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HYPERPOLARIZING MUSCARINIC RESPONSES OF FRESHLY DISSOCIATED RAT HIPPOCAMPAL CA1 NEURONES

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SUMMARY

1. Intracellular mechanisms of the muscarinic acetylcholine (ACh) response were investigated in pyramidal neurones freshly dissociated from the rat hippocampal CA1 region. Current recordings were made in the whole-cell mode using the nystatin 'perforated'-patch technique, by which the muscarinic ACh response can be continuously recorded without so-called 'run-down' phenomenon. The amount of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was fluorometrically measured using fura-2.

2. In current clamp conditions, ACh induced a transient hyperpolarization accompanied by a decrease in membrane input resistance.

3. Under voltage clamp conditions at a holding potential (V_h) of -40 mV, ACh induced two types of muscarinic currents observed either alone or together: a transient outward current and a slowly activating sustained inward current.

4. The ACh-induced transient outward current reversed the direction at K^+ equilibrium potential (E_K), and the reversal potential (E_{ACh}) shifted 56·7 mV for a tenfold change of extracellular K^+ concentration ($[K^+]_o$).

5. The ACh-induced transient outward current increased in a sigmoidal fashion with increase in ACh concentration, where the half-maximal concentration (EC_{50}) and the Hill coefficient (n) were 8×10^{-7} M and 1·9, respectively. Both muscarine and carbamylcholine mimicked the ACh response, but neither McN-A-343 (M1 agonist) nor oxotremorine (cardiac M2 agonist) induced any current.

6. Muscarinic antagonists reversibly blocked the ACh response in a concentration-dependent manner. The inhibitory potency was in the order of atropine > pirenzepine > AF-DX-116.

7. The ACh-induced transient outward current was never recorded when $[Ca^{2+}]_i$ was chelated by the acetoxymethyl ester form of 1,2-bis(*O*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA AM). On the other hand, in Ca^{2+}-free external solution containing 2 mM EGTA and 10 mM Mg^{2+}, the ACh response was elicited by the first application and successive ACh applications did not induce any response. Fura-2 imaging showed that $[Ca^{2+}]_i$ was increased when ACh was added to the external medium with or without Ca^{2+}, though in Ca^{2+}-free medium only the first application of ACh increased the $[Ca^{2+}]_i$.

8. The ACh response was not affected by pretreatment with pertussis toxin (PTX)

but the inhibitory effect of ACh on the high-threshold Ca^{2+} channel was abolished completely.

9. Pretreatment with Li^+ enhanced the amplitude of the transient outward current and the increase in $[Ca^{2+}]_i$ induced by ACh.

10. The calmodulin antagonists W-7, chlorpromazine and trifluoperazine reversibly inhibited the ACh response in a concentration-dependent manner. A novel Ca^{2+}–calmodulin-dependent protein kinase II inhibitor, KN-62, also reversibly blocked the ACh response. The protein kinase inhibitor, H-7, had no effect.

11. We tentatively conclude that ACh may bind the M3 receptor coupled to PTX-insensitive G protein (probably G_q) and stimulate the inositol lipid metabolism through phospholipase C. Consequently, the increase of inositol 1,4,5-trisphosphate ($InsP_3$) would release Ca^{2+} from the intracellular Ca^{2+} store sites. The released Ca^{2+} would then bind calmodulin, the complex of which activates Ca^{2+}–calmodulin-dependent protein kinase II (CaMKII), resulting in opening of K^+ channels.

INTRODUCTION

Acetylcholine (ACh) is one of the major neurotransmitters in the mammalian brain, and ACh receptors in the brain are largely muscarinic (Cole & Nicoll, 1984). Pyramidal neurones in the hippocampal CA1 region receive the cholinergic input originating in the medial septum (Lewis & Shute, 1967; Krnjević & Ropert, 1982). Recently, the improved cloning techniques indicated that there are at least five genes (m1, m2, m3, m4 and m5) which encode distinct muscarinic ACh receptors. The m1, m2, m3 and m4 subtypes correspond to the pharmacologically classified receptors M1, M2, M3 and M4, respectively (Watson & Abbot, 1992). In slice preparations of the rat nucleus parabrachialis, ACh activated the M2 receptor which increased K^+ conductance and elicited membrane hyperpolarization (Egan & North, 1986). The ACh-induced outward current was noted in fibroblast A9L cells and NG108-15 neuroblastoma–glioma hybrid cells expressing m1 and m3 subtypes (Brann, Buckley, Penelope, Jones & Bonner, 1987; Fukuda *et al.* 1988). In the hippocampal neurones, there seem to be no detailed reports on the ACh-induced hyperpolarization, although immunohistochemical studies revealed the presence of M1 and M3 receptors in hippocampal CA1 neurones (Spencer, Horvàth & Traber 1986; Buckley, Bonner & Brann, 1988). In addition, second messengers activated by ACh receptors remain obscure in the hippocampal neurones, though the activation of m1 or m3 subtypes increases inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) in NG108-15 cells (Fakuda *et al.* 1988). In human embryonic kidney cells, an increase in both cAMP and $Ins(1,4,5)P_3$ was reported (Peralta, Ashkenazi, Winslow, Ramachandran & Capon, 1988). In A9L cells the activation of phospholipase A_2 was reported (Conklin, Brann, Buckley, Ma, Bonner & Axelrod, 1988). This heterogeneity may be due to the type of host cells in which the receptors are expressed. In the present investigation we found that the hippocampal CA1 pyramidal neurones exhibit K^+ current induced by ACh through a subtype of muscarinic receptors using the nystatin 'perforated'-patch technique. The intracellular mechanisms of the muscarinic ACh receptor-mediated hyperpolarizing response were also clarified.

METHODS

Preparation

The dissociation technique of rat CNS neurones has been described elsewhere (Ito, Wakamori & Akaike, 1991). Briefly, 12- to 21-day-old Wistar rats were decapitated under ether anaesthesia and 350 μm transverse slices of brain were prepared using a microslicer (Model DTK-1000, D.S.K., Kyoto, Japan). These brain slices were incubated in Krebs solution containing 0·01% (w/v) pronase for 20 min at 31 °C, and subsequently exposed to 0·01% (w/v) thermolysin. The CA1 region of hippocampus was micro-punched out and dissociated mechanically with fine glass pipettes. After the dissociated neurones had adhered to the bottom of the Petri dish (usually within 30 min), we started the electrophysiological recordings. However, in the experiments using pertussis toxin (PTX) we did the electrophysiological recordings in dissociated neurones pretreated with or without PTX for 6–8 h. Neurones possessing original morphological features such as dendritic processes were used in the present study.

Solutions

The ionic composition of the external solution was (mM): NaCl, 150; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; glucose, 10; and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes), 10. The pH was adjusted to 7·4 with tris (hydroxymethyl) aminomethane base (Tris base). The external solutions containing 10, 20 and 50 mM K^+ were made by the substitution of equimolar Na^+ with K^+. The Ca^{2+}-free external solution was made by replacing 2 mM Ca^{2+} with 9 mM Mg^{2+} and adding 2 mM EGTA. The amount of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was measured in external solutions containing 10^{-7} M tetrodotoxin (TTX) and 2 mM Co^{2+} or 10^{-5} M La^{3+} in order to block both voltage-dependent Na^+ and Ca^{2+} currents. The ionic composition of the external solution with 10 mM Ba^{2+} was (mM): NaCl, 138; CsCl, 5; $MgCl_2$, 1; $BaCl_2$, 10; glucose, 10; TTX, 10^{-7} M; and Hepes, 10. The pH was adjusted to 7·4 with Tris base. A stock solution containing 10 mg ml^{-1} nystatin was prepared and was added in a final concentration of 75 μg ml^{-1} to the patch-pipette solutions, whose compositions were (mM): KCl, 150 and Hepes, 10 for recording the ACh-induced current; and CsCl, 150 and Hepes, 10; for recording high voltage-activated Ba^{2+} current (HVA-I_{Ba}). The pH was adjusted to 7·2 with Tris base.

Electrical recording

Electrical recordings were made using the nystatin perforated-patch technique (Horn & Marty, 1988). The resistance between the recording electrode filled with internal solution and the reference electrode was 4–8 MΩ. The current and voltage were measured with a patch clamp amplifier (EPC-7, List-Electronic, Germany), monitored on both a storage oscilloscope (R5113, Tektronix, Beaverton, OR, USA) and a pen-recorder (Recti Horiz 8K, San-ei, Tokyo, Japan), and recorded on video tape after digitization with a PCM processor (PCM 501 ESN, Nihon Kohden, Tokyo, Japan). Series resistance was compensated more than 70% and capacitance was also compensated. All experiments were carried out at room temperature (23–25 °C). For statistical analysis we used Student's *t* test, and values are expressed as means ± S.E.M.

Ca^{2+} fluorometric measurements

All procedures concerning Ca^{2+} fluorometric measurements were carried out in a dark room under illumination with dim red light. Dissociated neurones were loaded at room temperature with 5 μM fura-2 AM (acetoxymethyl ester form) in the external solution. The fura-2 AM solution was replaced with fresh external solution after 90 min incubation. The fluorescence with 340 and 380 nm excitation wavelengths was detected with a photomultiplier tube (R 1104, Hamamatsu, Japan). These intensity data were digitized with Interdec Ca^{2+} fluorometric measurement system (M-1000, Interdec, Osaka, Japan). The ratios of 340/380 nm were calculated after subtracting background fluorescence value from a cell-free region of the Petri dish. Then, the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated.

Drug application

Rapid application of drugs was performed with the 'Y-tube' method. Details of this technique have been reported elsewhere (Murase, Randic, Shirasaki, Nakagawa & Akaike, 1990). In brief, the small orifice (about 40 μm in diameter) of the Y-tube tip was placed near a neurone in a Petri dish,

with continuous perfusion with the external solution. One of the other two ends was immersed into the external solution in a test tube and the third was connected to a vacuum pump via an electromagnetic valve which was controlled with a stimulator (SEN-7103, Nihon Kohden). With opening of the valve for 1 s, the external solution was drawn from the test tube beyond the orifice by a negative pressure of −400 mmHg. Following closure of the valve, the external solution was flushed out from the Y-tube tip to the neurone by gravity. The exchange of external solution surrounding a dissociated neurone was completed within 10–20 ms. In the present study, cholinergic agonists were applied for 10–20 s at 3 min intervals.

Drugs

Drugs used in the present experiments were: acetylcholine chloride (ACh), muscarine chloride, pirenzepine, nystatin, thermolysin and apamin (Sigma, USA); pronase (Calbiochem, USA); glycine and 1-oleoyl-2-acetyl-glycerol (OAG) (Nakarai, Japan); pertussis toxin (PTX), H-7 and W-7 (Seikagaku Kogyo, Japan); atropine sulphate (Merck, USA); carbamylcholine chloride, tetraethylammonium chloride (TEA) and 4-aminopyridine (4-AP) (Tokyo Kasei, Japan); oxotremorine and McN-A-343 (RBI, USA); tetrodotoxin (TTX) (Sankyo, Japan); fura-2 AM and BAPTA AM (1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; Dojin, Japan); iberiotoxin (Peptide Institute, Japan). KN-62 (1-(*N*,*O*-bis-1,5-isoquinolinesulphonyl)-*N*-methyl-L-tyrosyl-4-phenylpiperazine) was synthesized in our laboratories (Tokumitsu, Chijiwa, Hagiwara, Mizutani, Terasawa & Hidaka, 1990). AF-DX-116 was a kind gift from Professor T. Kamiya (Fukuoka University). The drugs were dissolved in the external solution just before use.

RESULTS

Acetylcholine-induced currents

Whole-cell recordings were made on 422 pyramidal neurones freshly dissociated from the ventral part of the rat hippocampal CA1 region. When the cell membrane was patched with a pipette containing 75 μg ml^{-1} nystatin over 10 min, 10^{-5} M ACh induced a transient nicotinic inward current (36 out of 422 neurones, Fig. 4*A*) and two types of muscarinic responses such as a transient outward current (Fig. 1*Aa*) and a slow inward current (Fig. 1*Ab*) at a holding potential (V_h) of −40 mV under voltage clamp conditions. Two hundred and nine neurones (49·6%) had only a transient outward current accompanied by an increase in membrane conductance. Only three neurones (0·7%) had a slow inward current with the decrease in membrane conductance, and the current lasted for 3–4 min even after washing out the ACh (Fig. 1*Ab*). Two hundred and four neurones (48·3%) showed a combination of the two types of muscarinic currents consisting of a transient outward current followed by a slow inward current (Fig. 1*Ac*). Six neurones (1·4%) did not respond to ACh.

In the neurones patched with the nystatin patch pipette, a resting membrane potential of -62 ± 5 mV ($n = 10$) was obtained under current clamp conditions. Application of 10^{-5} M ACh induced a transient hyperpolarization with a decrease in input resistance (Fig. 1*Ba*). In the same neurone under voltage clamp conditions at a V_h of −60 mV, the ACh evoked a transient outward current (Fig. 1*Bb*). The hyperpolarizing command pulses of 10 mV (800 ms) applied every 3 s during a recording revealed an increase in membrane conductance in the presence of ACh.

The application of ACh at 3 min intervals evoked identical ACh-induced transient outward currents over 45 min by the use of the nystatin perforated-patch technique (Fig. 2*A*). However, use of a conventional patch pipette in the whole-cell mode (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) caused a 'run-down' of the transient outward current, and the ACh response completely disappeared within

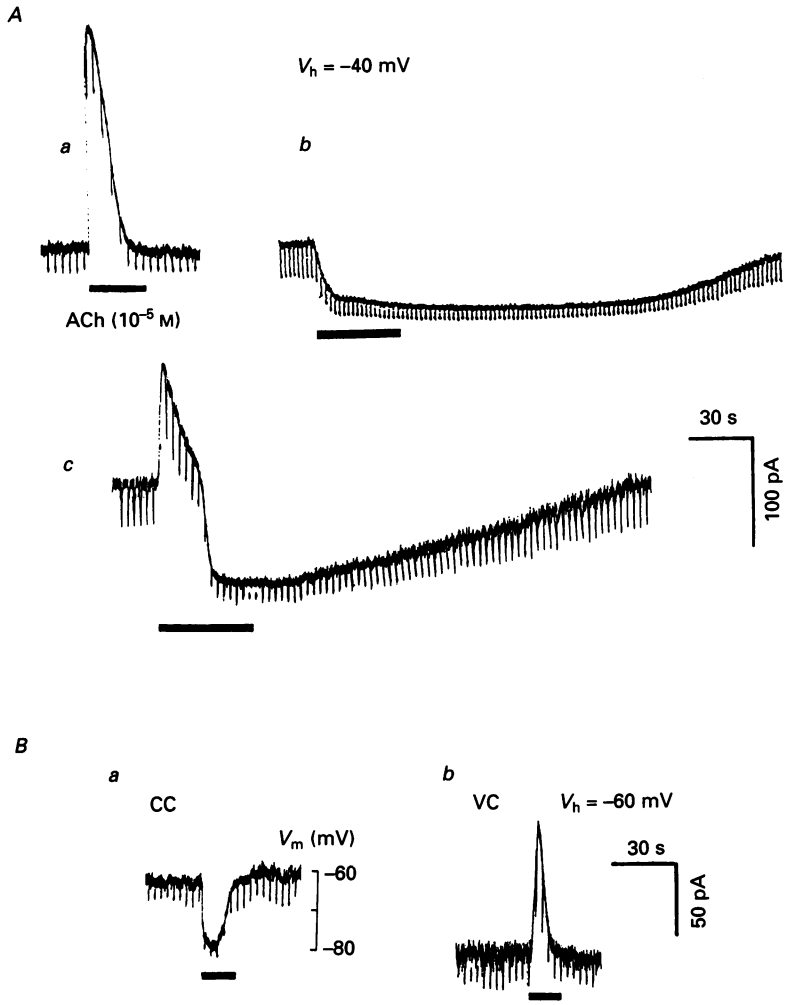

Fig. 1. ACh-induced muscarinic responses. *A*, various ACh-induced currents at a holding potential (V_h) of −40 mV. The three traces are typical cases from 422 neurones examined. *a*, ACh-induced transient outward current (I_{ACh}); *b*, a slow inward current; *c*, the combination of two types of currents. A brief hyperpolarizing pulse of 10 mV and 800 ms duration was applied every 3 min. *B*, ACh-induced response obtained from the same neurone under current clamp (CC) and voltage clamp (VC) conditions. Under current clamp conditions the application of ACh induced a transient hyperpolarization with a decrease in the input resistance. In voltage clamp conditions at a V_h of −60 mV the I_{ACh} was accompanied by an increase in conductance. Horizontal bars under each ACh response indicate the application period of 10^{-5} M ACh. V_m membrane potential.

10 min without alteration in the glycine-induced Cl^- currents (Fig. 2*B*). In subsequent experiments, therefore, the nystatin perforated-patch technique was used.

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

Neurones were perfused with external and internal (pipette) solutions containing 5 and 150 mM K^+, respectively. The ACh responses at various V_h between −100 and −20 mV are shown in Fig. 3*A*, in which the transient response reversed the direction

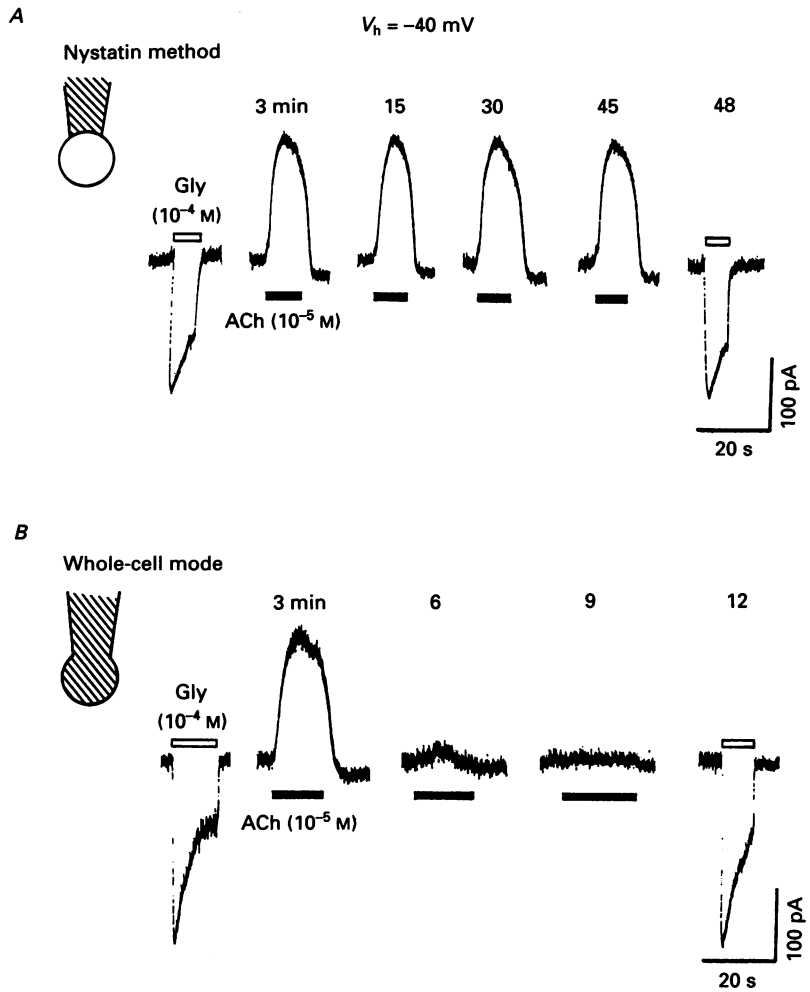

Fig. 2. Prevention of 'run-down' of I_{ACh} by the use of the nystatin perforated-patch technique. The V_h was −40 mV. *A*, nystatin perforated-patch preparation. Identical ACh responses were recorded over 45 min. *B*, conventional whole-cell recording of ACh response. Note no time-dependent change of Cl^- current induced by 10^{-4} M glycine (Gly) despite the time-dependent decrease in I_{ACh}. Horizontal bars above and below the responses indicate the application period of 10^{-4} M Gly and 10^{-5} M ACh, respectively.

between −100 and −80 mV. The reversal potential (E_{ACh}) estimated from the current–voltage (*I–V*) relationship was −84 mV. The value was close to the K^+ equilibrium potential (E_K) of −86 mV calculated with the Nernst equation for the given extra- and intracellular K^+ concentrations ($[K^+]_o$ and $[K^+]_i$, respectively). The

I–V relationships for ACh were examined in external solutions containing 10, 20 and 50 mM K^+ at a constant 150 mM $[K^+]_i$. All responses were normalized to the peak response of transient outward current at a V_h of −40 mV in an external solution containing 5 mM K^+ (asterisk in Fig. 3*B*). The E_{ACh} changed by 56·7 mV for a tenfold

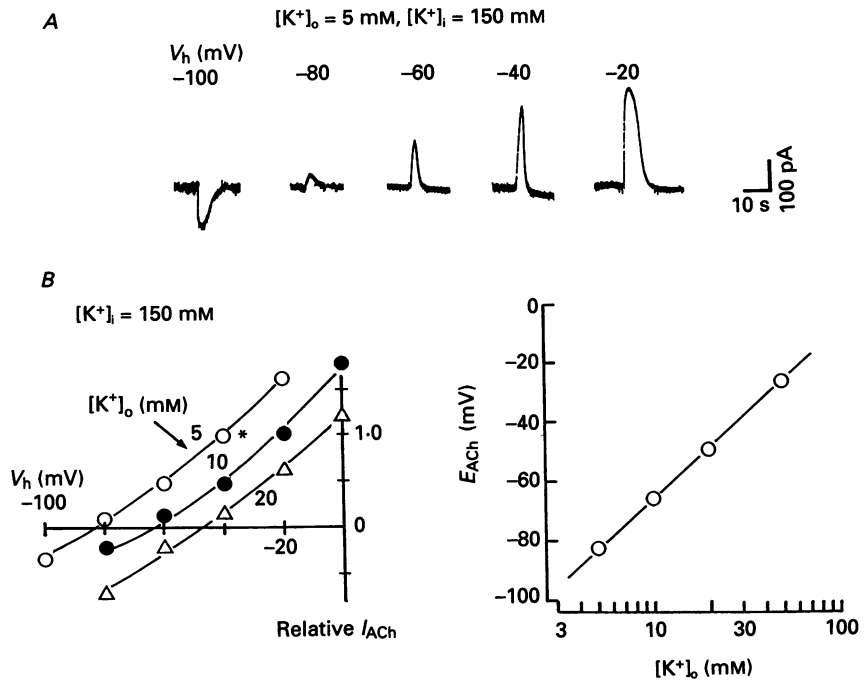

Fig. 3. Current–voltage (*I–V*) relationship for ACh-induced transient response. *A*, typical ACh responses at various V_h in the same neurone perfused with external and internal solutions containing 5 and 150 mM K^+, respectively. *B*, normalized *I–V* relationships for ACh responses in external solutions with 5, 10 and 20 mM K^+ at a constant 150 mM $[K^+]_i$. Currents were normalized to the peak response at a V_h of −40 mV in external solution containing 5 mM K^+. *C*, E_{ACh} plotted as a function of $[K^+]_o$. E_{ACh} changed 56·7 mV for a tenfold change of $[K^+]_o$. In *B* and *C*, each point is the mean ± S.E.M. of five neurones.

change in $[K^+]_o$ (Fig. 3*C*), indicating that the ACh-induced current is passing through K^+ channels. In support of this notion, K^+ channel blockers such as 4-aminopyridine (4-AP) and tetraethylammonium (TEA) blocked the ACh response in a concentration-dependent manner. The values of half-maximal inhibition (IC_{50}) were 1 mM for 4-AP and 2 mM for TEA. On the other hand, neither apamin (10^{-6} M) nor iberiotoxin (10^{-7} M) suppressed the ACh-induced transient outward current ($n = 6$; data not shown), indicating that the ACh current is not passing through small- or large-conductance Ca^{2+}-activated K^+ channels.

Effects of cholinergic agonists

ACh (10^{-5} M) induced an extremely rapid transient inward current followed by a transient outward current at a V_h of −40 mV (Fig. 4*A a*). In the same neurone, 10^{-4} M nicotine induced only a rapid inward current, indicating that nicotinic receptors exist in some hippocampal CA1 neurones and that the ACh-induced transient

outward current is not mediated by the nicotinic receptor (Fig. 4*Ab*). Figure 4*B* shows the effects of muscarinic agonists on a neurone at a V_h of −40 mV, Muscarine (Mus) and carbamylcholine (CCh) mimicked the ACh-induced transient outward current (I_{ACh}), but neither 10^{-4} M McN-A-343 (M1 agonist) nor 10^{-4} M oxotremorine

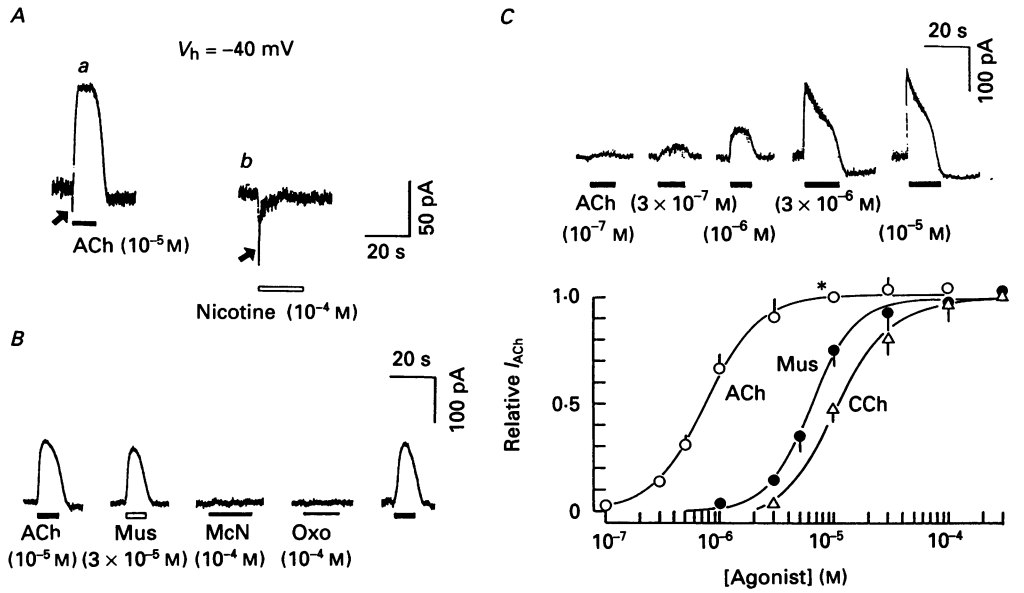

Fig. 4. Effects of cholinergic agonists. *A*, nicotinic and muscarinic ACh responses at a V_h of −40 mV. *a*, ACh induced an extremely rapid transient inward current (arrow) followed by a transient outward current. *b*, nicotine induced only a rapid inward current. The two traces were obtained from the same neurone. *B*, ACh and muscarine (Mus) induced transient outward currents. All recordings were obtained from the same neurone. Horizontal bars under the responses indicate the application period of the agonists. *C*, the concentration–response relationships for ACh, Mus and carbamylcholine (CCh). All currents were normalized to the peak amplitude of transient outward current induced by 10^{-5} M ACh (*). Each point represents the mean of ten to twelve neurones. Vertical bars indicate ± S.E.M. The continuous lines were drawn with a least-squares fitting routine using the following equation:

$$I = I_{max} C^n / (C^n + EC_{50}{}^n),$$

where I denotes the current observed with concentration C, I_{max} the maximum current, EC_{50} the half-maximal concentration and n the Hill coefficient.

(cardiac M2 agonist) induced current. These results indicate that the I_{ACh} is not mediated by M1 and M2 receptors. The I_{ACh} increased in a sigmoidal fashion with increasing ACh concentration (Fig. 4*C*). The threshold, half-maximal (EC_{50}) and maximal concentrations in the concentration–response curve were 10^{-7}, 8×10^{-7} and 10^{-5} M, respectively. The Hill coefficient was 1·9. The peak amplitudes of I_{ACh} induced by Mus and CCh were normalized to that induced by 10^{-5} M ACh, in which the EC_{50} values were 6×10^{-6} M for Mus and 10^{-5} M for CCh. However, the maximum current amplitudes in the respective concentration–response curves for these three cholinergic agonists were almost identical (Fig. 4*C*). At ACh concentrations beyond

3×10^{-6} M, about 50% of neurones induced additional long-lasting slow inward current. There were no differences in the ion selectivity and pharmacology of transient outward current in the presence or absence of the slow inward current. In this study, we did not further analyse the slow inward current.

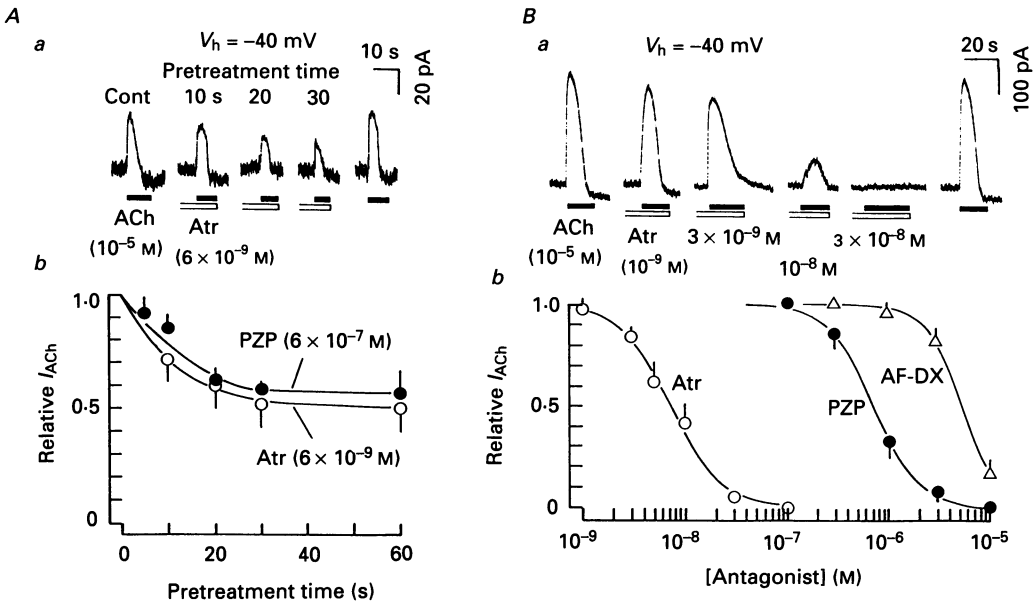

Fig. 5. Effects of muscarinic antagonists on the I_{ACh}. *A*, the time course of I_{ACh} inhibition by atropine (Atr) and pirenzepine (PZP). Antagonists were applied for various periods before the simultaneous application with 10^{-5} M ACh. The maximum inhibitory effect on the ACh response was obtained by pretreatment with antagonists for 30 s. *Ba*, the inhibitory effect of atropine at various concentrations on the ACh response. The pretreatment time with atropine was 30 s. *b*, concentration-dependent inhibition curves of atropine, PZP and AF-DX-116 (AF-DX) on the ACh response. Horizontal bars under the responses indicate the application period of ACh and atropine. Each symbol is the mean of ten to twelve neurones. Vertical bars indicate ± S.E.M. The continuous lines were drawn as in Fig. 4.

Effects of muscarinic antagonists

Atropine (Atr), a non-selective muscarinic antagonist, reversibly inhibited I_{ACh}, suggesting that the current is mediated by muscarinic ACh receptors. Muscarinic ACh receptors are pharmacologically classified into four subtypes (M1, M2, M3 and M4) (Watson & Abbot, 1992). To elucidate what type of muscarinic receptor mediates the ACh response, pirenzepine (PZP), a M1 antagonist, and AF-DX-116, a M2 antagonist, were tested. Figure 5*A* shows the time course of the inhibition of ACh response after adding 6×10^{-9} M Atr and 6×10^{-7} M PZP. Steady-state inhibition was observed within 30 s of the pretreatment with these antagonists. Therefore, the current measurements in the following experiments were made after 30 s of pretreatment with the individual antagonists. Concentration-dependent inhibition curves of Atr, PZP and AF-DX-116 at a V_h of −40 mV are summarized in Fig. 5*B*. The values of

IC_{50} were 7×10^{-9} M for Atr, 5×10^{-7} M for PZP and 5×10^{-6} M for AF-DX-116, indicating that the ACh response is not mediated by a M2 receptor.

Intracellular mechanisms of ACh-induced outward current (I_{ACh})

As shown in Fig. 6*A*, the muscarinic ACh response in about 50% of neurones examined consisted of a combination of a transient outward current and a slow

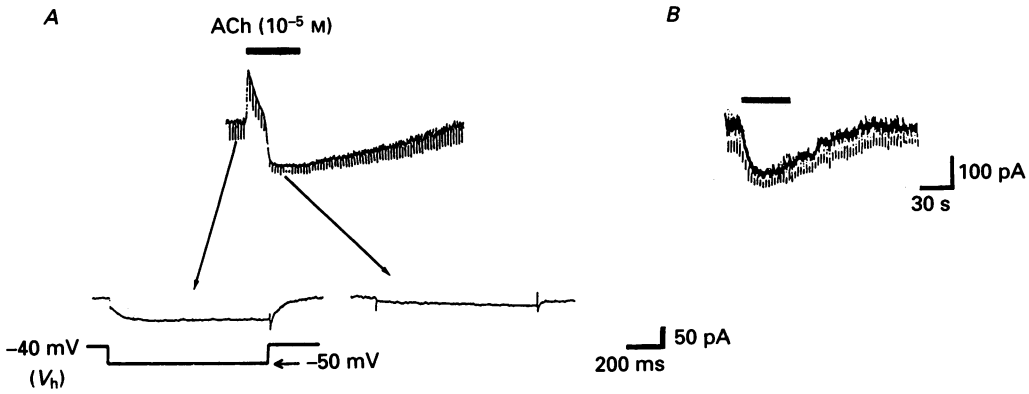

Fig. 6. Involvement of $[Ca^{2+}]_i$ in the I_{ACh}. *A*, ACh induced a transient outward current and a slow inward current in a neurone immersed in normal external solution containing 2 mM Ca^{2+}. The I_M was activated by a 10 mV hyperpolarizing command voltage step (800 ms duration) from a V_h of −40 mV. *B*, only slow inward current was evoked by ACh after pretreatment with 5 μM BAPTA AM for 2 h.

inward current. These neurones exhibited a slowly activating, non-inactivating K^+ current (M-current, I_M) which was suppressed by ACh (Halliwell & Adams, 1982). The lower panel in Fig. 6*A* shows the inward current relaxations produced by a hyperpolarizing voltage command from a V_h of −40 mV to −50 mV before and during the application of 10^{-5} M ACh. After the treatment of these neurones with 5 μM BAPTA AM for 2 h, the ACh could not induce any transient outward current but only slow inward current ($n = 8$; Fig. 6*B*). This result indicates that the rise in $[Ca^{2+}]_i$ contributes to the induction of I_{ACh}. Therefore, we further examined the source of the elevated $[Ca^{2+}]_i$. When ACh was applied to neurones perfused with external solutions with or without 2 mM Ca^{2+}, similar increases in the $[Ca^{2+}]_i$ were observed with fura-2 imaging ($n = 8$; Fig. 7*Aa* and *b*). As shown in Fig. 7*B*, the removal of extracellular Ca^{2+} by adding 2 mM EGTA also did not affect the peak amplitude of I_{ACh}, though the decay phase was accelerated (Fig. 7*Bb*). However, at 3 min after the first application of ACh in Ca^{2+}-free external solution, the successive application of ACh elicited neither increases in $[Ca^{2+}]_i$ nor current (Fig. 7*Ac* and *Bc*). After 2 min recovery in external solution containing 2 mM Ca^{2+}, both ACh response and $[Ca^{2+}]_i$ elevation recovered completely (Fig. 7*Ad* and *Bd*). These results indicate that the increase of $[Ca^{2+}]_i$ is an important factor to induce the I_{ACh} and that Ca^{2+} is released from the intracellular Ca^{2+} stores in the presence of ACh.

To identify the intracellular pathways from activation of ACh receptors to opening of K^+ channels via the increase in $[Ca^{2+}]_i$, the following experiments were carried out. First, we studied what type of guanine nucleotide-binding proteins (G proteins) may

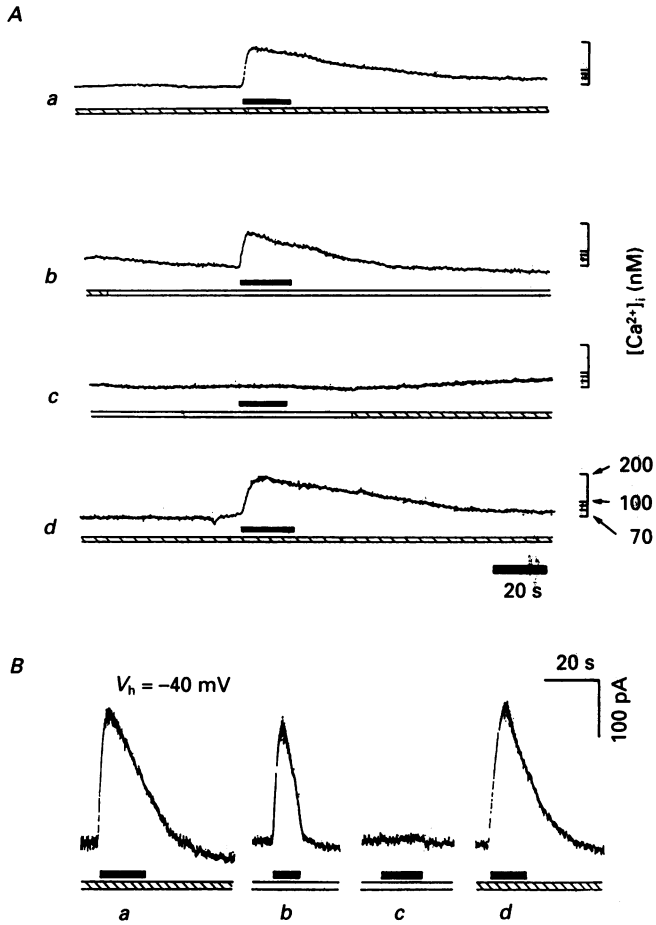

Fig. 7. Relationship between I_{ACh} and $[Ca^{2+}]_i$ elevation. *A*, effect of Ca^{2+}-free external solution containing 2 mM EGTA on intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) in the presence or absence of 10^{-5} M ACh. The $[Ca^{2+}]_i$ was measured by fura-2 fluorometry. *a*, the increase of $[Ca^{2+}]_i$ produced by adding 10^{-5} M ACh (filled bar) to normal external solution containing 2 mM Ca^{2+} (hatched bar). *b*, ACh increased the $[Ca^{2+}]_i$ even in Ca^{2+}-free external solution containing 2 mM EGTA (open bar). *c*, successive application of ACh in Ca^{2+}-free external solution did not increase the $[Ca^{2+}]_i$. *d*, recovery of ACh response in normal external solution with 2 mM Ca^{2+}. *Ba* and *b*, the removal of extracellular Ca^{2+} led to little decrease in the peak current amplitude of the I_{ACh}, as induced by the first application of ACh at a V_h of −40 mV. *c*, successive application of ACh at 3 min intervals in Ca^{2+}-free external solution produced no response. *d*, after 2 min recovery in external solution with 2 mM Ca^{2+}, the ACh response recovered completely. Horizontal bars under the responses indicate the application period of ACh and external solutions with or without Ca^{2+}. The fura-2 fluorometric traces in *A* and the I_{ACh} in *B* were obtained from different neurones.

be involved in generation of I_{ACh}. The dissociated hippocampal neurones were incubated in external solution with or without 200 ng ml^{-1} pertussis toxin (PTX) for 6–8 h. To ensure that PTX was effective in these dissociated neurones, the effect of ACh on the high voltage-activated Ba^{2+} inward current (HVA-I_{Ba}) was examined in K^+-free external solution containing 10 mM Ba^{2+} in the presence or absence of PTX.

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-threshold Ca^{2+} channel was completely inactivated. As shown Fig. 8*A a*, 10^{-5} M ACh suppressed the activation phase of HVA-I_{Ba} in control neurones 6 h after the dissociation whereas ACh had no effect on

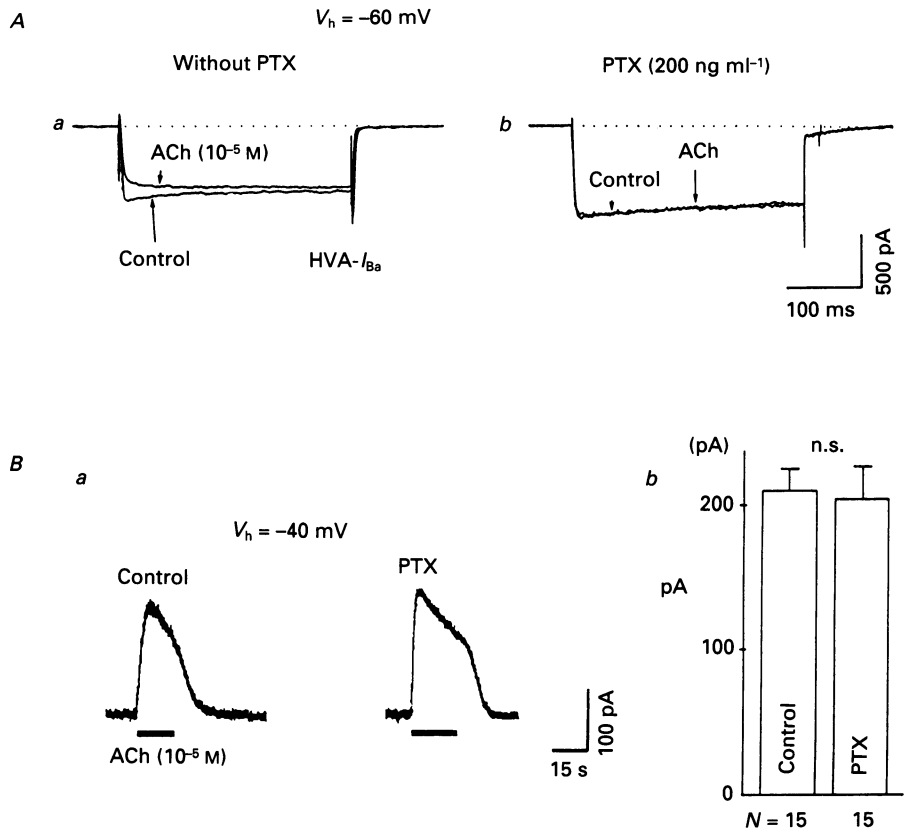

Fig. 8. Effects of PTX on HVA-I_{Ba} and I_{ACh}. The HVA-I_{Ba} was evoked by a 300 ms step pulse to −10 mV from a holding potential (V_h) of −60 mV. In these experiments, neurones were pretreated with ACh for at least 2 min. *A a*, 10^{-5} M ACh suppressed the HVA-I_{Ba} in a neurone without PTX treatment. *b*, ACh had no effect on the HVA-I_{Ba} in a neurone after treatment with 200 ng ml^{-1} PTX for 6 h. *Ba*, dissociated neurones were immersed in external solution with or without PTX for 6 h. *b*, data were obtained from neurones with or without PTX treatment for 6–8 h. Vertical bars show ±S.E.M.; n.s., no significant difference. *N*, the number of neurones tested.

HVA-I_{Ba} in neurones treated with PTX for 6 h (Fig. 8*A b*). Even in these PTX-treated neurones, however, the ACh could induce a transient outward current as well as that in control without PTX treatment (n = 15; Fig. 8*B*). The results suggest that PTX-sensitive G protein is not coupled to the muscarinic ACh receptor which induces transient outward current in rat hippocampal pyramidal neurones.

Calmodulin antagonists such as chlorpromazine (CPZ), trifluoperazine (TFP) and W-7 were applied for 60 s before the co-administration of 10^{-5} M ACh and one of the antagonists. These antagonists reversibly diminished I_{ACh} in a concentration-

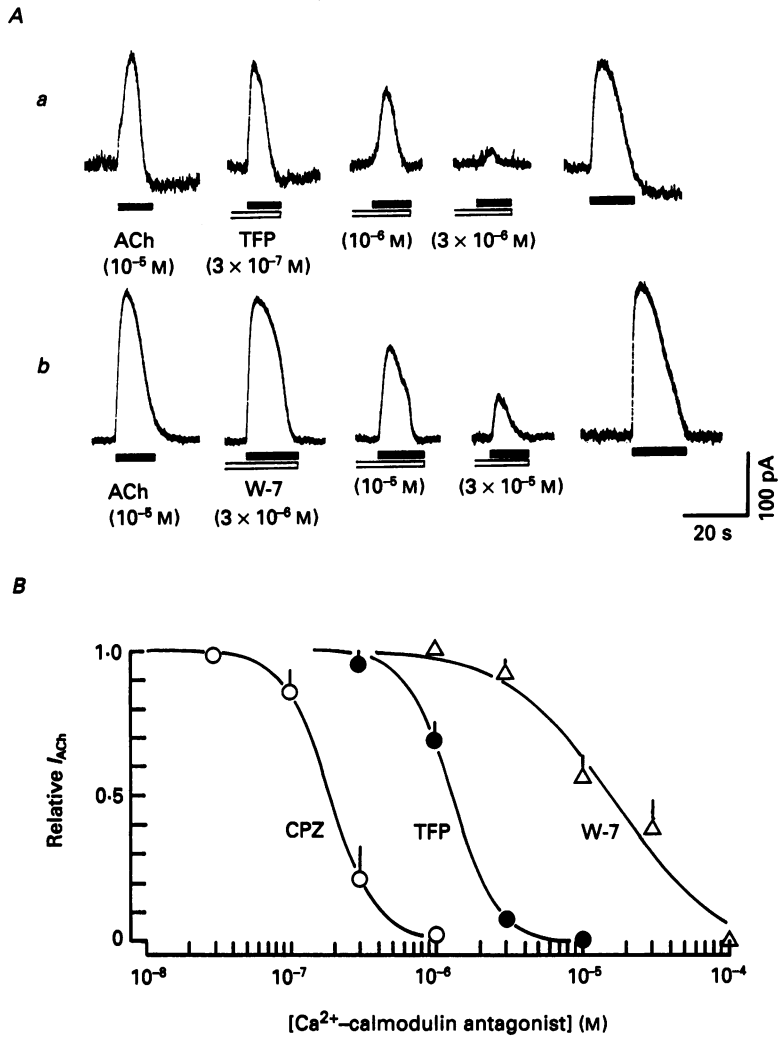

Fig. 9. Effects of Ca^{2+}–calmodulin antagonists on the I_{ACh}. *A*, trifluoperazine (TFP) and W-7 blocked the ACh response in a concentration-dependent manner. The antagonists were applied for 60 s prior to the simultaneous application of 10^{-5} M ACh at a V_h of −40 mV. Horizontal bars under the responses indicate the application period of ACh and the Ca^{2+}–calmodulin antagonists used. *B*, the concentration–inhibition curves of chlorpromazine (CPZ), TFP and W-7 on the ACh response. All currents were normalized to the peak current induced by 10^{-5} M ACh alone. Each symbol indicates the mean of seven to eight neurones. Vertical bars indicate ±S.E.M. The continuous lines were drawn as in Fig. 5*B*.

dependent manner with IC_{50} values of $2{\cdot}0 \times 10^{-7}$ M for CPZ, $1{\cdot}5 \times 10^{-6}$ M for TFP and $1{\cdot}3 \times 10^{-5}$ M for W-7 (Fig. 9). The complete recovery of the I_{ACh} took 2–5 min after washing out the antagonists. These results suggest that calmodulin participates in the ACh response. To clarify the involvement of Ca^{2+} calmodulin-dependent protein kinase II (CaMKII), we successively examined the effect of KN-62, a novel inhibitor

of CaMKII, on the ACh response. Figure 10 shows a typical record obtained from six neurones. The I_{ACh} was completely inhibited by 3×10^{-7} M KN-62 within 6 min and it gradually recovered to the control level after the wash-out of KN-62. However, the ACh response was not affected by 10^{-6} M KN-04, an inactive analogue of KN-62 (data not shown).

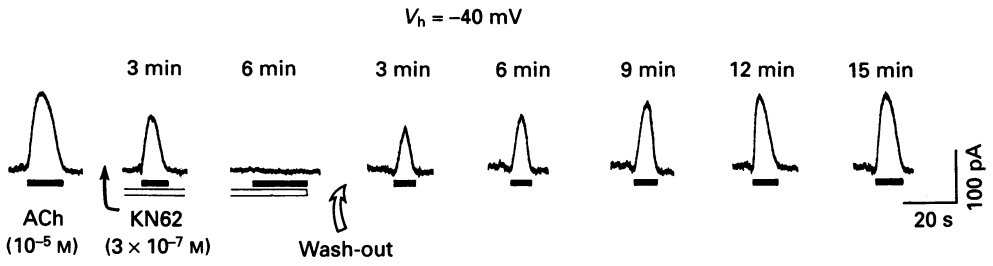

Fig. 10. The inhibition of I_{ACh} by 3×10^{-7} M KN-62. The inhibition was reversible. V_h was −40 mV.

We also tested the effect of H-7, an inhibitor for protein kinase C (PKC) and cAMP- and cGMP-dependent protein kinases, on the ACh response. In other tissues, the IC_{50} value of H-7 for these protein kinases is below $6{\cdot}0 \times 10^{-6}$ M (Hidaka, Inagaki, Kawano & Sasaki, 1984). In the present experiments, H-7 even at 10^{-5} M had no effect on the ACh response ($n = 5$). The membrane permeant PKC activator, 1-oleoyl-2-acetyl-glycerol (OAG) at 5×10^{-6} M, also had no effect ($n = 4$), indicating that PKC probably does not participate in this muscarinic ACh response in the hippocampal pyramidal neurones.

Since no membrane-permeant phospholipase C (PLC) blocker is available, we could not obtain direct evidence that G protein activates PLC. However, fura-2 imaging showed that 10^{-5} M ACh increased the $[Ca^{2+}]_i$ (Fig. 7*A*), suggesting that PLC may catalyse the inositol lipid turnover to produce inositol 1,4,5-trisphosphate (Ins(1,4,5)P_3). The Ins(1,4,5)P_3 binds to Ins(1,4,5)P_3 receptors on intracellular Ca^{2+} stores from which Ca^{2+} is released (Streb, Irvine, Berridge & Schulz, 1983). Biochemical and electrophysiological experiments show that Li^+ inhibits the inositol lipid turnover. For example, Li^+ decreases the content of Ins(1,4,5)P_3 and inositol 1,3,4,5-tetrakisphosphate (InsP_4) by diminishing PLC activation (Avissar, Shreiber, Danon & Belmarker, 1988; Worley, Heller, Snyder & Baraban, 1988). It is also reported that Li^+ has a reciprocal action. Li^+ increases inositol 1,3,4-trisphosphate (Ins(1,3,4)P_3) and inositol 1,4-bisphosphate (Ins(1,4)P_2) by inhibiting 1-phosphatase, resulting in the increase of Ins(1,4,5)P_3 (Inhorn & Majerus, 1988; Gee, Reid, Jackson, Barnaby & Ragan, 1988). In six neurones of the present experiment, pretreatment with 10^{-4} M Li^+ enhanced the I_{ACh}. In these neurones the responses to 10^{-5} M ACh was relatively smaller than those seen in most of the neurones (Fig. 11*A*). In addition, the facilitatory rise of $[Ca^{2+}]_i$ by adding ACh in the presence of Li^+ was observed by fura-2 fluorometric measurements (Fig. 11*B*). One may claim that the activation and the decay time course in I_{ACh} were not always coincident with that in fura-2 imaging (Figs 7 and 11). The discrepancy may relate to the fact that (1) the recordings were from different neurones, (2) the electrophysiological recording might be more

sensitive than the fluorometric recording we have used, since the fura-2 imaging shows the cytosolic Ca^{2+} concentration rather than the Ca^{2+} concentration of a compartment just beneath the plasma membrane, and (3) the I_{ACh} was not directly activated by Ca^{2+} such as small- and large-conductance Ca^{2+}-activated K^+ channels but the I_{ACh} involves CaMKII activation.

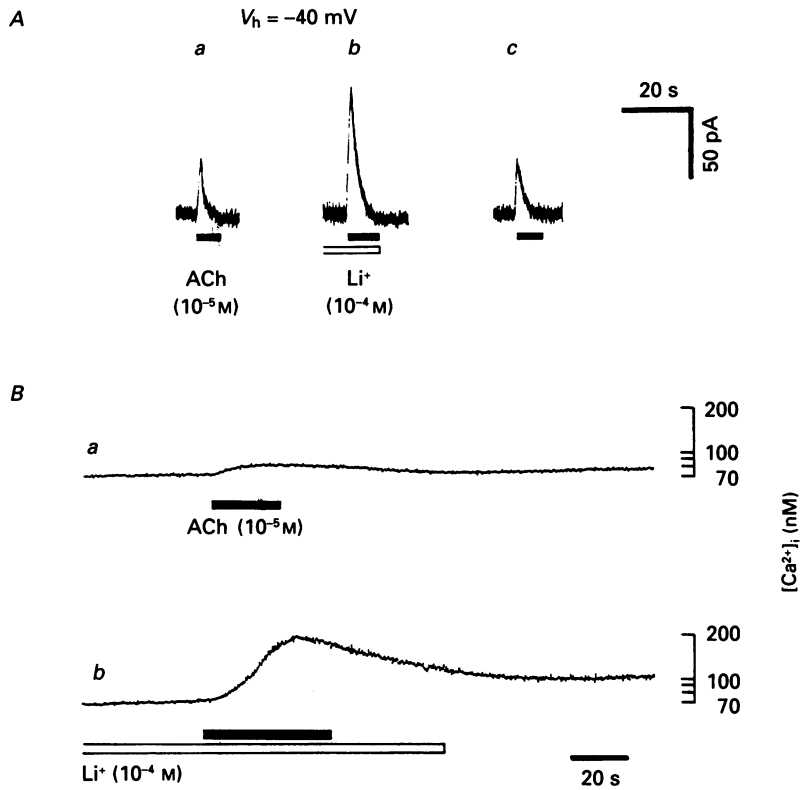

Fig. 11. Effects of Li^+ pretreatment on both I_{ACh} and $[Ca^{2+}]_i$ elevation. *A*, the reversible enhancement of I_{ACh} by pretreatment with 10^{-4} M Li^+ for 2 min. *B*, a further increase of $[Ca^{2+}]_i$ induced by ACh in the presence of Li^+, determined by fura-2 fluorometric measurements. Horizontal bars under the responses indicate the application period of ACh and Li^+. Traces in *A* and *B* were recorded in different neurones.

DISCUSSION

Regulation of K^+ conductance by ACh

Excitability of hippocampal CA1 neurones which receive cholinergic input from the medial septum is controlled by muscarinic receptors (Lewis & Shute, 1967; Krnjević & Ropert, 1982). Conventional microelectrode studies on ACh actions in hippocampal slice preparations showed (1) blockade of after-hyperpolarization (AHP) which accounts for spike frequency adaptation (Benardo & Prince, 1982), (2) depression of EPSPs by the decrease of glutamate release (Ben-Ari, Krnjević, Reinhardt & Ropert, 1981), (3) suppression of leak K^+ current (I_l) (Madison, Lancaster & Nicoll, 1987), (4) blockade of M-current (I_M) (Halliwell & Adams, 1982),

and (5) short-latency hyperpolarization with increase in K^+ conductance (Segal, 1982). Most of the reports on the muscarinic response focused on the depolarizing effect of ACh in the hippocampal neurones, including intracellular mechanisms and physiological roles (Brown, 1988; Agopyan & Agopyan, 1991). In the present study, our interest was drawn to the ACh-induced transient hyperpolarization in the hippocampal neurones. It had been thought that the hyperpolarizing response induced by ACh was due to an excitation of inhibitory neurones. However, the ACh could induce a transient outward current accompanied by an increase in K^+ conductance even in the dissociated hippocampal CA1 pyramidal neurones without any synaptic inputs (Fig. 1*Aa*). The E_{ACh} changed 56·7 mV for a tenfold change in $[K^+]_o$ (Fig. 3*C*). These results suggest that ACh directly hyperpolarizes the hippocampal neurones. This is compatible with the report of a hyperpolarizing postsynaptic muscarinic action in the rat hippocampal slice (Segal, 1982). Similar hyperpolarizations accompanied by a decrease of input resistance during the ACh application have been observed in many tissues such as sympathetic ganglion cells (Hartzell, Kuffler, Stickgold & Yoshikami, 1977), rabbit superior cervical ganglion cells (Cole & Shinnick-Gallagher, 1984), rat lateral geniculate neurones (McCormick & Prince, 1986), rat nucleus parabrachial neurones (Egan & North, 1986) and NG 108-15 cells (Neher, Marty, Fukuda, Kubo & Numa, 1988). Therefore, this type of muscarinic hyperpolarizing response may contribute to the inhibitory regulation of membrane excitability in the hippocampus.

Receptor type

Although the muscarnic ACh receptor subtypes cannot be differentiated clearly using the agonists and antagonists, these subtypes have different sensitivity to these drugs. The order of the blockade for the M1 receptor is pirenzepine (PZP) > AF-DX-116 whereas that for the M2 receptor is the reverse relationship in A9L cells (Brann *et al.* 1987) and *Xenopus* oocytes (Fukuda, Kubo, Akiba, Maeda, Mishina & Numa, 1987). The subtypes of muscarinic ACh receptors are distributed heterogenerously in the brain. *In situ* hybridization study shows that m1 (pharmacologically M1) and m3 mRNAs are rich in the pyramidal cell layer of the hippocampal CA1 region whereas m2 (pharmacologically M2) mRNA is low and m4 mRNA is at an intermediate level (Buckley *et al.* 1988). In neurones dissociated from the nucleus of Meynert, M2 receptors might be involved in the ACh-induced response, because the sensitivity of the ACh response was in the order of atropine > AF-DX-116 > PZP (Harata, Tateishi & Akaike, 1991). Neurones dissociated from the dentate gyrus, in which there are numerous M1 receptors but no M3 ones (Buckley *et al.* 1988; Nabekura & Akaike, 1992), are sensitive to the M1 agonist McN-A-343. In the present study, the order of blockade for the I_{ACh} was PZP > AF-DX-116, and McN-A-343 (M1 agonist) induced no current. Furthermore, the IC_{50} (5×10^{-7} M) for PZP was closer to that ($1{\cdot}6 \times 10^{-7}$ M) of m3 (pharmacologically M3) than m1 ($3{\cdot}5 \times 10^{-8}$ M) in binding studies (Bonner, Buckley, Young & Brann, 1987). Therefore, the I_{ACh} in hippocampal pyramidal neurones seems to be evoked by the binding of ACh to M3 receptors. However, it should be pointed out that at this moment we cannot still exclude the possibility of the existence of a molecular biological m5 subtype of muscarinic ACh receptor associated with the ACh response. Thus, we need further studies to identify

with certainty the subtypes coupled with the ACh response when more selective agonists and antagonists are available.

Intracellular pathway

The G protein mediates the muscarinic response, as determined by reconstituting muscarinic receptors together with G protein in phospholipid vesicles (Haga, Haga, Ichiyama, Katada, Kurose & Ui, 1985). The M2 and M4 ACh receptors activated the PTX-sensitive guanine nucleotide binding protein (G_i), and in turn inhibited the adenylate cyclase in Chinese hamster ovary cells (Ashkenazi *et al.* 1987). On the other hand, M1 and M3 receptors couple to the PTX-insensitive G protein in NG108-15 cells (Fukuda *et al.* 1988). In cultured rat hippocampal neurones, pretreatment of neurones with PTX abolished the inhibitory effect of ACh on the HVA-Ca^{2+} channel (Toselli, Lang, Costa & Lux, 1989). In the present study, the pretreatment of the dissociated rat hippocampal CA1 pyramidal neurones with PTX also blocked the modulatory effect via G proteins of ACh on the HVA-Ca^{2+} channel. However, even in the neurones pretreated with PTX, the ACh could induce the transient outward current. These results indicate that PTX-sensitive G protein is not involved in the I_{ACh}.

Since membrane-impermeant modulators of PLC cannot be applied to neurones by the use of the nystatin perforated-patch technique, there is no direct evidence that G protein activates PLC and releases Ins(1,4,5)P_3 in the dissociated rat hippocampal neurones. However, the Ins(1,4,5)P_3 is increased by the activation of M3 receptors in NG108-15 cells (Fukuda *et al.* 1988) and hippocampal neurones in primary culture (Kanterman, Ma, Briley, Axelrod & Felder, 1990). Neher *et al.* (1988) also showed a dramatic increase of $[Ca^{2+}]_i$ by the M_3 receptor of ACh in NG108-15 cells, suggesting that the activation of G protein in the presence of ACh may be linked with PLC, which catalyses phosphatidyl inositol bisphosphate to produce Ins(1,4,5)P_3 and diacylglycerol (DG). In the present experiments, Li^+ enhanced both the I_{ACh} and the $[Ca^{2+}]_i$ (Fig. 11). Therefore, Li^+ might block the phosphatase which cleaves the 1-phosphate from Ins(1,4)P_2 and Ins(1,3,4)P_3 yielding Ins(4)P and Ins(3,4)P_2, respectively. Consequently, the accumulation of Ins(1,4,5)P_3 may cause the enhancement of the ACh response in the dissociated hippocampal neurones. These electrophysiological and fluorometrical data indirectly suggest that Ins(1,4,5)P_3 is involved in the ACh-induced transient outward current. Recently, a PTX-insensitive and PLC-stimulating G protein was cloned and named G_q (Taylor, Chae, Rhee & Exton, 1991). Therefore, the ACh-induced transient outward current in the rat hippocampal pyramidal neurones may involve this G_q.

Ins(1,4,5)P_3 releases Ca^{2+} from intracellular Ca^{2+} stores (Streb *et al.* 1983). In the present experiments, the ACh could induce the transient outward current in the presence or absence of external Ca^{2+} (Fig. 7*B*), though the second application of ACh in Ca^{2+}-free medium induced no response. This result, in agreement with the fura-2 fluorometric measurements (Fig. 7*A*), suggests that the increase of $[Ca^{2+}]_i$ is due to the release of Ca^{2+} from intracellular Ca^{2+} stores by Ins(1,4,5)P_3 and that the stored Ca^{2+} ions are readily depleted without Ca^{2+} influxes from the extracellular side.

Calmodulin antagonists inhibited the ACh response in the order of chlorpromazine (CPZ) > trifruoperazine (TFP) > W-7. Binding studies showed that the rank order

of sensitivity to calmodulin was TFP > CPZ (Levin & Weiss, 1979), which is the reverse relationship from the present results. This discrepancy may be due to an anti-cholinergic action of CPZ, which would be added to the calmodulin antagonistic action. The value of IC_{50} for W-7 was $1{\cdot}3 \times 10^{-5}$ M that is nearly equal to the IC_{50} value at which concentration the W-7 inhibits cell proliferation (Hidaka *et al.* 1981). These results suggest that the intracellular free Ca^{2+} released from Ca^{2+} stores during the application of ACh may bind the calmodulin. Calmodulin was found to activate at least five different kinds of calmodulin-dependent protein kinases such as a phosphorylase kinase, a myosin light chain kinase and three kinds of Ca^{2+}–calmodulin-dependent protein kinases (CaMK) (Blackshear, Nairn & Kuo, 1988). KN-62 (3×10^{-7} M), a novel inhibitor of CaMKII, reversibly diminished the I_{ACh} at a concentration lower than an inhibition constant (K_i) value (9×10^{-7} M) in a binding study (Tokumitsu *et al.* 1990), indicating that CaMKII may be involved in the ACh response. Onozuka, Furuichi, Imai & Fukami (1991) proposed that CaMKII might act on the inner surface of the membrane to phosphorylate either K^+ channels or membrane associated proteins, resulting in the enhancement of channel activities in snail neurones. In the present preparation, however, it is unclear whether K^+ channels are directly phosphorylated by CaMKII or other proteins are phosphorylated to open the K^+ channels by CaMKII.

Since the protein kinase inhibitor (H-7) did not suppress the I_{ACh} in the present hippocampal pyramidal neurones, both DG and PKC probably do not play important roles in activating the ACh response. Persistent or repeated activation of PLC leads to an accumulation of DG. Excessive DG modulates G protein through PKC or disconnects the link between muscarinic receptor and G protein, and turns off the phosphoinositide metabolism in 1321N1 astrocytoma cells and pancreatic acinar cells (Orellana, Solski & Brown, 1987; Maruyama, 1989). In the present preparation, however, 1-oleoyl-2-acetyl-glycerol had no effect on the I_{ACh}, thereby ruling out such a negative feedback loop. In summary, ACh may bind to M3 receptors coupled to G_q and stimulate inositol lipid metabolism through PLC. Consequently, Ins(1,4,5)P_3 would release Ca^{2+} from intracellular Ca^{2+} stores and this Ca^{2+} may bind calmodulin which induces phosphorylated CaMKII. Consequently the K^+ channels would open.

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