA samples were treated with DNaseI for 12–15 min at 25°C to eliminate genomic DNA contamination. The resulting RNA was reverse-transcribed (RT) to cDNA using ReverTra Ace (Toyobo, Osaka, Japan) with random primers in a total volume of 20 μL. One-tenth of RT products were used as templates (2 μl) to perform PCR reaction. RT reaction with omission of reverse transcriptase was used as templates for negative control PCR. Primers used for RT-PCR were as follows: M1 forward 5’-CTGGTTTCCTTCGCTTCTG-3’ and reverse 5’-GCTGCTCTTCTCTCCTTGA-3’, M2 forward 5’-TACCCCCCTACGTGGATTGCG-3’ and reverse 5’-ATGATGACAGGCAGATAG-3’, M3 forward 5’-CACAGGCAAGACCTCTGCA-3’ and reverse 5’-TCAACTGGCCAAGAGGGG-3’, M4 forward 5’-AGTGCTCTCATCCAGTTCTTCA-3’ and reverse 5’-CACATTACCCGGTCTGTCTTTG-3’, and M5 forward 5’-CTCACTATTGGGCATCTTCTTC-3’ and reverse 5’-GGTCTCTTGGTCTGCTTCTGT-3’. The thermal cycling condition of RT-PCR was 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and then 72°C for 7 min. PCR amplified products were electrophoresed in 1.5% agarose gels, stained with ethidium bromide, and photographed.

RESULTS

Effects of cholinergic agonists on spontaneous firing in SCN neurons

To investigate the cholinergic effects on SCN neurons, we first determined the firing responses of SCN neurons to the nonspecific cholinergic agonist CCh at a saturating concentration of 100 μM (Fig. 1). This concentration has been shown to produce maximum phase shifts in firing rhythms of SCN neurons (Liu and Gillette 1996; Trachsel et al. 1995) and also produced maximum effects on excitation or inhibition in this study (see Fig. 1C). Figure 1A shows the CCh effects on spontaneous firing in three representative cells with each having its firing rate reduced (left), increased (middle), or not
altered (right) by CCh. Similar responses were also elicited by specific cholinergic agonists muscarine and nicotine albeit with different response proportions (see following text). To determine the receptor mechanisms for mediating the CCh effects on spontaneous firing, we compared the effects of CCh with muscarine and nicotine in the same cells (n = 79). For the 79 cells that were treated with all three agonists, 45 cells (57%) were inhibited by CCh, 19 cells (24%) were excited by CCh, and 15 cells (19%) were nonresponsive. Among the 45 cells that were inhibited by CCh, 87% (39/45) were also inhibited by muscarine, but only 24% (11/45) were also inhibited by nicotine, suggesting that the cholinergic inhibition of firing was mediated mainly via muscarinic receptors. Figure 1B shows such a representative cell, with its firing rate markedly inhibited by CCh and muscarine but not by nicotine. Consistently, CCh inhibition of firing was partially blocked by 0.1 μM atropine (mAChR antagonist) (n = 7) but not by 20 μM mecamylamine (nAChR antagonist; n = 7; data not shown). On the other hand, among the 19 cells that were excited by CCh, 58% (11/19) were also excited by muscarine, and 42% (8/19) were also excited by nicotine, suggesting that cholinergic enhancement of firing might be mediated via both muscarinic and nicotinic receptors. The inhibitory and excitatory effects of CCh and muscarine on spontaneous firing were dose-dependent and appeared to saturate at a concentration of 100 μM. As summarized in Fig. 1C, increasing concentrations of CCh (1, 10, and 100 μM) reduced the control firing rate to 83.1 ± 7.2% (n = 10; P < 0.05), 29.2 ± 25.7% (n = 9; P < 0.001), and 25.7 ± 9.5% (n = 8; P < 0.001), respectively (top left), or increased it to 114.7 ± 7.7% (n = 4; P > 0.05), 140.3 ± 14% (n = 4; P > 0.05), and 155.7 ± 18.7% (n = 4; P < 0.05), respectively (top right). Similarly increasing concentrations of muscarine (1, 10, and 100 μM) reduced the rate to 46.8 ± 9.9% (n = 11; P < 0.001), 22.8 ± 6.4% (n = 10; P < 0.001), and 18.6 ± 4.5% (n = 11; P < 0.001), respectively (bottom left), or increased it to 121.1 ± 6.9% (n = 4; P > 0.05), 166.7 ± 15.1% (n = 4; P < 0.05), and 186.3 ± 4.5% (n = 4; P < 0.05), respectively (bottom right).

Many more cells were sampled to determine the response proportions to cholinergic agonists (n = 535, including the 79 cells stated in the preceding text; data not shown). Among these neurons, many of them tested with multiple drugs, 228 cells were treated with CCh, 204 with muscarine, and 206 with nicotine. CCh inhibited 55% (125/228) and excited 21% (48/228) cells, leaving 24% (55/228) nonresponsive. Similarly, muscarine inhibited 55% (113/206) and excited 18% (36/206) cells, leaving 27% (55/206) nonresponsive (χ² = 0.99, P = 0.61; muscarine vs. CCh). In contrast, nicotine inhibited 18% (36/204) and excited 39% (79/204) cells with 44% (90/204) nonresponsive (χ² = 64.17, P < 0.0001; nicotine vs. CCh). The results also indicate that the response proportions produced by CCh are mimicked by muscarine but not by nicotine. Note that among the 48 cells that were excited by CCh, 4% (2/48) exhibited mixed responses of inhibition and excitation as opposed to 50% (9/18) in whole cell recordings (see Fig. 3).

A small portion of the cells were also treated with 1 μM McN-A-343 (n = 41), an agonist for M1/4 mAChR (Caulfield and Birdsall 1998), and with 200 μM choline (n = 30), a specific agonist for α7-nAChR (Mandelzys et al. 1995). The results indicate that McN-A-343, similar to muscarine and CCh, inhibited 54% (22/41) and excited 15% (6/41) cells, with
32% (13/41) nonresponsive ($\chi^2 = 0.48, P = 0.79$; McN-A-343 vs. muscarine; $\chi^2 = 1.51, P = 0.47$; McN-A-343 vs. CCh), and that choline, similar to nicotine, inhibited 20% (6/30) and excited 33% (10/30) cells, with 47% (14/30) nonresponsive ($\chi^2 = 0.32, P = 0.85$; choline vs. nicotine).

To determine whether there was temporal variation in response proportions produced by the cholinergic agonists, cells were grouped according to the time they were recorded during the day (ZT 0–12) or at night (ZT 12–24). The results indicate no day-night variation in the response proportions by CCh ($\chi^2 = 1.35, P = 0.51$; day vs. night), muscarine ($\chi^2 = 4.94, P = 0.08$; day vs. night), or nicotine ($\chi^2 = 2.71, P = 0.26$; day vs. night).

**CCh effects on membrane potentials of SCN neurons**

The CCh effects on the membrane potentials were studied with the whole cell current-clamp recording technique. As in cell-attached recordings, SCN neurons (n = 92) in whole cell recordings also displayed similar response proportions, with most cells (59%; 54/92) inhibited by 100 μM CCh. The inhibition manifested as a partial or total blockade of action potential with or without clearly visible membrane hyperpolarization (Fig. 2). Because the inhibitory effects of CCh rundown in whole cell recordings (see Fig. 4A), the whole cell data presented in the following text are mostly from the first applications of CCh.

We first examined the effects of CCh on the membrane potential (Fig. 2A). Figure 2A1 shows a typical experiment procedure with a 30-s recording in control, followed by a 30-s application of 100 μM CCh and then ≥30-s washout of the drug to allow recovery. For this particular cell, CCh hyperpolarized the membrane potential and completely and reversibly arrested the generation of action potentials. To better determine the membrane hyperpolarization by CCh, we selected cells of which the resting potentials could be clearly determined either due to low firing rate or with their action potentials blocked by 0.3 μM TTX. Figure 2A2 plots the CCh-induced hyperpolarization against the membrane potential of each individual cell. For a total of 38 cells, the CCh-induced hyperpolarization averaged 8.0 ± 0.9 mV (n = 38; the mean represented by the dotted line). Figure 2A3 plots the membrane potential in CCh against that in control. As indicated, in 45% (17/38) cells, the membrane potentials were hyperpolarized by CCh to potentials more negative than −70 mV with some approaching the predicted $K^+$ equilibrium potential of −87 mV (the bottom dotted line). The results suggest that CCh enhanced $K^+$ conductance(s) to hyperpolarize the membrane potentials, thereby inhibiting spontaneous firing.

**CCh effects on action potentials and interspike potentials**

In addition to membrane hyperpolarization, CCh appeared to alter the action potentials and slow the rate of depolarization during the interspike intervals (Fig. 2B). As indicated in Fig. 2B1, the action potentials become larger during the inhibition by CCh, reminiscent of those recorded in cell-attached conditions (Fig. 1A, leftmost panel). To better determine the CCh effects on action potentials and interspike potentials, 10 pairs of action potentials each in control (black) and in CCh (gray) are superimposed in Fig. 2B2. As indicated, CCh prolonged the interpulse intervals by slowing the rate of depolarization to the extent that only two pairs of action potentials (gray) were seen in the figure as opposed to ten pairs in control (black). Figure 2B3 superimposes the average pairs of action potentials to better demonstrate the effects of CCh on the rate of depolarization, which decreased from 0.075 mV/ms in control to 0.038 mV/ms in CCh as reflected by the slopes of fitted dotted lines. Furthermore, the voltage trajectory in CCh was more negative than in control as would be expected if the membrane potential was hyperpolarized by CCh. The average action potential (expanded in Fig. 2B4) also differed markedly in CCh with lower spiking threshold, faster rate of upstroke, larger peak overshoot potential, faster rate of downstroke, and more negative peak afterhyperpolarization potential, phenomena accounted for by the more hyperpolarized membrane potential. The results indicate that CCh inhibited spontaneous firing by hyperpolarizing the resting potentials and slowing the rate of depolarization with effects on action potentials likely due to more hyperpolarized resting potentials.

A few cells (n = 4) had their firing rates inhibited by CCh with no apparent change in the membrane potentials and action potentials. Figure 2C shows such an example. The application of CCh markedly reduced the firing rate from ~5 to <1 Hz without much effect on the membrane potential (Fig. 2C1). Figure 2C2 superimposes 10 pairs of action potentials each in control (black) and in CCh (gray), indicating that CCh slowed the rate of depolarization to prolong the interpulse interval. Figure 2C3 superimposes the average pairs of action potentials. The fitted dotted lines indicated a decrease in the rate of depolarization toward spiking threshold from 0.11 mV/ms in control to 0.057 mV/ms in CCh. Apparently for this cell CCh inhibited spontaneous firing mainly by slowing the rate of depolarization. Note that while CCh markedly altered the average interspike potential, it virtually had no effect on the average action potential (Fig. 2C4).

**Excitatory effects of CCh on SCN neurons**

Compared with 59% cells inhibited by CCh, only 19% (18/92) cells were excited by CCh. Among these cells, half of them exhibited excitation only, and another half showed mixed responses of inhibition and excitation (Fig. 3). In excitation-only cells, the excitation was moderate in terms of increase in spontaneous firing (Fig. 3A1) or in resting potential (A2). For a total of nine cells, the averaged depolarization was 5.3 ± 0.9 mV (n = 9). By contrast, in mixed response cells the excitation was considerably larger, often to the extent of producing depolarization block (Fig. 3B, left panel). The mixed CCh effects could be clearly seen by expanding the voltage response time course (Fig. 3B, right). For a total of nine cells, the initial hyperpolarization averaged 6.6 ± 1.2 mV (n = 9), and the delayed depolarization amounted to 17.3 ± 3.0 mV (n = 9). Figure 3C summarizes the results. The hyperpolarization of 6.6 ± 1.2 mV (n = 9) in mixed response cells is similar to those exhibiting only inhibitory response of hyperpolarization 8.0 ± 0.9 mV (n = 38; $P = 0.46$; Student’s t-test), whereas the depolarization in mixed response cells is approximately three times ($P < 0.005$; Student’s t-test) as large as in excitation-only cells. Note that among the 18 cells excited by CCh, five (all excitation-only) cells were recorded in the presence of picrotoxin to block GABAergic neurotransmission, suggesting that ≥5% (5/92) cells were excited by CCh via postsynaptic mechanisms.
FIG. 2. CCh effects on the membrane potential of SCN neurons. A1: a representative cell to demonstrate a complete block by CCh of spontaneous firing. A2: plot of CCh-induced hyperpolarization against membrane potentials in a total of 38 cells with (open circles) or without (full circles) the addition of TTX. Dotted line, mean of CCh hyperpolarization. A3: plot of membrane potentials in CCh vs. in control with (open circle) or without (full circle) the addition of TTX. The membrane potentials in CCh approached the predicted reversal potential (~87 mV, represented by the bottom dotted line) for K^+-selective channels. Note that 45% (17/38) cells in CCh were hyperpolarized to potentials more negative to ~70 mV (data points between the 2 dotted lines). B1–B4: a representative cell to demonstrate an incomplete block by CCh of spontaneous firing (B1). CCh hyperpolarized the cell and increased its spike amplitude. Superimposition of 10 pairs of action potentials each in control (black) and in CCh (gray; B2). Note the prolongation of interpulse intervals in CCh, to the extent that only two out of ten pairs are seen in the figure. Superimposition of averaged paired action potentials (over ten pairs) in control (black) and in CCh (gray; B3). The dotted lines drawn to the interspike potentials were 0.075 mV/ms in control and 0.038 mV/ms in CCh. Superimposition of averaged action potentials in control (black) and in CCh (gray; B4). C1–C4: a representative cell to demonstrate the inhibition of spontaneous firing by CCh with no apparent effect on the resting potential (C1). Superimposition of the averaged paired action potentials in control (black) and in CCh (gray; C3). The dotted lines fits to the slopes of the interpulse potential were 0.11 and 0.057 mV/ms for control and CCh, respectively. Superimposition of averaged action potentials in control (dotted black curve) and in CCh (solid gray curve) to indicate a lack of effect of CCh on the action potential (C4). Broken horizontal lines represent the 0 levels of voltage unless specified otherwise.
only 3 mV (from –59 to –62 mV; H11011) response for application of CCh at potential by 16 mV (from –54 to –70 mV; H11011).

Furthermore, the proportions of cells expressing inhibitory and excitatory response, but not the proportion of nonresponsive neurons, were also altered in perforated patch recordings, being 78% (94/121), 3% (4/121), and 19% (23/121), respectively.

We compared the CCh effects on the resting potential and the membrane current in the same cells by switching between current- and voltage-clamp modes (Fig. 4B). For the voltage-clamp experiments, the membrane currents were activated by holding the cell at −52 mV (after correction of −12 mV junction potential), and voltage pulses were stepped to potentials between −42 and −132 mV in 10-mV decrements. Figure 4B shows the effects of CCh on the membrane potential (top), membrane currents (middle), and I-V relations (bottom) from a representative cell. As indicated, CCh reversibly hyperpolarized the cell and shifted the membrane currents in both out- and inward direction depending on the membrane potentials. Note the CCh-induced outward shift of the holding current at −52 mV. The I-V relations were constructed by plotting against the membrane potentials the leak current amplitude averaged over the duration of 20–50 ms after the capacitative transient (marked by ●, ■, and ○). For this particular cell, the CCh-induced inward shift in the background current occurred at voltages more negative to −102 mV. The CCh-induced inward current at these voltages was most likely an inward rectifier, because it was blocked by 1 mM Cs⁺ and 0.3 mM Ba²⁺ (Yang and Huang, unpublished results). Figure 4C summarizes the averaged I-V relation of the CCh-induced currents from a total of 11 cells. The zero-current voltage of the CCh-induced background currents was close to −102 mV, some 10–15 mV more negative than the predicted reversal potential (−87 mV) for K⁺-selective channels, suggesting the involvement of other mechanisms in addition to enhancement of K⁺ conductance.

Expression of mACHr mRNAs in SCN

We used RT-PCR to determine the expression of mACHr transcripts in SCN (Fig. 5). Positive control reactions were performed using cDNA of rat brain (M1–M5) to determine the primer efficiency and anneal temperature. These primers were then used to examine the gene transcription of M1–M5 in SCN. The RT-PCR of SCN showed positive signals with primers of all five mACHr subtypes.

CCh hyperpolarization is mediated by multiple muscarinic receptors

To determine the mACHr subtypes involved in mediating the CCh effects, we first used five relatively selective mACHr antagonists: pirenzepine (M1), gallamine (M2), himbacine and AF-DX 384 (M2/4), and 4-DAMP (M1/3/4/5) (see Caufield and Birdsall 1998). Figure 6, A–D, shows the effects of these drugs on ΔV (2 leftmost panels) and ΔI_{50} (2 rightmost panels), and Fig. 6, E and F, summarizes the results. As indicated in Fig. 6A, 1 μM piren-
pirenzepine partially block ΔV and ΔI_{52} in a representative cell. On average 1 μM pirenzepine reduced ΔV and ΔI_{52} to 66.0 ± 9.8% (n = 7; P < 0.05; paired t-test; Fig. 6E) and 51.5 ± 3.2% (n = 7; P < 0.0001; paired t-test; Fig. 6F), respectively. In contrast, 25 μM gallamine (IC_{50} = 1.97 μM for M2 receptor, (Ehler and Griffin 2008)) had little effect on the CCh effects as indicated in a different cell (Fig. 6F). On average ΔV and ΔI_{52} were 105.2 ± 14.5% (n = 6; P = 0.73; paired t-test; Fig. 6E) and 105.7 ± 5.3% (n = 6; P = 0.33; paired t-test; Fig. 6F), respectively, in the presence of 25 μM gallamine. While the M2 antagonist did not alter the CCh effects, the M2/4 antagonists, himbacine and AF-DX 384, almost completely blocked them. Figure 6C shows the himbacine effects on CCh responses in a representative cell. On average 1 μM himbacine reduced ΔV and ΔI_{52} to 10.9 ± 5.3% (n = 6; P < 0.0001; paired t-test; Fig. 6E) and 10.9 ± 3.5% (n = 6; P < 0.0001; paired t-test; Fig. 6F), respectively. Similar results were also obtained with 1 μM AF-DX 384, which reduced ΔV and ΔI_{52} to 11.1 ± 4.4% (n = 6; P < 0.0001; paired t-test; Fig. 6E) and 12.9 ± 3.5% (n = 5; P < 0.0001; paired t-test; Fig. 6F), respectively. Likewise 1 μM 4-DAMP also nearly completely blocked the CCh responses in another cell (Fig. 6D). On average 1 μM 4-DAMP reduced ΔV and ΔI_{52} to 10.8 ± 2.8% (n = 8; P < 0.0001; paired t-test; Fig. 6E) and 15.2 ± 4.3% (n = 6; P < 0.0001; paired t-test; Fig. 6F), respectively. The lack of effect of M2 antagonist and the nearly complete block of CCh responses by the non-M2 antagonist indicate a lack of involvement of M2 mAChR in mediating CCh effects. The rank order of potency (4-DAMP ~ AF-DX 384 ~ himbacine > pirenzepine) suggests that the CCh effects are mediated mainly via M4 mAChR and, to a lesser extent, via M1 mAChR.

The involvement of M4 mAChR in mediating CCh effects was further demonstrated with the highly selective M4 antagonist, the green mamba toxin MT3 (Caufield and Birdsall 1998; Jolkkonen et al. 1994). The results indicate that 100 nM MT3 reduced ΔV and ΔI_{52} to 56.8 ± 7.7% (n = 6; P < 0.005; paired t-test; Fig. 6E) and 55.9 ± 6.2% (n = 6; P < 0.001; paired t-test; Fig. 6F), respectively. In contrast, 20 nM J104129, a highly selective antagonist for M3 mAChR (Mitsuya et al. 1999) (Ki values being 4.2, 19, and 490 nM for human M3, M1, and M2, respectively), was without effect, changing ΔV and ΔI_{52} to 98.1 ± 3.4% (n = 7; P = 0.60; paired t-test; Fig. 6E) and 97.3 ± 4.4% (n = 4; P = 0.59; paired t-test; Fig. 6F), respectively. However, J104129 at a concentration of 100 nM reduced ΔV and ΔI_{52} to 67.6 ± 6.3% (n = 6; P < 0.005; paired t-test) and 73.8 ± 6.7% (n = 4; P < 0.05; paired t-test), respectively, suggesting an action on the M1 mAChR. Together the results also suggest that both M4 and M1 receptors were involved in the CCh inhibitory effects. It should be noted that no reversal of CCh responses, from inhibition to excitation, was ever observed in these experiments.

![Figure 4](http://jn.physiology.org/)

**CHOLINERGIC MODULATION OF SCN EXCITABILITY**

**FIG. 4.** Carbachol effects on SCN neurons in perforated patch recordings. A. 1 and 2: 2 representative cells for comparison of CCh effects in whole cell (A1) and perforated-patch (A2) recordings. A1: the CCh-induced hyperpolarization has decreased from a magnitude of 16 mV (left) to only 3 mV (right) after 9 min in the whole cell recording, demonstrating rundown of CCh inhibition during the course of experiment. Note that the resting membrane potential also became more hyperpolarized. A2: the CCh-induced hyperpolarization remained about the same after ~25 min in perforated-patch recording and so was the resting membrane potential. B and C: CCh effects on the background membrane current. B: a representative cell to demonstrate the reversible effects of CCh on the resting potential (top), the membrane current (middle), and the I-V relations (bottom). Note the rapid onset but slow washout of CCh-induced hyperpolarization. C: plot of the averaged I-V relation of CCh-activated background currents (n = 11). ⋯⋯, the 0 current levels.


**CCh hyperpolarization is mediated by activation of background K⁺ currents**

We then determine the effects of K⁺ channel blockers on CCh-induced hyperpolarization (ΔV) and CCh-induced outward shift of the holding current at −52 mV (ΔI_{-52}; Fig. 7). The effects of 0.3 mM Ba²⁺, 10 mM TEA, 5 mM 4-aminopyridine (4-AP), combined presence of 0.3 mM Ba²⁺ and 10 mM TEA, and 1 mM Cs⁺ on representative cells are shown in Fig. 7, A–E, respectively, and the statistics summarized in Fig. 7, F and G. As indicated in Fig. 7A, 0.3 mM Ba²⁺ reduced ΔV (2 leftmost panels) and ΔI_{-52} (2 rightmost panels) in a representative cell. On average, 0.3 mM Ba²⁺ reduced ΔV and ΔI_{-52} to 50.5 ± 5.3% (n = 10; P < 0.0001; paired t-test; Fig. 7F) and 52.2 ± 2.6% (n = 7; P < 0.0001; paired t-test; Fig. 7G), respectively. TEA at a concentration of 10 mM also reduced ΔV and ΔI_{-52} in a different cell (Fig. 7B). On average, 10 mM TEA reduced ΔV and ΔI_{-52} to 74.8 ± 3.4% (n = 6; P < 0.005; paired t-test; Fig. 7F) and 66.7 ± 11.2% (n = 6; P < 0.05; paired t-test; Fig. 7G), respectively. In contrast, 5 mM 4-AP slightly reduced ΔV and ΔI_{-52} in another cell (Fig. 7C). On average, 5 mM 4-AP reduced ΔV and ΔI_{-52} to 85.4 ± 6.8% (n = 6; P = 0.09; paired t-test; Fig. 7F) and 83.9 ± 8.3% (n = 6; P = 0.11; paired t-test; Fig. 7G), respectively. The combined presence of 10 mM TEA and 0.3 mM Ba²⁺ blocked most of ΔV and ΔI_{-52} in a fourth representative cell (Fig. 7D). On average, the combined presence of 10 mM TEA and 0.3 mM Ba²⁺ reduced ΔV and ΔI_{-52} to 29.5 ± 4.3% (n = 7; P < 0.0001; paired t-test; Fig. 7F) and 31.3 ± 5.3% (n = 6; P < 0.0005; paired t-test; Fig. 7G), respectively. In addition, 1 mM Cs⁺ slightly reduced ΔV and ΔI_{-52} in a fifth cell (Fig. 7E). On average, 1 mM Cs⁺ changed ΔV and ΔI_{-52} to 104.8 ± 19.1% (n = 6; P = 0.81; paired t-test; Fig. 7F) and 97.6 ± 7.0% (n = 6; P = 0.75; paired t-test; Fig. 7G), respectively. Similar to the lack of effect of Cs⁺, 100 nM tert-leu-Q, a blocker for G-protein-regulated K⁺ channel (GIRK), also had no effect on CCh inhibition (data not shown). Ten times lower concentrations of Ba²⁺ (30 μM), TEA (1 mM), or 4-AP (0.5 mM) were also without effect (data not shown). Together the results suggest that CCh activates at least two background K⁺ currents, one sensitive to submillimolar concentrations of Ba²⁺ and the other to millimolar concentrations of TEA.

**CCh depolarization is mediated via inward shift of the background currents**

As stated in the preceding text, CCh inhibited 78% (94/121) but excited only 3% (4/121) SCN neurons in perforated-patch recordings. We have managed to obtain both current- and voltage-clamped data from three cells (Fig. 8). Figure 8A shows the CCh effects on the membrane potential (top), membrane currents (middle), and the I-V relations (bottom) from one of these cells. For this particular cell, 100 μM CCh depolarized the cell by ~5 mV and produced inward shift of holding current at ~52 mV by ~2 pA. On average, CCh depolarization was 6.0 ± 0.9 mV (n = 3) with perforated-patch recordings, similar to depolarization of 5.3 ± 0.9 mV (n = 9; P = 0.72; Student’s t-test) in excitation-only cells with whole cell recordings (Fig. 3C). The I-V relations indicate reversible inward shifts in the background currents at least at voltages positive to ~92 mV. The CCh-induced inward shifts in the background currents is likely
mediated by inhibition of the background K⁺ currents because in two cells recorded in the whole cell mode, 10 mM TEA completely blocked the CCh-induced depolarization (data not shown). In an additional cell also recorded in the whole cell mode, the CCh-induced depolarization was not altered by 0.3 mM Ba²⁺ (data not shown). Together the results suggest that CCh-induced depolarization is likely mediated by TEA-sensitive, but Ba²⁺-insensitive, background K⁺ currents. Figure 8B shows the averaged I-V relation of the CCh-blocked background currents.

DISCUSSION

This study demonstrates that activation of mAChR or nAChR can regulate spontaneous firing in SCN neurons and that the CCh effects on SCN neuron excitability are apparently mediated by mAChRs. RT-PCR analysis demonstrates the presence of all five mAChR mRNAs in SCN, but the majority of cells (~60% in cell-attached and whole cell and ~80% in perforated-patch recordings) were hyperpolarized by CCh acting on postsynaptic M4 and M1 mAChRs to activate Ba²⁺- and TEA-sensitive back-
FIG. 7. Effects of K⁺ channel blockers on CCh-induced hyperpolarization (ΔV) and outward shift of the holding current at \(-52\) mV (ΔI_{-52}). A–E: 5 representative cells to demonstrate the effects of 0.3 mM Ba²⁺ (A), 10 mM TEA (B), 5 mM 4-aminopyridine (4-AP, C), combined presence of 0.3 mM Ba²⁺ and 10 mM TEA (D) and 1 mM Cs⁺ (E) on ΔV (left 2 panels) and ΔI_{-52} (right 2 panels). F and G: summaries of the K⁺ channel blocker effects on ΔV (F) and ΔI_{-52} (G). *, **, *** the 0 current levels. *P < 0.05; **P < 0.01; ***P < 0.001.
Mechanisms for CCh inhibition of firing

CCh inhibited ~60% cells in cell-attached and whole cell but ~80% cells in perforated-patch recordings. The reason for the difference is not clear at this moment but could be due to the addition of TTX and picrotoxin or change in the cellular responses in perforated-patch recordings. Nevertheless, whole cell and perforated-patch recordings indicate that CCh inhibited firing by hyperpolarizing the membrane potential as a result of activation of background K⁺ currents. However, the reversal potential for the CCh-activated K⁺ conductance was some 10–15 mV more negative to the predicted value, suggesting the involvement of yet unknown mechanisms.

While RT-PCR analysis demonstrates the presence of all five mAChR subtypes, studies with antagonists for mAChRs suggest the involvement of M4 and M1, but not M2 or M3, mAChRs in mediating CCh inhibition. The lack of involvement of M2 subtype is concluded from the observations that the M2 agonist gallamine was without effect and non-M2 antagonist 4-DAMP nearly completely blocked the CCh effects, whereas the lack of effect of selective M3 antagonist J104129 (20 nM, ~5 times of a Kᵢ value of 4.2 nM for M3) (Mitsuya et al. 1999) suggests that M3 subtype was probably not involved. The rank order of potency of 4-DAMP ~ himbacine ~ AF-DX 384 > pirenzepine (all at 1 μM) suggests that CCh inhibition is mediated mainly via M4 and to a lesser extent via M1 mAChR. Further evidence for the involvement of M4 mAChR comes from the observation that CCh inhibition was partially (~45%) blocked by the highly selective M4 antagonist MT3. The partial (~30%) block of CCh inhibition by J104129 at a concentration of 100 nM (~5 times of a Kᵢ value of 19 nM for M1) (Mitsuya et al. 1999) also suggests the involvement of M1 mAChR. The lack of reversal from CCh inhibition to CCh excitation in the presence of mAChR antagonists suggest that CCh excitation, if present in the same cells, is weak compared with CCh inhibition, a conclusion consistent with the rare occurrence (3%) of CCh-induced depolarization in perforated patch recordings. Because no day-night difference was observed in response proportions by CCh or muscarine, the signaling mechanisms from receptors to effectors might be similar at day and at night. This is contrary to the cholinergic modulation of circadian timing, which is mediated via M1 mAChR and is restricted to the nocturnal phase to advance circadian rhythms (Gillette et al. 2001; Liu and Gillette 1996; but see Bina and Rusak 1996).

CCh most likely activates at least two types of background K⁺ currents to produce membrane hyperpolarization. One background K⁺ current was sensitive to Ba²⁺ but not to TEA or 4-AP, suggesting an origin of two-pore domain K⁺ channels (K2P), which are known to be responsible for the resting

depolarizations were produced by muscarine and McN-A-343 but not by nicotine and choline. The inability of nicotine and choline to reproduce the CCh effects may be accounted for by the nature of nAChR subunits in SCN neurons. In adult SCN, a7-nAChR is the most abundant nAChR subunits (O’Hara et al. 1999). Because CCh is a poor agonist for a7-nAChR, ~1,000 times weaker than nicotine in displacing α-bungarotoxin binding (Anand et al. 1993), CCh may not be able to activate a7-nAChR as it does to mAChR. The ability of atropine and inability of mecamylamine to block CCh effects on firing rate are consistent with this view.

CCh acts on muscarinic receptors to regulate SCN firing rate

Cell-attached recordings suggest that the CCh effects on firing rate are mediated by mAChR because similar response proportions were produced by muscarine and McN-A-343 but
membrane potential in a wide variety of cells (Patel and Honoré 2001). In situ hybridization studies reveal high expression levels of TASK3 in SCN (Karschin et al. 2001; Talley et al. 2001) with moderate levels of TASK1 and TRAAK (Talley et al. 2001) or undetectable level of TASK1 and TASK5 (Karschin et al. 2001). Our RT-PCR analysis confirmed the presence of TASK1, TASK3, and TRAAK mRNA in SCN (Hsu and Huang, unpublished results), and experiments are underway to determine the involvement of two-pore domain K⁺ channels in mediating the CCh inhibition. The other background K⁺ current was sensitive to millimolar concentrations of TEA (10 mM) and perhaps 4-AP (5 mM), but not to 1 mM TEA or 0.5 mM 4-AP. The identity of this background current remained to be determined, but its activation by CCh at subthreshold voltages suggests an origin of a voltage-dependent subthreshold K⁺ current in these cells. Although CCh also activated an inward rectifier, the lack of effect of 1 mM Cs⁺ and 100 nM tertiapin-Q on ΔV and ΔI_lick suggests that the action did not contribute to CCh-induced hyperpolarization.

Mechanisms for CCh enhancement of firing

CCh excited ~20% cells in cell-attached and whole cell but only 3% cells in perforated-patch recordings. Again the reason could be due to the addition of TTX and picrotoxin or change in the cellular responses in perforated-patch recordings. The change in cellular responses in different recording modes is suspected, however, at least for the different percentage of mixed response cells in cell-attached and whole cell recordings: only 4% (2/48) CCh-excited cells exhibited mixed responses of inhibition and excitation in cell-attached recordings, compared with 50% (9/18) CCh-excited cells in whole cell recordings. Results from whole cell and perforated-patch recordings indicate that the postsynaptic origin of CCh depolarization might be mediated by inhibition of a TEA-sensitive, and perhaps Ba²⁺-insensitive, background K⁺ current. However, due to the nature of very infrequent encounter, we were only able to obtain both current- and voltage-clamped data from three cells with even fewer cells for channel blocker studies (2 cells blocked by 10 mM TEA, and one cell insensitive to 0.3 mM Ba²⁺).

Comparison with previous studies

Our cell-attached recordings (55% inhibition and 21% excitation) are more consistent with previous results made also from in vitro, but not in vivo, studies in rat SCN: acetylcholine is more inhibitory than excitatory with in vitro single-unit recordings [26% inhibition and 7% excitation (Shibata et al. 1983); 39% inhibition and 1% excitation (Kow and Pfaff 1984)] but is predominantly excitatory in an in vivo study (11% inhibition and 80% excitation) (Nishino and Koizumi 1977). While the apparent discrepancy between in vitro and in vivo results remains unresolved, this study has elucidated the mechanisms for CCh inhibition of firing and, to a lesser extent, for CCh enhancement of firing. We also do not know the relationship between the different subsets of SCN neurons based on cholinergic responsiveness as in this study and the functionally characterized four groups of SCN neurons based on dendritic structures (Jiang et al. 1997).

Muscarinic modulation of SCN neuronal excitability

It remains to be determined how activation of M4 and M1 receptors leads to activation of background currents to inhibit SCN neuronal excitability. The activation of second-messenger-mediated pathways may be involved as suggested by the slow washout of CCh-induced hyperpolarization (Fig. 4B). On the one hand, coupling of M1 to G_i inhibits the TASK subfamily of K2P channels or M current and increases neuronal excitability (Mathie 2007; Suh and Hille 2005). Because TASK1 and TASK3 are known to be present in SCN (Karschin et al. 2001; Talley et al. 2001; unpublished results), it is possible that the M1/G_Q/TASK signaling pathway might contribute to CCh excitation. Although we do not know how activation of M1 receptor might lead to inhibition, our unpublished results (Yang and Huang) suggest a partial involvement of PKC in CCh inhibition. On the other hand, while coupling of M4 to G_q could potentially activate GIRK, the lack of effect of Cs⁺ and tertiapin-Q did not support its participation in CCh inhibition. Instead G_q-mediated activation of K2P channels has been shown to occur in TREK (Mathie 2007) and TWIK channels (Deng et al. 2007). Although in situ hybridization study did not support the presence of these K2P channels in SCN (Talley et al. 2001), it remains an interesting possibility for further investigations.

Functional implications

This study demonstrates direct modulation of SCN neuron excitability by cholinergic activation of nAChR and mAChR; however, the different response proportions by nicotine and muscarine suggest different roles of mAChR and nAChR. Specifically, the abundant presence of α7-nAChR in adult SCN (O’Harra et al. 1999) and the ability of choline to mimic nicotine effects suggest that acetylcholine and its metabolite choline may act on α7-nAChR to regulate SCN neuron excitability.

Importantly our data have drawn a consistent picture regarding the actions of CCh. All three recordings indicate ~20% cells being refractory to CCh stimulation irrespective of the presence of GABAergic neurotransmission. Most SCN neurons were inhibited postsynaptically by CCh acting on M4 and M1 mAChRs to activate background K⁺ currents. The remaining cells were excited by CCh through membrane depolarization likely mediated by postsynaptic inhibition of background K⁺ currents. As such acetylcholine may enhance or reduce SCN neuron excitability in different physiological settings via acting on different populations of neurons. In this context, our findings may help shed light on the positive correlation between the firing rates of SCN neurons with sleep states (Deboer et al. 2003). The presumably increased release of acetylcholine in SCN during REM sleep and waking may act on populations of CCh (muscarine)- and/or nicotine-excited cells to increase firing rate. Experimental confirmation of the idea may require identification of active cholinergic neurons in the brain areas projecting to the SCN and of the responsive SCN neurons. Along the line of thinking, the cholinergic basal forebrain neurons have been shown to fire at high rates during REM sleep and waking and virtually cease firing during NREM sleep (Lee et al. 2005).

This study also raises an interesting question whether cholinergic modulation of neuronal excitability plays a role in the
cholinergic regulation of circadian timing. Recently Buchanan and Gillette (2005) resolves the issue of site-dependent effect of CCh on circadian timing (the so-called “CCh paradox”) (Colwell et al. 1993) by demonstrating that CCh acts on cholinergic receptors in SCN to produce phase advance at night but acts on extra-SCN cholinergic sites to produce light-like biphasic phase shifts. Because the CCh effects on firing rates were similar between day and night, in contrast to its night-time effects on phase shifts, it appears that acetylcholine may be independently involved in excitability and circadian timing. Nonetheless the involvement of M1 receptors in regulating both excitability and circadian timing suggests that M1 (along with M4-) mediated changes in neuronal excitability, particularly at night, could potentially participate in the M1-mediated phase shifting.

Finally, the observations of ~80% SCN neurons being responsive to postsynaptic CCh stimulation and of all five subtypes of mACHr being expressed in SCN suggest cholinergic functions beyond modulation of excitability and circadian timing. Results from deletion of basal forebrain cholinergic inputs to SCN suggest that acetylcholine also plays a trophic role in maintaining SCN integrity, with a bearing on understanding circadian rhythm dysfunctions associated with Alzheimer’s disease (Madeira et al. 2004). Further elucidation of receptor, signaling, and ionic mechanisms involved in cholinergic actions should help better understand their functional implications.

GRANTS

This work was supported by Taiwan National Science Council Grants NSC96-2745-B-182-002-URD and NSC96-2330-B-182-028-MY2 to R. C. Huang and Chang Gung Medical Research Foundation Grant CMRP160251 to R. C. Huang and by Molecular Medicine Research Center.

REFERENCES


