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Muscarinic acetylcholine response in pyramidal neurones of rat cerebral cortex

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1 The effects of acetylcholine (ACh) on pyramidal neurones acutely dissociated from the rat cerebral cortex were studied in the whole-cell mode, by use of the nystatin-perforated patch recording configuration.

2 ACh induced a net inward current (I_{ACh}) accompanied by a membrane conductance decrease at a holding potential (V_H) of -40 mV. I_{ACh} increased in a concentration-dependent manner with a half-maximum concentration (EC_{50}) of 8.7×10^{-7} M.

3 I_{ACh} mainly resulted from the suppression of the voltage- and time-dependent K^+ current (M-current).

4 Muscarine and muscarinic agonists such as McN-A-343, oxotremorine and oxotremorine-M mimicked the ACh response. The potency was in the order of oxotremorine-M > McN-A-343 ≥ muscarine > oxotremorine.

5 Pirenzepine shifted the concentration-response curve for ACh to the right and the corresponding Schild plot yielded a pA_2 value of 7.81. Other muscarinic antagonists also reversibly blocked I_{ACh} in a concentration-dependent manner. The inhibitory potency was in the order of atropine > 4-DAMP > pirenzepine > AF-DX-116.

6 I_{ACh} could be induced normally even after pre-incubation of dissociated neurones in external solution with 200 ng ml^{-1} pertussis toxin (PTX) for 8 h, whereas the inhibitory effect of ACh on high-voltage-activated Ca^{2+} channels was completely abolished by the PTX treatment.

Keywords: Rat cortical pyramidal neurone; muscarinic acetylcholine receptor; M-current

Introduction

The cholinergic innervation to the cerebral cortex has intrigued investigators for many years because of its possible roles in higher brain functions such as arousal, memory and learning (Squire & Davis, 1981). Interest in the cortical cholinergic system has been stimulated further by the fact that there are deficits of this system in patients with Alzheimer's disease (Coyle *et al.*, 1983). One major step toward understanding this system was the study of the muscarinic acetylcholine receptors in the central nervous system (McKinney & Richelson, 1984). The activation of muscarinic receptors has been shown to evoke a wide variety of responses, suggesting that multiple subtypes might exist. Molecular biological studies have confirmed the existence of at least five distinct subtypes (m1–m5) of muscarinic receptor genes (Bonner, 1989). The m1–m4 genes correspond most closely to the pharmacologically defined M_1–M_4 subtypes, respectively (Hulme *et al.*, 1990; Lazareno *et al.*, 1990).

Many biochemical and electrophysiological studies have addressed the ACh response in the mammalian cortex. In the rat cortex, the activation of muscarinic receptors stimulates phosphoinositide hydrolysis (Downes, 1982) and evokes a synaptic inhibition and a slow excitation (Krnjevic *et al.*, 1971; Krnjevic, 1974). In the guinea-pig cortex, the muscarinic receptor with high affinity for pirenzepine depolarizes the pyramidal neurones, while that with low affinity for pirenzepine is involved in the activation of inhibitory interneurones (McCormick & Prince, 1985). The ACh-induced depolarization of pyramidal neurones is mainly due to a decrease in a voltage-dependent K^+ current known as the M-current (I_M; Brown & Adams, 1980). However, the muscarinic receptor subtypes and the pharmacological properties involved in these responses have not been fully elucidated in this brain area.

In the present study, therefore, we investigated quantitatively the ACh-induced response and its pharmacological properties in mammalian cortical neurones under voltage-clamp conditions. For this purpose we used pyramidal neurones acutely dissociated from the rat cerebral cortex. The nystatin perforated patch recording configuration was used to maintain, intact, the intracellular second messenger systems.

Methods

Preparation

Single pyramidal neurones of the cerebral cortex were acutely dissociated as reported previously (Ito *et al.*, 1991). Briefly, 1- to 2-week-old Wistar rats were decapitated under deep ether anaesthesia. The area between 2 and 4 mm from the anterior tip of the frontal lobe was cut into coronal slices (350 μm thick) with a microslicer (D.S.K., Model DTK-1000), and pre-incubated in incubation solution saturated with 95% O_2:5% CO_2 for 40 min at room temperature (22–25°C). Thereafter, the slices were treated with enzymes. The enzyme treatments were made first in an oxygenated standard external solution with 0.015% pronase for 15 min at 31°C and successively in 0.015% thermolysin under the same conditions. The slice preparations were then rinsed with Ca^{2+}-free EGTA external solution and incubated for another 15 min in standard solution. The dorsomedial portion of the cortex was micro-punched out and dissociated mechanically with fine fire-polished glass Pasteur pipettes in a small plastic culture dish (Falcon) filled with the standard solution. Dissociated neurones adhered to the bottom of the dish within 30 min, allowing electrophysiological studies to be conducted. The neurones having the original morphological features of pyramidal neurones such as the pyramidal somatic shape and the prominent apical dendritic process were used.

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Electrical measurement

Electrical recordings were performed by use of the nystatin perforated patch recording with some modifications (Wakamori *et al.*, 1993). Patch pipettes were fabricated from glass capillaries (Narishige, 1.5 mm outer diameter) on a two-state puller (Narishige, PB-7). The resistance between the patch-pipette filled with the internal soluton and the reference electrode was 4 to 8 MΩ. After formation of the stable perforated patch, the series resistance ranged from 16 to 20 MΩ. Ionic currents and voltages were measured with a patch-clamp amplifier (List Electronic, EPC-7). Signals were filtered with a low-pass filter (NF Electronic Instruments, FV-665) at a cut off frequency of 1 kHz and were monitored simultaneously on a storage oscilloscope (Iwatsu, MS-5100A) and a pen-recorder (San-ei, RECTI HORIZ 8K). Records were stored on a video cassette recording system (Mitsubishi, HV-F93) via a pulse coded modulation processor (SONY, PCM 501) for subsequent analysis using the pCLAMP system (Axon Instruments). All the experiments were performed at room temperature (22–25°C).

Statistical analysis

The data are presented as the mean ± standard error of the mean (s.e.mean) in the text, and the s.e.mean is indicated by a vertical bar in figures.

The continuous theoretical curves for concentration-response relationships were constructed according to a modified Michaelis-Menten equation (1) using a least-squares fitting routine (Kijima & Kijima, 1980):

$$I = I_{max} \frac{C^n}{C^n + EC_{50}{}^n} \qquad (1)$$

where I is the drug-induced current amplitude and C is the corresponding drug concentration. EC_{50} and n denotes the half-maximum concentration and Hill slope, respectively. The equation for the concentration-inhibition curve is the mirror image of the Michaelis-Menten equation:

$$I = 1 - \frac{C^n}{C^n + IC_{50}{}^n} \qquad (2)$$

where I is the current amplitude normalized by that of the control and IC_{50} denotes the half-inhibition concentration of antagonists.

Solutions and their application

The ionic composition of the standard external solution was (in mM): NaCl 150, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid (HEPES) 10 and glucose 10. The pH was adjusted to 7.4 with tris (hydroxymethyl) aminomethane base (Tris-base). The ionic composition of the incubation medium was (in mM): NaCl 125, KCl 5, KH_2PO_4 1.2, $MgSO_4$ 1.3, $CaCl_2$ 2.4, $NaHCO_3$ 26 and glucose 10, aerated with 95% O_2:5% CO_2 gas to a final pH of 7.4. The ionic composition of the external solution with 10 mM Ba^{2+} was (in mM): NaCl 138, CsCl 5, $MgCl_2$ 1, $BaCl_2$ 10, HEPES 10, glucose 10 and tetrodotoxin 0.001. The pH was adjusted to 7.4 with Tris-base. The ionic composition of the pipette solution was (in mM): KCl 150 and HEPES 10 for recording the ACh-induced current, and CsCl 150 and HEPES 10 for recording the high-voltage activated Ba^{2+} current (HVA I_{Ba}). The pH was adjusted to 7.2 with Tris-base. A fresh stock solution of 10 mg ml^{-1} nystatin in methanol was prepared every day and kept at − 20°C. This was then dissolved into the pipette solution to produce a final nystatin concentration of 150 μg ml^{-1}. The final pipette solution was made fresh every 2 or 3 h.

Drugs were applied by a rapid application system termed the 'Y-tube' method, as described elsewhere (Murase *et al.*, 1990). With the use of this technique, the solution surrounding a dissociated neurone could be completely exchanged within 10 to 20 ms.

Drugs

The drugs used in the present experiments were acetylcholine chloride (ACh), muscarine chloride, nicotine, nystatin and thermolysin (Sigma), tetrodotoxin (TTX) (Sankyo), atropine sulphate (Merck), pertussis toxin (PTX) (Seikagaku Kougyo), pronase (Calbiochem), oxotremorine-M, 4-diphenylacetoxy-N-methylpiperidine (4-DAMP) methiodide and 4-(m-chlorophenylcarbamoyloxy)2-butynyltrimethylammonium chloride (McN-A-343) (RBI). 11-2[[2-[(dietylamino)methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one (AF-DX-116) was kindly given by Prof. T. Kamiya (Fukuoka University, Japan). All drugs were dissolved in external solution just before use.

Results

ACh-induced currents

In the perforated patch recording configuration, 10^{-5} M ACh evoked a net inward current (I_{ACh}) associated with a 30 to 60% decrease in membrane conductance in 75 out of 110 cortical pyramidal neurones at a holding potential (V_H) of − 40 mV (Figure 1a, upper panel). The inward currents were long-lasting and typically persisted for 4 to 5 min after the application of ACh for 30 to 40 s. The lower panel in Figure 1a illustrates the effect of ACh on currents evoked by 300 ms hyperpolarizing step pulses from − 40 to − 50 mV. The step pulse represented a leak current plus the M-current (I_M; Brown & Adams, 1980; Constanti & Brown, 1981) before the application of ACh. During the steady-state of I_{ACh}, the step pulse represented the deactivation of I_M. The I_M was reactivated by the removal of ACh. A small transient outward current was observed in 35 out of 110 neurones (31.8%), which was followed by a slow inward current (Figure 1b).

During the perforated patch whole-cell recording, the application of ACh at 10 min intervals could elicit I_{ACh} with stable current amplitudes over 1 h (Figure 1c(i)), whereas the conventional whole-cell recording (Akaike *et al.*, 1978; Hamill *et al.*, 1981) caused a considerable 'run-down' of I_{ACh} within 15 min after rupture of the membrane patch (Figure 1c(ii)). It is known that I_M in rat sympathetic ganglion cells slowly declines over a period of 15 min in a conventional whole-cell patch recording (Brown *et al.*, 1989). ATP added to the pipette solution can reduce the run-down (Pfaffinger, 1988). However, this may alter the responsiveness for ACh since the muscarinic ACh response is mediated by multiple steps of the intracellular signal transduction system. In the present study, therefore, the nystatin perforated patch recording configuration was used in subsequent experiments to prevent wash-out of the ACh response.

Current-voltage (I-V) relationship for ACh response

The concentrations of K^+ in the external and internal (pipette) solutions were 5 and 150 mM, respectively. The voltage-dependent Ca^{2+} and Na^+ channels were suppressed by adding 10^{-5} M La^{3+} and 10^{-6} M tetrodotoxin, respectively. Figure 2a illustrates the inward currents evoked by 10^{-5} M ACh at various V_Hs. As shown in Figure 2a and summarized in Figure 2b, the I_{ACh} had distinct voltage-dependency. The I_{ACh} increased as the membrane depolarized and was not observed at potentials more negative than − 80 mV without showing any reversal of current direction, being consistent with that of I_M. Figure 2c shows the current-voltage (*I-V*) relationships to asymmetrical hyperpolarizing ramp voltage between − 20 and − 100 mV from a V_H of − 40 mV, before (control) and during the application of ACh. In this case, the *I-V* property was also identical to that of I_M.

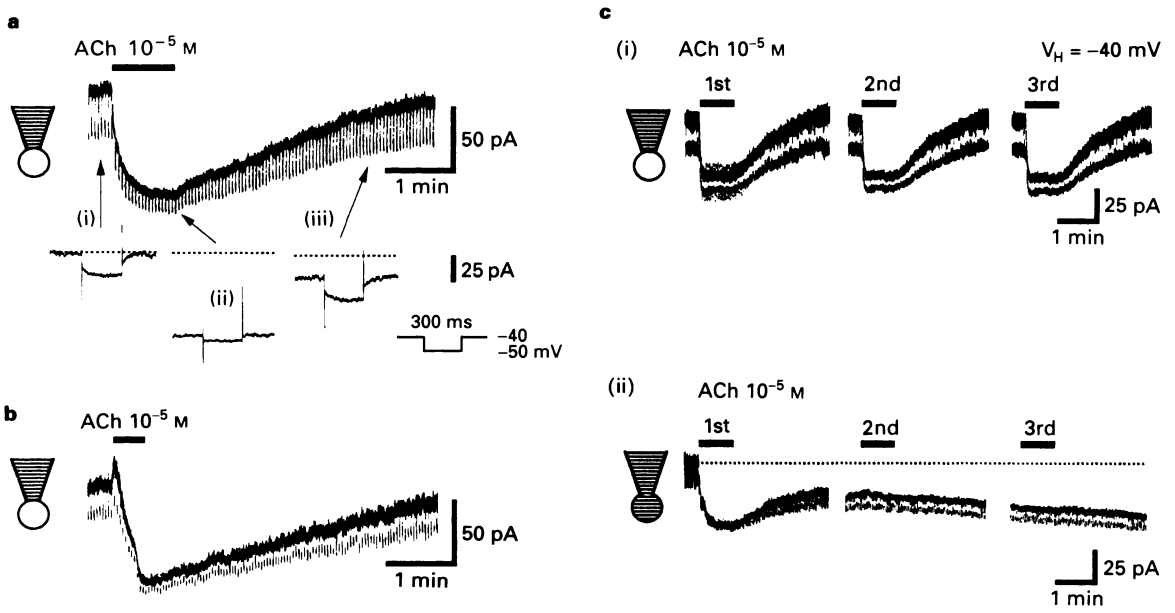

Figure 1 Effects of ACh (10^{-5} M) on the pyramidal neurones freshly dissociated from the rat cerebral cortex. (a) ACh-induced inward current (I_{ACh}) recorded by the nystatin perforated patch technique. The neurone was held at a holding potential (V_H) of − 40 mV and a brief hyperpolarizing pulse of 10 mV with 300 ms duration was applied every 2 s. ACh was applied during the period indicated by the horizontal bar above each current recording. Inward current relaxations produced by a hyperpolarizing voltage command from − 40 to − 50 mV, before, during and after the application of 10^{-5} M ACh are expanded for detail and shown in the lower panel of (a). (b) ACh elicited a small transient outward current followed by a long-lasting inward current in 31.8% of neurones tested. (c) (i) In nystatin perforated patch mode, I_{ACh} was constantly recorded by successive applications of ACh. (c) (ii) I_{ACh} recorded in the conventional whole-cell mode at a V_H of − 40 mV. The I_{ACh} showed run-down with time. Recordings (i) and (ii) were obtained from different neurones.

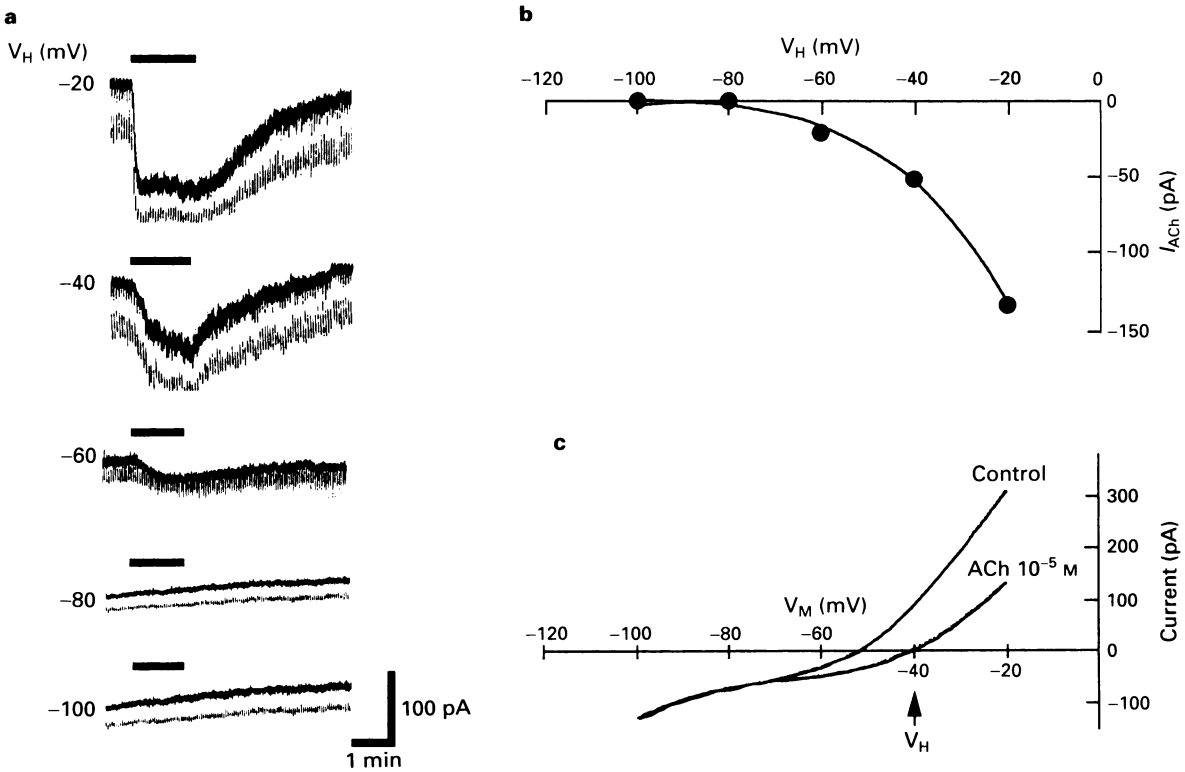

Figure 2 ACh response and its current-voltage (*I-V*) relationship. (a) ACh-induced currents at various V_Hs. Horizontal bars indicate the period of application of 10^{-5} M ACh. (b) *I-V* relationship of the I_{ACh} at various V_Hs. The data were obtained from (a). Similar results were obtained from five cells. (c) *I-V* relationships obtained by ramp-commands between − 20 and − 100 mV in 2 s in the presence or absence of 10^{-5} M ACh. V_M; membrane potential. Similar results were obtained from six cells.

Concentration-response relationships for ACh and its agonists

Figure 3a(i) and (ii) show typical inward currents induced by ACh and muscarine of various concentrations at a V_H of -40 mV, respectively. Nicotine evoked no response at concentrations up to 10^{-3} M. Figure 3b summarizes the concentration-response relationships for ACh and its agonists. The ACh and muscarine responses increased in a concentration-dependent manner. Muscarine was approximately 10 times less potent than ACh. The half maximum concentration (EC_{50}) of ACh in the concentration-response curve was 8.7×10^{-7} M. The Hill coefficient was 0.9. The EC_{50} value and Hill coefficient of the muscarine response were 8.6×10^{-6} M and 0.8, respectively. These results suggest that the ACh response is mediated by the muscarinic ACh receptors. Therefore, the effects of muscarinic agonists such as McN-A-343, oxotremorine and oxotremorine-M were tested in the cortical pyramidal neurones. Figure 3a(iii) shows the inward currents evoked by McN-A-343 in a concentration-dependent manner. Figure 3b also summarizes the concentration-response relationships for McN-A-343 and two other muscarinic agonists. The EC_{50} of McN-A-343 was 4.5×10^{-6} M. Oxotremorine, a cardiac M_2 agonist (Brann *et al.*, 1987), was about 30 times less potent than muscarine. The order of potency was ACh > oxotremorine-M > McN-A-343 $\geqslant$ muscarine > oxotremorine.

Effects of muscarinic antagonists

The effects of muscarinic antagonists (atropine, pirenzepine, 4-DAMP and AF-DX-116) on the I_{ACh} were examined. Pirenzepine shifted the concentration-response curve for ACh to the right. Figure 4a(i) shows the concentration-response relationships for ACh obtained under control condition (●), and in the presence of 10^{-7} (▲), 3×10^{-7} (◆) and 10^{-6} M (■) pirenzepine ($n = 4-5$ in each case), with EC_{50} values of 8.49×10^{-7}, 7.17×10^{-6}, 1.64×10^{-5} and 7.64×10^{-5} M, respectively. Figure 4a(ii) illustrates the corresponding Schild plot (Arunlakshana & Schild, 1959). The estimated pA_2 value was 7.81. Figure 4b summarizes the concentration-inhibition curves of various antagonists on I_{ACh} elicited by 10^{-5} M ACh. Atropine, pirenzepine, 4-DAMP and AF-DX-116 reversibly blocked I_{ACh} in a concentration-dependent manner. The half-inhibition concentrations (IC_{50}) were 2.49×10^{-9} M for atropine, 1.18×10^{-8} M for 4-DAMP, 2.07×10^{-7} M for pirenzepine and 3.35×10^{-6} M for AF-DX-116.

Effect of pertussis toxin (PTX)

It is well established that muscarinic receptors are linked to G-proteins (Bonner, 1989). To investigate what kind of G-proteins may mediate the I_{ACh}, the dissociated cortical neurones were incubated in standard external solution with or without PTX 200 ng ml^{-1} for 8 h, because PTX is able to ADP-ribosylate the α-subunit of G_i and G_o proteins, resulting in the inhibition of their activation by the receptors (Kurose *et al.*, 1983; Murayama & Ui, 1983). The PTX-treated and non-treated neurones were voltage-clamped at a V_H of -40 mV and tested with 10^{-5} M ACh. The amplitude and response pattern of I_{ACh} in the PTX-treated neurones did not differ from those of the control without PTX treatment (Figure 5a, $n = 8$). To ensure that PTX was effective, the effect of ACh on the high-voltage activated Ba^{2+} inward

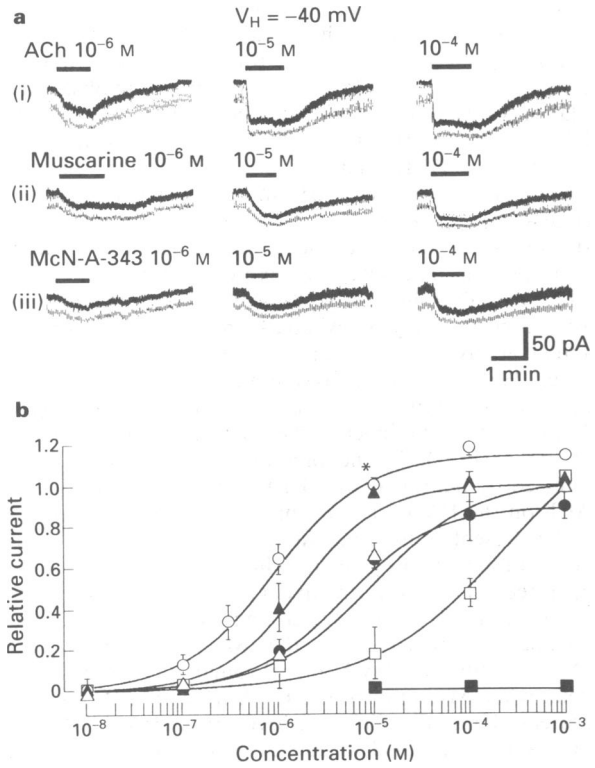

Figure 3 Concentration-response relationships for ACh agonists. (a) The inward currents induced by ACh, muscarine and McN-A-343 of various concentrations at a V_H of -40 mV. The neurones were exposed to each agonist for the period indicated by a horizontal bar. (b) Concentration-response relationships for the peak inward current induced by ACh (○), muscarine (△), McN-A-343 (●), oxotremorine (□) and oxotremorine-M (▲). Nicotine (■) elicited no response. All currents were normalized to the peak current amplitude induced by 10^{-5} M (*). Each point represents the mean (± s.e.mean) of six to eight neurones.

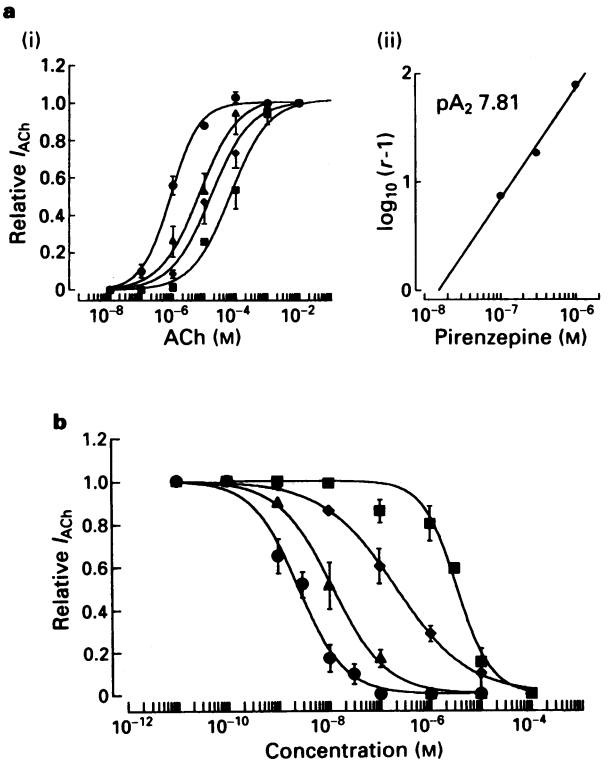

Figure 4 Effects of muscarinic antagonists on I_{ACh}. (a) (i) Shift of the concentration-response curves for ACh (●) by 10^{-7} (▲), 3×10^{-7} (◆) and 10^{-6} M (■) pirenzepine. (ii) Corresponding Schild plot where r is the agonist concentration ratio. The line was fitted by linear regression and yielded a pA_2 for pirenzepine of 7.81 with a unconstrained slope of 1.07 ($R^2 = 0.99$). (b) Concentration-dependent inhibition curves of I_{ACh} in the presence of atropine (●), 4-DAMP (▲), pirenzepine (◆) and AF-DX-116 (■). Neurones were pre-treated for 2 min with each antagonist. Each symbol represents the mean (± s.e.mean) of five to seven neurones.

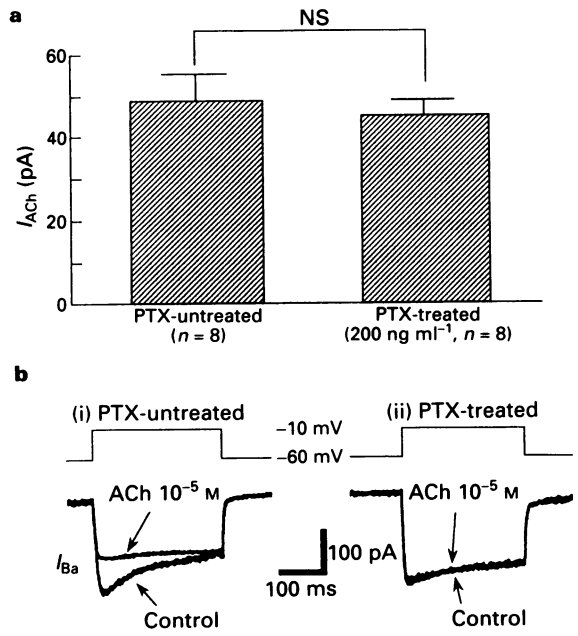

Figure 5 Effects of pertussis toxin (PTX) on I_{ACh} and HVA I_{Ba}. (a) Dissociated neurones were immersed in external solution with or without PTX for 8 h. NS: no significant difference. *n*: the number of neurones tested. (b) HVA I_{Ba} was evoked by a 300 ms step pulse to − 10 mV from a V_H of − 60 mV. In these experiments, neurones were pretreated with ACh for at least 2 min. (b) (i) ACh 10^{-5} M suppressed HVA I_{Ba} in neurones without PTX treatment. (ii) ACh had no effect on HVA I_{Ba} in neurones treated with 200 ng ml^{-1} PTX for 8 h ($n = 12$).

current (HVA, I_{Ba}) passing through the Ca^{2+} channel was simultaneously examined in K^+-free Cs^+ external solution containing 10 mM-Ba^{2+} in the presence or absence of PTX (Figure 5b). The HVA I_{Ba} was induced by a 300 ms depolarizing command step to − 10 mV from a V_H of − 60 mV, at which potential the low-voltage activated Ca^{2+} channel was inactivated (Akaike *et al.*, 1989). Here, 10^{-5} M ACh suppressed the activation phase of I_{Ba} in control neurones 8 h after the dissociation ($n = 8$), whereas ACh had no effect on I_{Ba} in neurones treated with PTX for 8 h ($n = 12$).

Discussion

In the present study, ACh induced a long-lasting net inward current (I_{ACh}) in rat cortical pyramidal neurones. This inward current resulted from the decrease in a voltage- and time-dependent K^+ conductance known as the M-current (I_M; Brown & Adams, 1980). This reduction of I_M is widely observed in various neurones such as neurones in the sympathetic ganglia (Brown & Adams, 1980; Constanti & Brown, 1981), the hippocampus (Benardo & Prince, 1982; Halliwell & Adams, 1982), the cerebral cortex (McCormick & Prince, 1985; Constanti & Sim, 1987) and the amygdala (Womble & Moises, 1992). The inhibition of I_M by ACh in these neurones may greatly enhance the neuronal excitability, thereby regulating functions of neuronal systems.

Molecular cloning studies have identified five genes for muscarinic ACh receptors denoted as m 1–m 5 (Bonner *et al.*, 1988) and the receptors expressed for m 1–m 4 may correspond pharmacologically to M_1–M_4 (Hulme *et al.*, 1990). Although muscarinic receptors cannot be differentiated exclusively with the agonists and antagonists, the receptors do show different sensitivity to these drugs. In the present study, pirenzepine effectively inhibited I_{ACh}, and the pA_2 value for pirenzepine was 7.81. This high affinity of pirenzepine is closer to those of M_1 and M_4 than that of M_2, M_3 and the receptor expressed for m 5. Atropine, 4-DAMP and AF-DX-116 also suppressed I_{ACh}. The sensitivity of I_{ACh} to these antagonists was in the order of atropine > 4-DAMP > pirenzepine > AF-DX-116, whereas AF-DX-116 has higher affinity for M_2 than pirenzepine (Micheletti *et al.*, 1987; Buckley *et al.*, 1989). Oxotremorine, the cardiac M_2 agonist, could induce inward current only at high concentrations. Thus, M_2 was not involved in the present response. McN-A-343 is functionally potent in stimulating the M_1 receptor and has less activity at M_2 and M_3, though this compound displays little selectivity for muscarinic receptors in the binding assay (Birdsall *et al.*, 1983; Freedman *et al.*, 1988; Lambrecht *et al.*, 1993). McN-A-343 mimicked the ACh response in the present preparation, while this agonist could not activate M_3 in CA1 pyramidal neurones (Wakamori *et al.*, 1993). Therefore, the candidate subtypes responsible for the I_{ACh} may be M_1 or M_4. In transfected NG108-15 cells, expressed receptors for m 1 and m 3 are capable of inhibiting I_M, while m 2 and m 4 are not (Fukuda *et al.*, 1988; Robbins *et al.*, 1991). In rat sympathetic ganglion cells M_1 also could couple to I_M but M_4 could not (Marrion *et al.*, 1989; Bernheim *et al.*, 1992). In addition, M_4 is known to link to PTX-sensitive G-protein, while the present response was PTX-insensitive. Taking these facts into account, the M_1 subtype may be predominantly responsible for the reduction of I_M in rat cortical pyramidal neurones. Immunocytochemistry (Levey *et al.*, 1991; Wall *et al.*, 1991) and *in situ* hybridization studies (Buckley *et al.*, 1988) showed the presence of multiple subtypes of muscarinic ACh receptors in the rat neocortex. The m 1 subtype, most abundant in this brain area, may regulate I_M. The m 2 protein was also recognized by immunocytochemistry, whereas mRNA for the m 2 gene is low. This is consistent with the suggestion that this subtype possibly functions as an autoreceptor at the ends of cholinergic fibres projected from the basal forebrain (Levey *et al.*, 1991). The proteins and mRNA for m 3 and m 4 were also detected. Although the M_3 receptor can be involved in the regulation of I_M (Fukuda *et al.*, 1988; Robbins *et al.*, 1991) and M_4 can be coupled to the high-voltage-activated (HVA) Ca^{2+} channel (Bernheim *et al.*, 1992), the functional significances of these subtypes in the cortex remain to be elucidated.

Multiple G-proteins are known to mediate the muscarinic ACh responses. The receptors expressed for m 1, m 3 and m 5 link to a PTX-insensitive G-protein and regulate inositol phospholipid hydrolysis, whereas those for m 2 and m 4 inhibited adenylyl cyclase through PTX-sensitive G-protein (Bonner, 1989). In electrophysiological studies, the inhibition of I_M in various types of neurones is PTX-insensitive (Dutar & Nicoll, 1988; Pfaffinger, 1988; Brown *et al.*, 1989; Bernheim *et al.*, 1992). In the present study, the I_{ACh} was not suppressed by treatment with PTX, while the inhibitory effect of ACh on the HVA Ca^{2+} channel was abolished, suggesting that the present muscarinic ACh receptor also regulated I_M via PTX-insensitive G-protein, while the HVA Ca^{2+} channel is operated by a PTX-sensitive G-protein. Recently, Bernheim *et al.* (1992) demonstrated that M_1 and M_4 coexist in rat sympathetic neurones and that M_1 suppresses I_M and the HVA Ca^{2+} current (HVA I_{Ca}) through PTX-insensitive G-protein, while M_4 inhibited the HVA I_{Ca} with a PTX-sensitive G-protein. Thus, the PTX-sensitive muscarinic inhibition of the HVA Ca^{2+} channel in cortical pyramidal neurones may be also mediated by another co-existing receptor subtype such as M_2 or M_4 rather than by M_1. In the present study, a PTX-insensitive reduction of the HVA Ca^{2+} channel was not observed. This may reflect differences between species or the brain region.

Of the neurones tested, 31.8% showed a small transient outward current, although its physiological implication is unknown. In hippocampal CA1 neurones, many inositol hydrolysis-mediated responses were accompanied by a transient outward K^+ current (Uneyama *et al.*, 1993; Wakamori *et al.*,

1993; Shirasaki *et al.*, 1994). Here, ACh and 5-hydroxytryptamine facilitated inositol triphosphate formation and thereby mobilized Ca^{2+} from intracellular stores. Then, Ca^{2+}/calmodulin-dependent protein kinase activated a K^+ conductance, resulting in an outward current. In the cortical region, ACh facilitated inositol hydrolysis via a muscarinic receptor with high affinity for pirenzepine, possibly M_1 (Downes, 1982). Hence, the present outward current may be activated through the inositol hydrolysis pathway.

Alzheimer's disease is considered to be associated with a marked depletion of cortical acetylcholinesterase and the loss of cortical cholinergic innervation (Mesulam & Geula, 1988). Furthermore, M_1-selective antagonists impair memory in animals (Messer *et al.*, 1990). Therefore, the understanding of the actions of ACh on cortical pyramidal neurones may facilitate the development of pharmacological therapies for Alzheimer's disease and other neurological disorders in this brain area.

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References

AKAIKE, N., KOSTYUK, P.G. & OSIPCHUK, Y.V. (1989). Dihydropyridine-sensitive low-threshold calcium channels in isolated rat hypothalamic neurones. *J. Physiol.*, **412**, 181–195.

AKAIKE, N., LEE, K.S. & BROWN, A.M. (1978). The calcium current of Helix neurone. *J. Gen. Physiol.*, **71**, 509–531.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BENARDO, L.S. & PRINCE, D.A. (1982). Cholinergic excitation of mammalian hippocampal pyramidal cells. *Brain Res.*, **249**, 315–331.

BERNHEIM, L., MATHIE, A. & HILLE, B. (1992). Characterization of muscarinic receptor subtypes inhibiting Ca^{2+} current and M current in rat sympathetic neurons. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 9544–9548.

BIRDSALL, N.J.M., BURGEN, A.S.V., HULME, E.C. & STOCKTON, J.M. (1983). The effect of McN-A-343 on muscarinic receptors in the cerebral cortex and heart. *Br. J. Pharmacol.*, **78**, 257–259.

BONNER, T.I. (1989). The molecular basis of muscarinic receptor diversity. *Trends Neurosci.*, **12**, 148–151.

BONNER, T.I., YOUNG, A.C., BRANN, M.R. & BUCKLEY, N.J. (1988). Cloning and expression of the human and rat m 5 muscarinic acetylcholine receptor genes. *Neuron*, **1**, 403–410.

BRANN, M.R., BUCKLEY, N.J., JONES, S.V.P. & BONNER, T.I. (1987). Expression of a cloned muscarinic receptor in A9 L cells. *Mol. Pharmacol.*, **32**, 450–455.

BROWN, D.A. & ADAMS, P.R. (1980). Muscarinic suppression of a novel voltage-sensitive K^+-current in a vertebrate neurone. *Nature*, **283**, 673–676.

BROWN, D.A., MARRION, N.V. & SMART, T.G. (1989). On the transduction mechanism for muscarine-induced inhibition of M-current in cultured rat sympathetic neurones. *J. Physiol.*, **413**, 469–488.

BUCKLEY, N.J., BONNER, T.I. & BRANN, M.R. (1988). Localization of a family of muscarinic receptor mRNAs in rat brain. *J. Neurosci.*, **8**, 4646–4652.

BUCKLEY, N.J., BONNER, T.I., BUCKLEY, C.M. & BRANN, M.R. (1989). Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol. Pharmacol.*, **35**, 469–476.

CONSTANTI, A. & BROWN, D.A. (1981). M-currents in voltage-clamped mammalian sympathetic neurones. *Neurosci. Lett.*, **24**, 289–294.

CONSTANTI, A. & SIM, J.A. (1987). Muscarinic receptors mediating suppression of the M-current in guinea-pig olfactory cortex neurones may be of the M_2-subtype. *Br. J. Pharmacol.*, **90**, 3–5.

COYLE, J.T., PRICE, D.L. & DELONG, M.R. (1983). Alzheimer's disease: a disorder of cortical cholinergic innervation. *Science*, **219**, 1184–1190.

DOWNES, C.P. (1982). Receptor-stimulated inositol phospholipid metabolism in the central nervous system. *Cell Calcium*, **3**, 413–428.

DUTAR, P. & NICOLL, R.A. (1988). Classification of muscarinic responses in hippocampus in terms of receptor subtypes and second-messenger systems: electrophysiological studies *in vitro*. *J. Neurosci.*, **8**, 4214–4224.

FREEDMAN, S.B., BEER, M.S. & HARLEY, E.A. (1988). Muscarinic M_1, M_2 receptor binding. Relationship with functional efficacy. *Eur. J. Pharmacol.*, **156**, 133–142.

FUKUDA, K., HIGASHIDA, H., KUBO, T., MAEDA, A., AKIBA, I., BUJO, H., MISHINA, M. & NUMA, S. (1988). Selective coupling with K^+ currents of muscarinic acetylcholine receptor subtypes in NG108-15 cells. *Nature*, **335**, 355–358.

HALLIWELL, J.V. & ADAMS, P.R. (1982). Voltage-Clamp analysis of muscarinic excitation in hippocampal neurons. *Brain Res.*, **250**, 71–92.

HAMILL, J.V., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F.J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.*, **391**, 85–100.

HULME, E.C., BIRDSALL, N.J.M. & BUCKLEY, N.J. (1990). Muscarinic receptor subtypes. *Annu. Rev. Pharmacol. Toxicol.*, **30**, 633–673.

ITO, C., WAKAMORI, M. & AKAIKE, N. (1991). Dual effect of glycine on isolated rat suprachiasmatic neurons. *Am. J. Physiol.*, **260**, C213–C218.

KIJIMA, H. & KIJIMA, S. (1980). Cooperative response of chemically excitable membrane. II. Two state models and their limitations. *J. Theor. Biol.*, **82**, 425–463.

KRNJEVIC, K. (1974). Chemical nature of synaptic transmission in vertebrates. *Physiol. Rev.*, **54**, 418–540.

KRNJEVIC, K., PUMAIN, R. & RENAUD, L. (1971). The mechanism of excitation by acetylcholine in the cerebral cortex. *J. Physiol.*, **215**, 247–268.

KUROSE, H., KATADA, T., AMANO, T. & UI, M. (1983). Specific uncoupling by islet-activating protein, pertussis toxin, of negative signal transduction via α-adrenergic, cholinergic, and opiate receptors in neuroblastoma × glioma hybrid cells. *J. Biol. Chem.*, **258**, 4870–4875.

LAMBRECHT, G., MOSER, U., GRIMM, U., PFAFF, O., HERMANNI, U., HILDEBRANDT, C., WAELBROECK, M., CHRISTOPHE, J. & MUTSCHLER, E. (1993). New functionally selective muscarinic agonists. *Life Sci.*, **52**, 481–488.

LAZARENO, S., BUCKLEY, N.J. & ROBERTS, F.F. (1990). Characterization of muscarinic M_4 binding sites in rabbit lung, chicken heart and NG108-15 cell. *Mol. Pharmacol.*, **38**, 805–815.

LEVEY, A.I., KITT, C.A., SIMONDS, W.F., PRINCE, D.L. & BRANN, M.R. (1991). Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. *J. Neurosci.*, **11**, 3218–3226.

MARRION, N.V., SMART, T.G., MARSH, S.J. & BROWN, D.A. (1989). Muscarinic suppression of the M-current in the rat sympathetic ganglion is mediated by receptors of the M_1-subtype. *Br. J. Pharmacol.*, **98**, 557–573.

MCCORMICK, D.A. & PRINCE, D.A. (1985). Two types of muscarinic response to acetylcholine in mammalian cortical neurones. *Proc. Natl. Acad. Sci. U.S.A.*, **82**, 6344–6348.

MCKINNEY, M. & RICHELSON, E. (1984). The coupling of the neuronal muscarinic receptor to responses. *Annu. Rev. Pharmacol. Toxicol.*, **24**, 121–146.

MESSER, W.S., BOHNETT, M. & STIBBE, J. (1990). Evidence for a preferential involvement of M1 muscarinic receptors in representational memory. *Neurosci. Lett.*, **116**, 184–189.

MESULAM, M.M. & GEULA, C. (1988). Nucleus Basalis (ch4) and cortical cholinergic innervation in the human brain: observations based on the distribution of acetylcholinesterase and choline acetyltransferase. *J. Comp. Neurol.*, **275**, 216–240.

MICHELETTI, R., MONTAGNA, E. & GIACHETTI, A. (1987). AF-DX 116, a cardioselective muscarinic antagonist. *J. Pharmacol. Exp. Ther.*, **241**, 628–634.

MURASE, K., RANDIC, M., SHIRASAKI, T., NAKAGAWA, T. & AKAIKE, N. (1990). Serotonin suppresses N-methyl-D-aspartate responses in acutely isolated spinal dorsal horn neurons of the rat. *Brain Res.*, **525**, 84–91.

MURAYAMA, T. & UI, M. (1983). Loss of the inhibitory function of the guanine nucleotide regulatory component of adenylate cyclase due to its ADP ribosylation by islet-activating protein, pertussis toxin, in adipocyte membranes. *J. Biol. Chem.*, **258**, 3319–3326.

PFAFFINGER, P.J. (1988). Muscarine and t-LHRH suppress M-current by activating an IAP-insensitive G-protein. *J. Neurosci.*, **8**, 3343–3353.

ROBBINS, J., CAULFIELD, M.P., HIGASHIDA, H. & BROWN, D.A. (1991). Genotypic m3-muscarinic receptors preferentially inhibit M-currents in DNA-transfected NG108-15 neuroblastoma × Glioma hybrid cells. *Eur. J. Neurosci.*, **3**, 820–824.

SHIRASAKI, T., HARATA, N. & AKAIKE, N. (1994). Metabotropic glutamate response in acutely dissociated hippocampal CA1 pyramidal neurones of the rat. *J. Physiol.*, **475**, 439–453.

SQUIRE, L.R. & DAVIS, H.P. (1981). The pharmacology of memory: a neurobiological perspective. *Annu. Rev. Pharmacol.*, **21**, 323–356.

UNEYAMA, H., UENO, S. & AKAIKE, N. (1993). Serotonin-operated potassium current in CA1 neurones dissociated from rat hippocampus. *J. Neurophysiol.*, **69**, 1044–1052.

WAKAMORI, M., HIDAKA, H. & AKAIKE, N. (1993). Hyperpolarizing muscarinic responses of freshly dissociated rat hippocampal CA1 neurones. *J. Physiol.*, **463**, 585–604.

WALL, S.J., YASUDA, R.P., HORY, F., FLAGG, S., MARTIN, B.M. & GINNS, E.I. (1991). Production of antisera selective for m1 muscarinic receptors using fusion proteins: distribution of m1 receptors in rat brain. *Mol. Pharmacol.*, **39**, 643–649.

WOMBLE, M.D. & MOISES, H.C. (1992). Muscarinic inhibition of M-current and a potassium leak conductance in neurones of the rat basolateral amygdala. *J. Physiol.*, **457**, 93–114.

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