Functional nicotinic ACh receptors on interneurones in the rat hippocampus

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1. Neuronal nicotinic ACh receptors (nAChRs) were studied in the rat hippocampal slice preparation using whole-cell patch-clamp recording techniques.

2. Responses to ACh (100 μM) were detected on inhibitory interneurones in the CA1 field of the hippocampus proper and in the dentate gyrus, but not on principal excitatory neurones in either region. The different neuronal types were identified based on their morphology and location.

3. ACh excited interneurones in the hippocampus and dentate gyrus in current-clamp recordings. In voltage-clamp recordings, ACh-activated inward currents were recorded from interneurones in the presence of blockers of synaptic transmission and the muscarinic ACh receptor antagonist atropine. The zero current potential for this response to ACh was near 0 mV.

4. The effect of ACh was mimicked by the nAChR-selective agonists nicotine (100 μM) and 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP, 100 μM). The response to ACh was reversibly antagonized by the neuronal nAChR antagonist mecamylamine (10 μM). The nAChR α7 subunit-selective antagonists α-bungarotoxin (100 nM) and methyllycaconitine (10 nM) also inhibited the response to ACh.

5. These observations demonstrate the presence of functional nAChRs on inhibitory interneurones in the rat hippocampus. Thus, a novel mechanism by which ACh can regulate neuronal activity in the hippocampus is revealed.

In the hippocampal formation of the rat, principal excitatory neurones and local inhibitory interneurones receive cholinergic innervation from the medial septum–diagonal band complex (MSDB) of the basal forebrain (Frotscher & Léránth, 1985). This cholinergic projection appears to be required for the characteristic rhythmical slow activity of hippocampal neurones, which is thought to underlie hippocampal functions in learning and memory (Stewart & Fox, 1990). Rhythmic excitation of hippocampal inhibitory interneurones by MSDB cholinergic projections leads to feed-forward inhibition of principal cells, and serves to synchronize hippocampal neuronal activity (Stewart & Fox, 1990; Cobb, Buhl, Halasy, Paulsen & Somogyi, 1995; Buzsáki & Chrobak, 1995).

Although muscarinic acetylcholine (ACh) receptor-mediated cholinergic function has been well studied in the hippocampus (Nicoll, 1988; Stewart & Fox, 1990), the function of nicotinic acetylcholine receptors (nAChRs) is less clear. Effects of nAChR ligands on mammalian hippocampal neuronal activity detected by extracellular recording techniques have previously been reported (Ropert & Krnjevic, 1982; Rovira, Ben-Ari, Cherubini, Krnjevic & Ropert, 1983; Freund, Jungschaffer, Collins & Wehner, 1988), although the target cells and mechanism of these effects have not been elucidated. Recently, nAChRs have been reported to modulate glutamate release from the terminals of mossy fibres of granule cells in the rat dentate gyrus which synapse onto pyramidal cells in the CA3 field of the hippocampus (Gray, Rajan, Radcliffe, Yakehiro & Dani, 1996). This report substantiated the current hypothesis that nAChRs may modulate, rather than mediate, fast synaptic transmission in the brain (McGehee & Role, 1995). Hippocampal neurones express mRNA for a variety of subunits which can form functional neuronal nAChRs (Wada *et al.* 1989; Séguéla, Wadiche, Dineley-Miller, Dani & Patrick, 1993) and bind nAChR ligands (Lindstrom, 1996). Hippocampal neurones, when dissociated and cultured, express functional nAChRs (Alkondon & Albuquerque, 1991).

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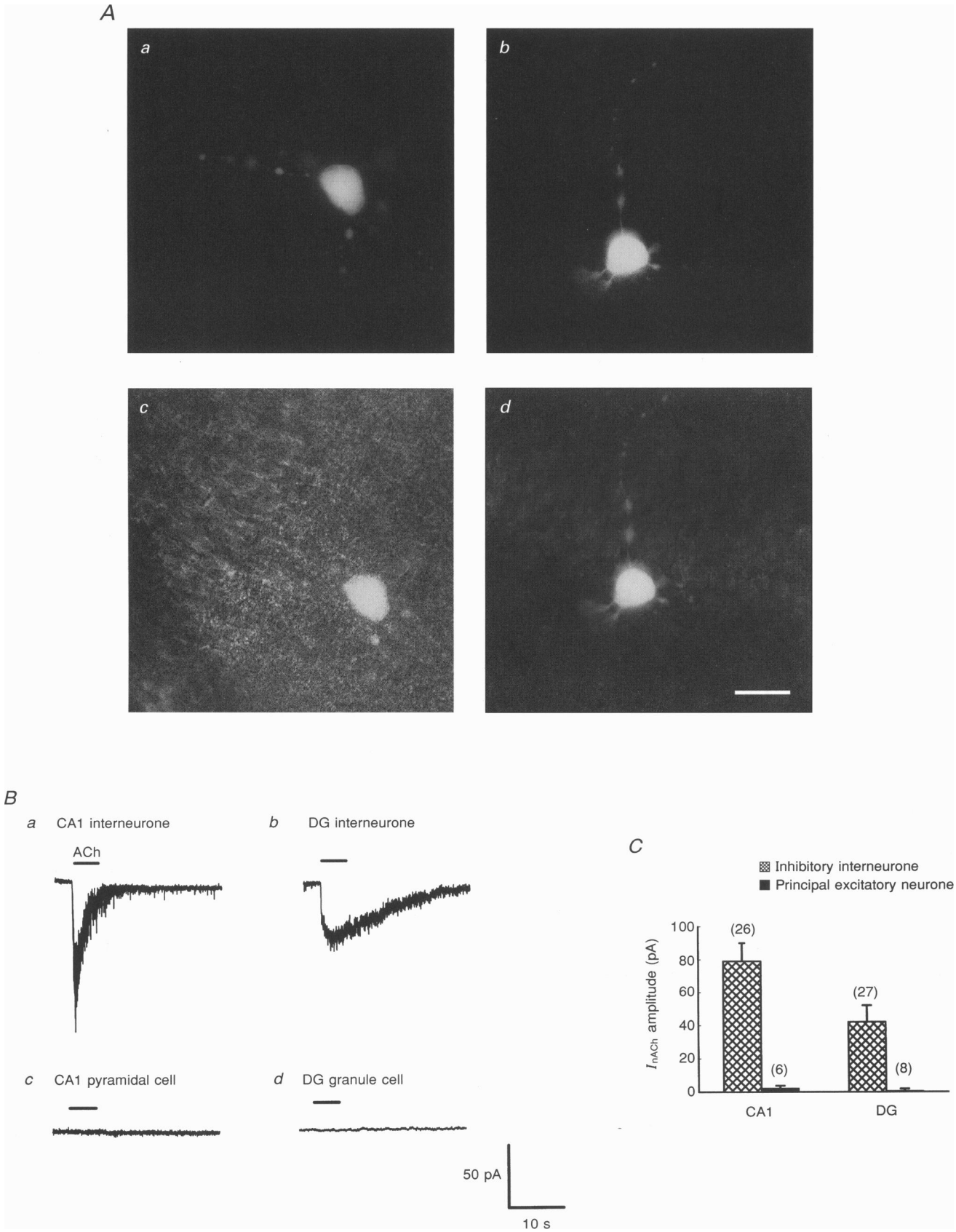

Figure 1. For legend see facing page.

In this study, the acute *in vitro* rat hippocampal slice preparation has been used to record from visually identified neurones in the hippocampus and dentate gyrus. Inhibitory interneurones in both regions, but not the principal excitatory cells of the s. pyramidale and s. granulosum, were excited by acetylcholine via activation of nAChRs. Because inhibitory interneurones can regulate the output of large numbers of principal excitatory neurones in the hippocampus (Sik, Penttonen, Ylinen & Buzsáki, 1995; Cobb *et al.* 1995; Buzsáki & Chrobak, 1995), this study reveals a novel mechanism by which nAChRs can regulate the flow of information through the hippocampus.

METHODS

Hippocampal slice preparation

Wistar rats (12–18 days old) were anaesthetized with halothane and decapitated, and the brain removed into ice cold artificial cerebrospinal fluid (ACSF). Coronal slices, 300 μm thick, were cut using a Vibratome (Series 1000, Ted Pella, Inc., Redding, CA, USA). Slicing ACSF contained (mM): 126 NaCl, 3·5 KCl, 6 $MgCl_2$, 2 $CaCl_2$, 1·2 NaH_2PO_4, 25 $NaHCO_3$, 11 glucose; gassed with 95% O_2–5% CO_2. Slices were immediately transferred to an incubation chamber containing experimental ACSF (in which [$MgCl_2$] was 1·3 rather than 6 mM) at 30 °C and bubbled with 95% O_2–5% CO_2.

Electrophysiology

After a minimum incubation time of 1 h, hippocampal slices were placed in a recording chamber, held in place using a platinum bridge with nylon cross fibres, and perfused with experimental ACSF (room temperature, bubbled with 95% O_2–5% CO_2) at 2–3 ml min^{-1}. Blockers of synaptic transmission (tetrodotoxin (TTX), 1 μM; 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 10 μM; 2-amino-5-phosphonovaleric acid (APV), 10 μM; and bicuculline, 10 μM, or picrotoxin, 50 μM), and a high concentration of the muscarinic AChR antagonist atropine (10 μM) were routinely included in the experimental ACSF. Whole-cell patch-clamp recordings were made from hippocampal CA1 interneurones in the stratum radiatum and pyramidal cells, and dentate gyrus interneurones and granule cells; cells were visually identified, based on their morphology using differential interference contrast optics and by their location in the slice. Patch pipettes were fabricated from Corning 7052 filamented glass (Garner Glass Co., Claremont, CA, USA) and had resistances of 3–5 MΩ (polished) when filled with the intracellular solutions detailed below. Neurones were voltage clamped to a holding membrane potential of −70 mV (unless otherwise stated) via an Axopatch 200-A amplifier (Axon Instruments). Holding potential values have been corrected for a 10 mV junction potential. Uncompensated series resistances were 7–15 MΩ; only cells maintaining stable series resistance were used. Intracellular solution contained (mM): 140 caesium gluconate, 10 Hepes, 2 $MgCl_2$, 0·5 $CaCl_2$, 5 BAPTA and 2 Mg-ATP; pH 7·2, adjusted with CsOH. In experiments to investigate the excitatory action of ACh on interneurones, caesium gluconate was replaced with potassium gluconate. In some experiments, Lucifer Yellow (0·3%) was included in the intracellular solution in order to confirm the identity of the different cell types.

ACh and other nAChR agonists were rapidly applied every 3 min to interneurone cell bodies via a quartz tube (i.d., 320 μm) positioned about 150 μm from the cell; drug delivery was controlled by a computer-driven valve (General Valve Co., Fairfield, NJ, USA). Responses to nAChR agonists were variable in amplitude and decay times, probably reflecting the variable rapidity of application and removal of nAChR agonists to interneurones at different depths within the slice. All antagonists were perfused via the ACSF for 9–15 min, until no further inhibition of the response was observed, except for experiments with the irreversible antagonist α-bungarotoxin, in which slices were pre-incubated for at least 10 min. All data were acquired using pCLAMP 6 software (Axon Instruments). Percentages are expressed as means ± S.E.M.; significance was determined using Student's t test, where $P < 0·05$ was considered significant.

Chemicals

Reagents for preparing ACSF and intracellular solutions were obtained from Sigma. All agonists and antagonists were obtained from Research Biochemicals International. Tetrodotoxin was obtained from Calbiochem.

RESULTS

Hippocampal inhibitory interneurones, but not principal excitatory cells, respond to ACh

The effect of acetylcholine (ACh) on four neuronal cell types in the rat hippocampal formation was investigated. Figure 1*A* shows a typical hippocampal CA1 interneurone (*a* and *c*) and dentate gyrus interneurone (*b* and *d*) representative of the cell types exhibiting responses to ACh, which have been filled with Lucifer Yellow by diffusion from a patch pipette and visualized using a fluorescence microscope. Hippocampal CA1 interneurones were typically 10–20 μm in diameter, and were found sparsely located throughout the stratum radiatum (Fig. 1*Ac*). Interneurones in the dentate gyrus (DG) were typically 15–20 μm in diameter, and were located close to the stratum granulosum–hilus border (Fig. 1*Ad*).

Figure 1. Effect of ACh on visually identified, voltage-clamped neurones in the rat hippocampus

A, fluorescence photomicrographs of representative hippocampal interneurones in the hippocampus CA1 field (*a*), and DG (*b*); cells were filled with Lucifer Yellow by diffusion of the intracellular solution from the patch pipette. Double exposure light/fluorescence photomicrographs illustrate the location of these same cells in the CA1 s. radiatum (*c*), close to the s. pyramidale (top left of *c*), and in the DG (*d*) at the border of the s. granulosum (middle, *d*) and hilar region (bottom, *d*). Scale bar, ~20 μm (applies to *a*–*d*). *B*, typical responses to ACh (100 μM, 5 s, bars) in voltage-clamp recordings (membrane potential, −70 mV) from the four neuronal types studied: CA1 s. radiatum interneurone (*a*), DG interneurone (*b*), CA1 pyramidal cell (*c*) and DG granule cell (*d*). Scale bars apply to all recordings. *C*, comparison of the amplitudes of responses to ACh in the four cell types. Numbers of cells given in parentheses. Responses of DG interneurones were significantly smaller than responses of CA1 interneurones.

ACh (100 μM) was applied to visually identified cells voltage clamped to near-resting membrane potentials (−70 mV), and typical responses of each cell type are shown in Fig. 1*B*. Interneurones in both the hippocampus and DG showed inward current responses to ACh (Fig. 1*Ba* and *Bb*, respectively). All interneurones tested showed responses to ACh; the range of response amplitudes for CA1 interneurones was −16 to −265 pA ($n = 26$), and for DG interneurones the range was −12 to −235 pA ($n = 27$). However, the principal excitatory neurones, which were

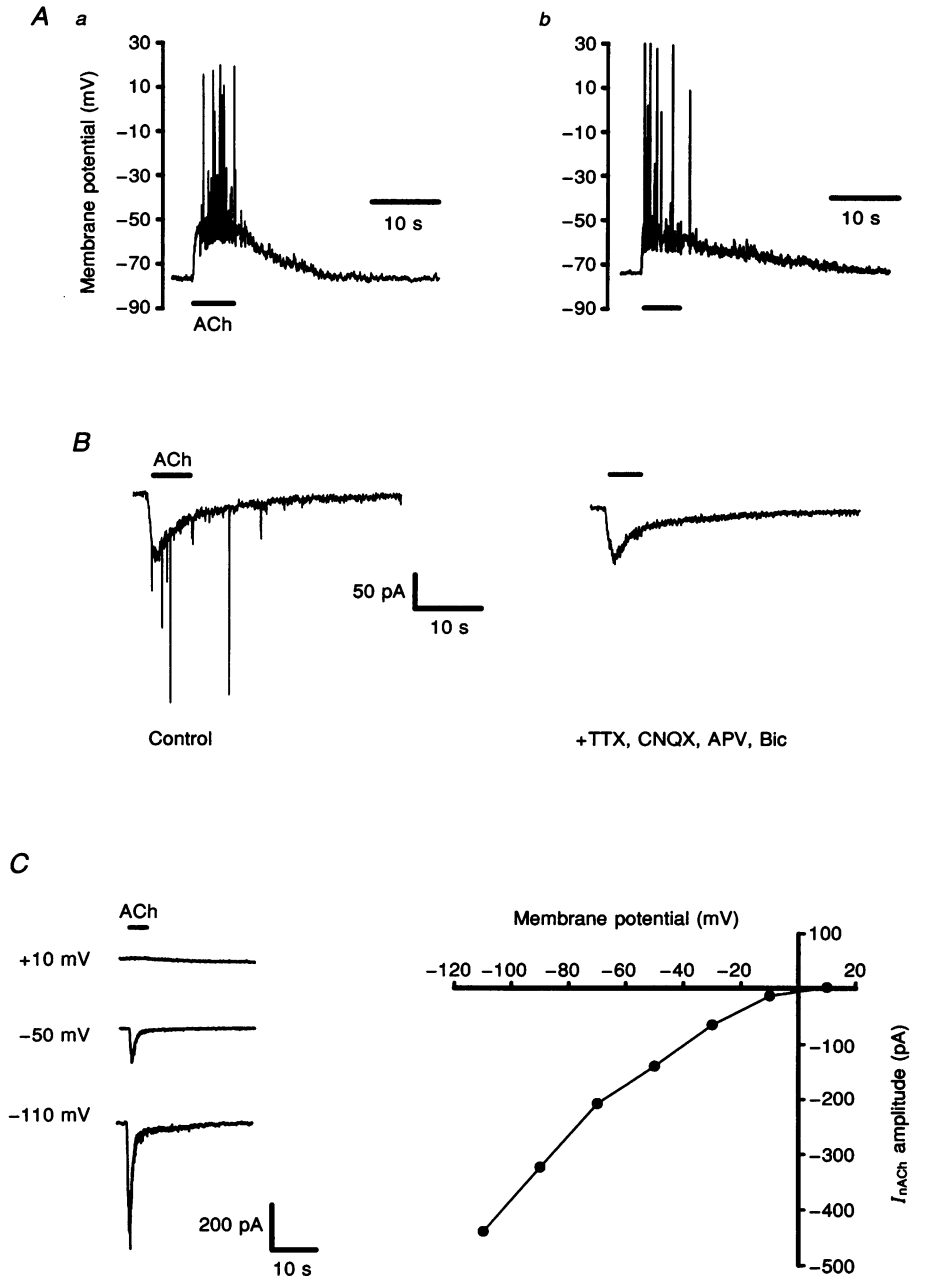

Figure 2. Direct excitation of hippocampal interneurones by ACh-activated cation current

A, current-clamp recordings (using potassium gluconate intracellular solution) from a CA1 interneurone (*a*) and a DG interneurone (*b*), showing the depolarization and action potential firing in response to ACh (100 μM, 5 s, bars). *B*, voltage-clamp recording (CA1 interneurone, cell held at −70 mV) demonstrating that the effect of ACh (100 μM, 5 s, bar) persists in the presence of inhibitors of synaptic transmission (TTX, CNQX, APV, Bic). *C*, voltage-clamp recording from a CA1 interneurone. Membrane potential was changed from −110 to 10 mV in order to obtain a steady-state current–voltage relationship for the effect of ACh (100 μM, 5 s, bar) on interneurones. Example traces at membrane potentials −110, −50 and 10 mV are shown (left). In this cell, the zero current potential was ~0 mV.

identified by the location of their cell bodies in the stratum pyramidale of the hippocampus CA1 field or s. granulosum of the DG, did not respond to ACh (Fig. 1*Bc* and *Bd*, respectively). The mean peak amplitude data for these cell types is summarized in Fig. 1*C*. The response to ACh in DG interneurones was significantly smaller than in hippocampal CA1 interneurones.

Acetylcholine directly excites hippocampal interneurones

Figure 2*Aa* shows a current-clamp recording from a hippocampal CA1 s. radiatum interneurone illustrating the membrane potential change in response to ACh. ACh evoked a 25 mV depolarization of the interneurone which was sufficient to bring the cell to threshold for action potential

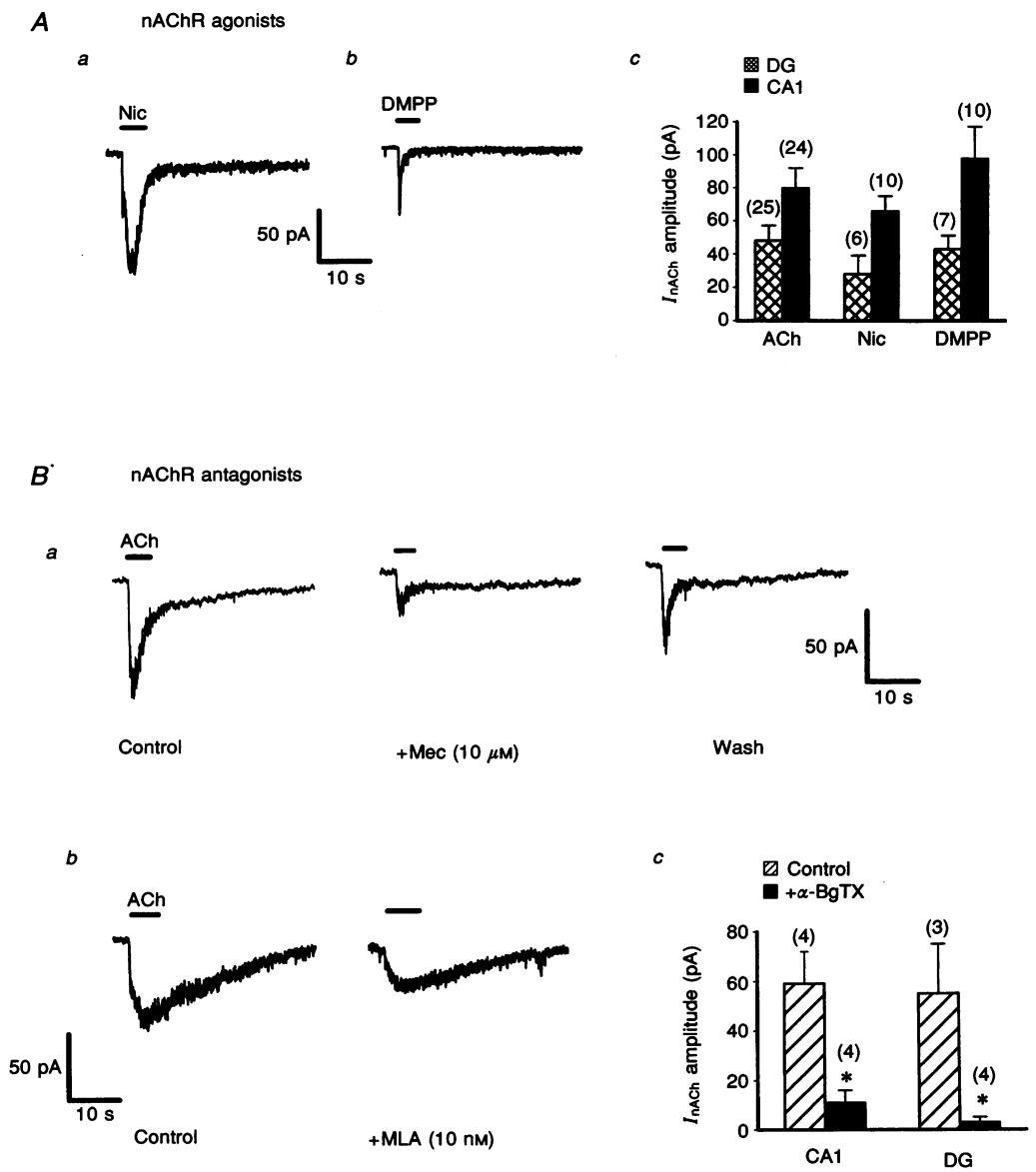

Figure 3. Pharmacological characterization of the effect of ACh on hippocampal interneurones

A, effect of the nAChR agonist nicotine (100 μM, 5 s) on a CA1 interneurone (*a*) and the nAChR agonist DMPP (100 μM, 5 s) on a DG interneurone (*b*), both voltage clamped to −70 mV. Both agonists mimic the effects of ACh on interneurones from the two regions (*c*); responses to all agonists by DG interneurones were significantly smaller than responses by CA1 interneurones (numbers of cells shown in parentheses). *B*, effect of nAChR antagonists on the responses of interneurones to ACh. Mecamylamine (Mec, 10 μM) reversibly inhibited the response to ACh (100 μM, 5 s, bars) in this voltage-clamped CA1 interneurone by 75% (*a*). Methyllycaconitine (MLA, 10 nM) inhibited the response to ACh (100 μM, 5 s, bars) in this voltage-clamped DG interneurone by 60% (*b*). Pre-treatment of slices with α-bungarotoxin (100 nM, 10 min minimum) significantly reduced ACh responses of CA1 and DG interneurones; control slices were matched from the same rats (*c*). * $P < 0{\cdot}05$.

firing. When this interneurone was voltage clamped to −70 mV, ACh evoked an inward current of −100 pA amplitude (data not shown). Figure 2*Ab* shows an identical experiment carried out in a DG interneurone, where ACh caused a 20 mV depolarization, inducing action potential firing, and evoked an inward current (−100 pA; data not shown) under voltage-clamp control. These data demonstrate that ACh can excite inhibitory interneurones of the hippocampal formation by activation of an inward current at near-resting membrane potentials. This effect of ACh is by direct action on the interneurones because it was not inhibited by blockers of synaptic transmission, including tetrodotoxin, glutamate receptor antagonists (CNQX and APV), and $GABA_A$ receptor antagonists (bicuculline or picrotoxin) as demonstrated in Fig. 2*B* (CA1 interneurone). All slices were routinely perfused with ACSF containing these blockers of synaptic transmission to eliminate any indirect effects of ACh.

The inward current response to ACh and the resulting excitation suggested the involvement of the nAChR (a ligand-gated ion channel). To further investigate this, ACh was applied to interneurones voltage clamped to different membrane potentials to obtain a current–voltage relationship. Figure 2*C* shows the relationship of ACh-evoked current *versus* membrane potential for a hippocampal CA1 s. radiatum interneurone. The membrane potential at which ACh produced zero net current flow was between −10 and 0 mV in hippocampal interneurones ($n = 3$ cells), and between −20 and +10 mV in DG interneurones ($n = 4$ cells); this is consistent with the activation of a non-selective cation conductance by ACh.

Functional nicotinic acetylcholine receptors on hippocampal interneurones

To investigate the pharmacology of the response to ACh in hippocampal interneurones, nicotinic receptor-selective ligands were utilized. These data are illustrated in Fig. 3. Application of the nAChR agonists nicotine (100 μM; hippocampal CA1 interneurone; Fig. 3*Aa*), and 1,1-dimethyl-4-phenyl-piperazinium iodide (DMPP, 100 μM; DG interneurone; Fig. 3*Ab*) mimicked the effect of ACh in activating inward current responses. A comparison of the responses to these nAChR agonists by the two cell types is summarized in Fig. 3*Ac*; responses to all three agonists by each cell type were similar, although the response to each agonist in DG interneurones was significantly smaller than the response in hippocampal CA1 interneurones.

The effects of nAChR antagonists are shown in Fig. 3*B*. Mecamylamine (10 μM) caused a marked inhibition of the inward current response to ACh. An example recording of the reversible effect of mecamylamine is shown in Fig. 3*Ba* (CA1 interneurone); the mean inhibition was 75 ± 5% ($n = 8$). The effect of mecamylamine on the response of DG interneurones to ACh was similar (88 ± 4%, $n = 5$). In contrast the muscarinic receptor antagonist atropine (10 μM) had no effect on the inward current response to ACh (data not shown). These data are consistent with the involvement of nAChRs in mediating the response to ACh.

The possible involvement of the α7 nAChR subunit in mediating this response was investigated. The presence of the α7 subunit confers significant Ca^{2+} permeability on nAChRs (Séguéla *et al.* 1993). The α7 subunit contains a binding site for α-bungarotoxin and methyllycaconitine, and these antagonists have been useful tools in identifying functional nAChRs containing α7 subunits (Alkondon, Pereira, Wonnacott & Albuquerque, 1992). Perfusion with methyllycaconitine (MLA, 10 nM, 9–15 min) significantly inhibited the response to ACh in DG interneurones (70 ± 3% inhibition, $n = 4$; example trace shown in Fig. 3*Bb*) and hippocampal CA1 interneurones (54 ± 8% inhibition, $n = 7$); this inhibition was partially reversible. The response to ACh was also inhibited by α-bungarotoxin (α-BgTX, 100 nM) in both cell types. Due to the irreversible effect of α-BgTX, hippocampal slices were pre-incubated in α-BgTX for 10 min, and responses to ACh in these slices were compared with control slices from the same rat. These data are summarized in Fig. 3*Bc*. In this series of experiments, the response to ACh by CA1 interneurones averaged −59 ± 13 pA ($n = 4$) in control slices and −11 ± 5 pA ($n = 4$) in α-BgTX-treated slices, corresponding to approximately 80% inhibition; this is similar to the 73% inhibition seen in one CA1 interneurone recording during which 100 nM α-BgTX was perfused onto the slice. For DG interneurones, responses to ACh averaged −55 ± 20 pA ($n = 3$) in control slices and −3 ± 2 pA ($n = 4$) in α-BgTX-treated slices, corresponding to approximately 90% inhibition; this is similar to the inhibition seen in two recordings from DG interneurones during which 100 nM α-BgTX was perfused onto the slice (84% and 80% inhibition). Thus, functional nAChRs in CA1 and DG interneurones contain the α7 subunit.

DISCUSSION

The whole-cell patch-clamp technique has been used to demonstrate the presence of functional nAChRs on visually identified interneurones in the rat hippocampus and DG. Interneurones in the CA1 field of the hippocampus were similar in morphology and distribution to those described by Lacaille, Kunkel & Schwartzkroin (1989). Interneurones in the DG resemble the basket cells described by Sloviter & Nilaver (1987) both in their morphology and location at the granule cell–hilus border. ACh, acting at nAChRs, excited interneurones by activation of a non-selective cation current. In contrast to inhibitory interneurones, no effect of ACh was seen on voltage-clamped principal excitatory cells in s. pyramidale, which were readily identified by their characteristic morphology and location. In the DG, visually identified principal excitatory cells in s. granulosum did not respond to ACh. Thus, ACh may regulate the activity of neuronal circuits in the hippocampal formation in part via activation of nAChRs located on interneurones in the hippocampus proper and dentate gyrus.

The involvement of nAChRs in mediating the excitatory effect of ACh on hippocampal interneurones was confirmed by a number of observations: the ability of the nAChR agonists nicotine and DMPP to mimic ACh, the antagonistic effect of mecamylamine, the lack of effect of atropine on the response to ACh, and the current–voltage relationship for the ACh-activated response. A particularly interesting observation was the inhibition of the response to ACh by the $\alpha7$ antagonists α-BgTX and MLA, suggesting that the functional nAChRs on interneurones in both the CA1 and DG may contain the Ca^{2+}-permeable $\alpha7$ subunit. This is consistent with the observation of Freedman, Wetmore, Strömberg, Leonard & Olson (1993) that [^{125}I]α-BgTX binds to GABA-containing neurones in the CA1 field of the hippocampus and the inner blade of the DG granule cell layer. The present results do not indicate the number of $\alpha7$ subunits present; moreover, α-BgTX also inhibits currents carried by the Ca^{2+}-permeable rat $\alpha9$ subunit (McGehee & Role, 1995), and therefore we cannot rule out a contribution of $\alpha9$ subunits to functional nAChRs in hippocampal interneurones, although there is currently no evidence that these neurones express the $\alpha9$ subunit. Thus, our results illustrate an α-BgTX- and MLA-sensitive effect of ACh on hippocampal interneurones, providing a putative mechanism for ACh-evoked Ca^{2+} entry into these cells.

Previous studies have reported effects of nAChR ligands on hippocampal neuronal activity. These reports have largely come from extracellular field potential recordings, and in contrast with the present data, facilitatory effects of nAChR agonists on excitatory activity in the s. pyramidale have been reported in rats (Ropert & Krnjevic, 1982; Rovira *et al.* 1983) and mice (Freund *et al.* 1988). In guinea-pig hippocampal slices, intracellular recordings revealed an inhibition of pyramidal cell activity by ACh, with an accompanying excitation of interneurones (Benardo & Prince, 1982); the pharmacology of the receptor mediating this response was not investigated. Therefore, a clear demonstration that inhibitory interneurones, rather than principal excitatory cells, are directly excited by nAChR activation has not been provided, and the present data illustrate a novel function for nAChRs in the rat hippocampus.

The observation that principal excitatory neurones in the hippocampus do not respond to ACh is in contrast to the demonstration of functional nAChR responses in cultured hippocampal neurones, which consist primarily of pyramidal cells (Alkondon & Albuquerque, 1991). One possible explanation for this disparity is that expression of functional nAChRs on the soma of pyramidal cells occurs as a consequence of dissociating and culturing hippocampal neurones. In the present studies, the absence of effects of ACh on granule cells in the DG may reflect an inability of recordings at the granule cell soma to detect responses to nicotine at distant terminals; Gray *et al.* (1996) have previously demonstrated enhancement of glutamate release by nAChRs at the granule cell mossy fibre terminal.

Interneurones in the CA1 field and DG of the rat hippocampal formation receive cholinergic innervation from the MSDB complex (Frotscher & Léránth, 1985). Because of the proposed role of the MSDB cholinergic projection in pacing the activity of hippocampal neurones (Stewart & Fox, 1990), the possible participation of functional nAChRs in this pathway may now be considered. MSDB regulation of rhythmical slow activity in the hippocampus is partially atropine sensitive, and also has a GABA-ergic component: these regulatory effects are mediated at least in part by excitation and inhibition, respectively, of hippocampal inhibitory interneurones, which in turn inhibit and synchronize the activity of the principal excitatory neurones (Stewart & Fox, 1990). In the hippocampal CA1 field, interneurones are capable of inhibiting excitatory cells in s. pyramidale as well as excitatory and inhibitory cells in the DG (Lacaille *et al.* 1989). A single interneurone is estimated to innervate thousands of pyramidal cells and therefore can simultaneously inhibit large populations of cells (Sik *et al.* 1995; Cobb *et al.* 1995; Buzsáki & Chrobak, 1995). In the DG, the axons of basket cells branch to form a plexus of fibres in the s. granulosum and mediate recurrent inhibition of excitatory granule cells (Sloviter & Nilaver, 1987); by analogy with CA1 inhibitory basket cells (Lacaille *et al.* 1989), DG basket cells may also participate in feed-forward inhibition. Thus, the finding that functional nAChRs mediate excitation of inhibitory interneurones in the CA1 and in the DG provides a putative mechanism for rapid excitation of interneurones by MSDB projections, leading to feed-forward inhibition of other hippocampal neurones, and possibly contributing to the rhythmical slow activity in the hippocampus. The precise location of functional nAChRs on interneurones in the CA1 and DG remains to be determined: it is not yet known whether nAChRs are positioned at specialized postsynaptic sites to receive ACh released from cholinergic terminals. However, in chick autonomic ganglia, perisynaptic α-BgTX-sensitive AChRs have been shown to mediate postsynaptic responses (Zhang, Coggan & Berg, 1996); thus, nAChRs in the hippocampus may also mediate postsynaptic responses, in addition to mediating presynaptic modulation of glutamate release (Gray *et al.* 1996).

In conclusion, the present experiments have demonstrated functional nAChRs on inhibitory interneurones in the hippocampal formation. Activation of nAChRs excites interneurones which can in turn inhibit large numbers of hippocampal excitatory and inhibitory neurones; thus nAChRs could participate in the cholinergic regulation of hippocampal neuronal activity. Alterations of nicotinic binding sites in the hippocampus have been reported in neurodegenerative disorders such as Alzheimer's and Parkinson's diseases; moreover, nicotine enhances cognitive function in some Alzheimer's patients (Nordberg, 1994). Thus, the identification of functional nAChRs in the hippocampus may illuminate a mechanistic basis for nAChR involvement in certain neurodegenerative disorders.

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