Muscarinic receptors mediating depression and long-term potentiation in rat hippocampus

Jonathan M. Auerbach and Menahem Segal

Department of Neurobiology, The Weizmann Institute, Rehovot 76100, Israel

1. Two concentration-dependent effects of the muscarinic agonist carbachol (CCh) were characterized in submerged slices of rat hippocampus using extracellular recordings of excitatory postsynaptic potentials (EPSPs): muscarinic long-term potentiation (LTP_m) and depression.

2. LTP_m of the EPSP slope was seen following long exposure (20 min) of the slice to low concentrations of CCh (0·2–0·5 μM). This LTP_m was not accompanied by a change in the size of the afferent fibre volley or by a change in paired-pulse potentiation, consistent with a postsynaptic locus of CCh action.

3. Intracellular recordings from voltage-clamped neurons of inward current evoked by iontophoretically applied α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and *N*-methyl-D-aspartate (NMDA) revealed that, while cellular responses to NMDA rose transiently upon superfusion with 0·5 μM CCh, responses to AMPA increased gradually and remained potentiated after washout of CCh.

4. LTP_m is mediated by an M2 muscarinic receptor. Two M2 muscarinic receptor antagonists, methoctramine and AFDX-116, blocked LTP_m. The M2 agonist oxotremorine induced LTP_m at low agonist concentrations. None of the M1 and M3 receptor agonists and antagonists tested affected LTP_m.

5. Muscarinic fast onset depression of the EPSP was seen in response to higher concentrations of CCh (2–5 μM). This depression was accompanied by an increase in paired-pulse potentiation, indicating a possible presynaptic locus of action. The M3 muscarinic receptor antagonist 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) blocked the muscarinic depression of the EPSP slope. M1, M2 and M4 muscarinic antagonists did not block this response.

6. Blockade of the muscarinic depression by 4-DAMP did not uncover a suppressed LTP_m. However, addition of picrotoxin facilitated the expression of LTP_m induced by high concentrations of CCh, indicating an involvement of interneurons in regulation of LTP_m.

7. Cholinergic denervation produced by fimbria–fornix transection resulted in supersensitivity of both M2- and M3-mediated effects, indicating that the receptors mediating these effects are not located on presynaptic cholinergic fibres. In the presence of 4-DAMP and picrotoxin the dose–response curve for CCh-induced effects in slices from lesioned animals was shifted to the left relative to that of normal animals, indicating a supersensitivity of both receptor types.

Long-term potentiation (LTP) is a long lasting increase in the efficacy of synaptic transmission (Bliss & Lomo, 1973; Bliss & Collingridge, 1993) typically induced in pyramidal cells of area CA1 in the hippocampal slice by tetanic stimulation of the Schaffer collateral–commissural afferent fibres. LTP can also be induced by exposure of the slice to various compounds which affect synaptic transmission or mobilization of intracellular calcium (Aniksztejn & Ben-Ari, 1991; Bortolotto & Collingridge, 1993). LTP has been accepted as a cellular model for learning and memory and, therefore, is used for the study of processes that underlie plastic changes in the brain (Doyere & Laroche, 1992; Bliss & Collingridge, 1993).

Acetylcholine (ACh) is known to be involved in cognitive processes of learning and memory (Buresova, Bures, Bohdanecky & Weiss, 1964; Bartus, Reginald, Dean, Beer & Lippa, 1982), but its role at the cellular and molecular level in these processes is still unclear. There are five

known subtypes of the muscarinic receptor expressed in mammalian brain, named M1–M5 (Waelbroeck, Tastenoy, Camus & Christophe, 1991). Several muscarinic actions of ACh in the hippocampus, including blockade of potassium channels (Cole & Nicoll, 1983; Madison, Lancaster & Nicoll, 1987), have been well documented. One effect of particular interest to us is the ACh-induced suppression of the excitatory postsynaptic potential (EPSP) evoked by stimulation of the Schaffer collateral–commissural fibres (Segal, 1982; Sheridan & Sutor, 1990). This effect is assumed to be mediated by presynaptic muscarinic receptors and is seen with increasing concentrations of ACh or the muscarinic agonist carbachol (CCh). The specific receptor subtype associated with this action is still controversial.

Insight into the role of ACh in the cellular and molecular aspects of plasticity has been gained by examining the effects of ACh on LTP. In the dentate gyrus of rat hippocampus, muscarinic activation facilitates LTP induction (Burgard & Sarvey, 1990) and physostygmine, an inhibitor of acetylcholinesterase, causes potentiation of population spikes resembling LTP (Ito, Miura & Kadokawa, 1988; Levkovitz & Segal, 1994). In area CA1, CCh can enhance LTP (Blitzer, Gil & Landau, 1990) and the muscarinic antagonist atropine can suppress associative LTP (Sokolov & Kleschevnokov, 1995). We have recently shown that bath application of low concentrations of CCh induces LTP (Auerbach & Segal, 1994) and application of an even lower concentration, while having no observable effect of its own, reduces the threshold for tetanic LTP induction. This muscarinic LTP (LTP_m) shares similarities with tetanic LTP which lies downstream of the involvement of the NMDA receptor. In the present study we used the hippocampal slice to explore LTP_m further. Here we verified that LTP_m has a postsynaptic locus of expression, characterized the muscarinic receptor subtype involved in LTP_m, and examined the relation between LTP_m and the cholinergic depression of the EPSP.

METHODS

Hippocampal slices were prepared from adult male Wistar rats (150–200 g) obtained from a local breeding colony. Animals were rapidly decapitated using a standard guillotine and the right hippocampus was dissected from the brain, sliced with a McIlwain tissue chopper (350 μm) and incubated for at least 1·5 h in a recovery chamber at room temperature. The chamber was filled with 10 ml artificial cerebrospinal fluid (ACSF) containing (mM): 124 NaCl, 4 KCl, 26 $NaHCO_3$, 1·25 NaH_2PO_4, 2 $CaCl_2$, 2 $MgSO_4$ and 10 glucose, pH 7·4, unless otherwise specified. The ACSF was saturated with a 95% O_2–5% CO_2 gas mixture (flow rate 0·4 l min^{-1}). Experiments were performed in a submerged slice chamber. The recording chamber was heated to 30 °C and superfused at a rate of 2·5 ml min^{-1} with ACSF. A bipolar stainless steel stimulating electrode was placed in stratum radiatum of CA1. Stimulation was delivered at a rate of 0·033 Hz, 50 μs pulse duration, with intensity adjusted such that evoked responses were about half-maximal. The extracellular recording electrode, containing 2 M NaCl (2–4 MΩ tip resistance), was placed in the stratum radiatum of the CA1 region for recording of EPSPs. Drugs were applied via the superfusion medium beginning 10 min after the establishment of a stable baseline response. A drug-naive hippocampal slice was used for each experiment, i.e. each slice was exposed to only one drug concentration.

Intracellular recordings were performed in a single-electrode voltage clamp configuration at a discontinuous sampling frequency of 3–4 kHz, in slices treated with 1 μM tetrodotoxin (TTX). Cells were clamped at their resting membrane potential (ranging from approximately −65 to −75 mV). The recording electrode, containing 4 M potassium acetate (40–80 MΩ), was placed in the pyramidal cell layer of CA1. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA; 25 mM) and *N*-methyl-D-aspartate (NMDA; 100 mM) were applied by current-balanced iontophoresis through a triple-barrelled pipette placed in stratum radiatum close to the soma of the recorded cell.

Bilateral fimbria–fornix transections were performed as follows: 6-week-old male rats anaesthetized with xylazine–ketamine (10 and 50 mg kg^{-1}, respectively, I.P.) and placed in a stereotactic instrument. Two small (< 1·0 mm diameter) holes were carefully drilled in the skull and bilateral knife cuts were made stereotactically 1 mm posterior to bregma, 1 mm lateral of mid-line, at a depth of 4·5 mm. The knife was moved laterally (45 deg) to transect all fimbria–fornix fibres. All surgery was performed under semi-sterile conditions, i.e. surgical equipment was soaked in 70% ethanol prior to use and the wound area was cleaned with 70% ethanol as well. Following surgery the wound was sutured and animals were placed in a clean, warmed cage. Animals were closely monitored until complete recovery, upon which they were transferred to a clean home cage where they had free access to food and water in a temperature- and light-controlled room. Under these conditions, no infection or abnormal behaviour was noticed in any of the rats at any time. Animals were used about 2 weeks after the operation. The resulting cholinergic denervation was verified in some rat brains stained for acetylcholinesterase, as detailed elsewhere (Biegon, Greenberger, & Segal, 1986).

Stock solutions were prepared in distilled water and stored at −20 °C. All working solutions were diluted in ACSF immediately prior to use. All drugs were obtained from RBI except for dicyclomine hydrochloride and picrotoxin, which were obtained from Sigma, and AMPA and NMDA, which were obtained from Tocris Cookson (Bristol, UK). AF-267 was a gift from Dr A. Fisher (Israel Institute for Biological Research, Israel). Signals were amplified with an Axoclamp-2A amplifier and digitized with a DMA interface (Axon Instruments). Data were collected, stored and analysed on an IBM-compatible computer (Asyst 3.1, Asyst Software Tech., Inc., Rochester, NY, USA). Intracellular data were collected on a continuous chart recorder. Cells recorded had a resting membrane potential below −60 mV and an input resistance greater than 30 MΩ. Data were pooled and normalized with respect to the steady baseline values (baseline = 1·00) and expressed as means ± standard error of the mean.

RESULTS

LTP_m is a postsynaptic phenomenon

LTP_m is not accompanied by an increase in the presynaptic volley. As Fig. 1 shows, superfusion of a submerged hippocampal slice with 0·5 μM CCh for 20 min resulted in LTP_m – a gradual, long lasting increase in the slope of the EPSP (measured as mV ms^{-1}) evoked by

stimulation of the Schaffer collateral–commissural afferents. CCh also blocks several K^+ conductances (Cole & Nicoll, 1983; Madison *et al.* 1987), which may reduce the threshold for activation of the Schaffer collateral pathway. In order to determine if LTP_m involves an increase in the presynaptic volley (PSV) resulting from such a reduction in activation threshold, we investigated the relationship between the potentiated EPSP slope seen in LTP_m and the presynaptic fibre volley. To this end, we simultaneously monitored PSV height and EPSP slope while superfusing slices with 0·5 μM CCh. The EPSP slope in these slices exhibited a typical LTP_m (potentiation of 1·698 ± 0·057 of baseline; $n = 6$; Fig. 1*A* and *B*), while the PSV showed no significant change throughout the experiment (Fig. 1*C*).

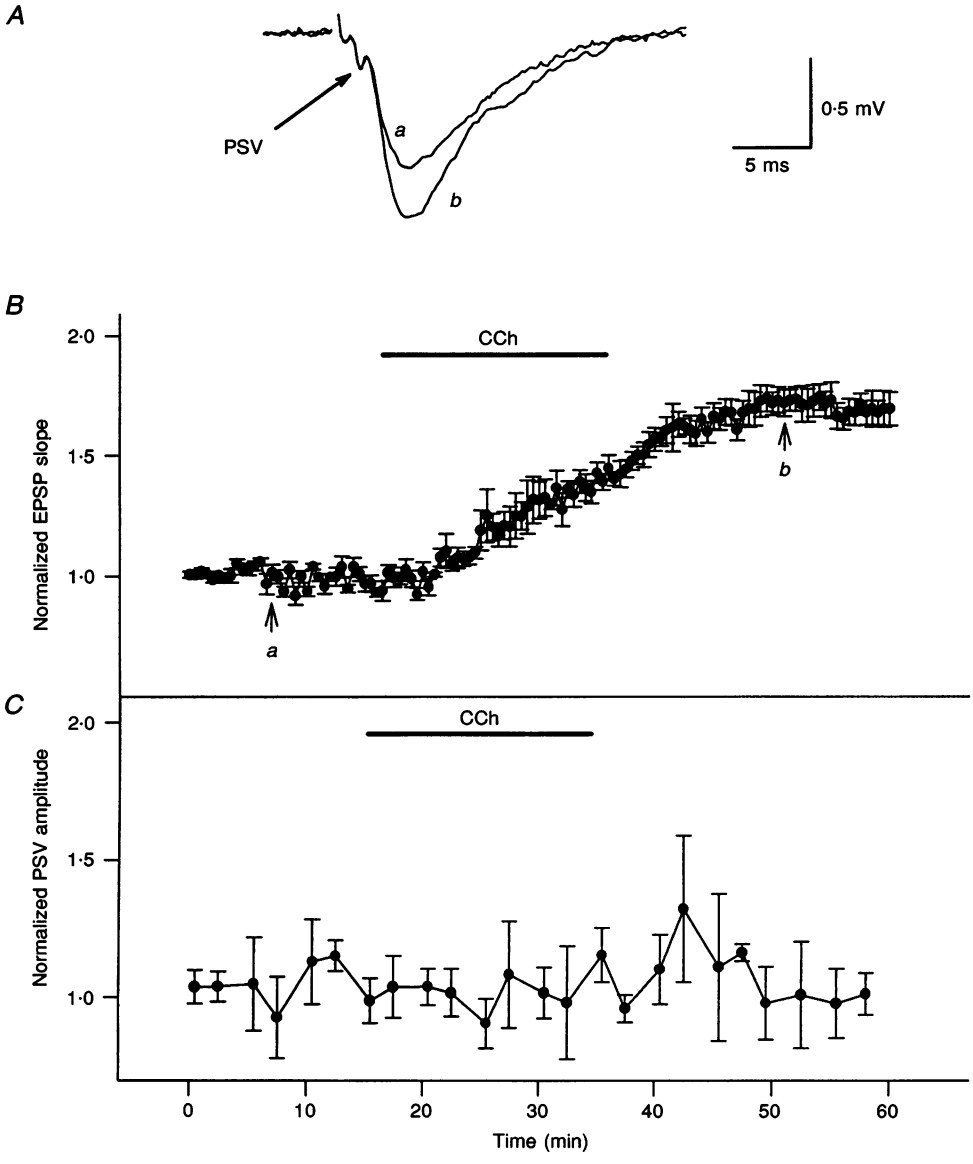

Figure 1. Carbachol (CCh) enhances EPSP slope without affecting presynaptic fibre volley (PSV)

A, superimposition of sample traces of two EPSPs from time points of 5 min (*a*) and 50 min (*b*) after the start of the experiment. The EPSP was potentiated by superfusion of slices with 0·5 μM CCh for 20 min, but there was no significant change in the size of the PSV (arrow). *B*, CCh (0·5 μM) induced LTP_m of the EPSP evoked by stimulation of the Schaffer collateral axons at a frequency of 0·033 Hz. The EPSP slope reached a level of 1·698 ± 0·057 of baseline values ($n = 6$ slices). For this and the following figures, the bar above the graph indicates duration of drug application. *a* and *b* refer to the time points at which the EPSPs shown in *A* were taken. *C*, the presynaptic volley amplitude did not change significantly during the experiments in which LTP_m was induced. The PSV from every fourth EPSP was measured, normalized, averaged across experiments and plotted.

Paired-pulse potentiation (PPP) is unaffected by LTP_m induction. PPP is considered to be a presynaptic form of short-term plasticity (Del Castillo & Katz, 1954; Leung & Fu, 1994), and in area CA1 of the hippocampal slice it is related to an increase in the number of quanta released from presynaptic terminals (Hess, Kuhnt & Voronin, 1987). Since PPP is assumed to be mediated by presynaptic mechanisms, a change in PPP following LTP_m induction would indicate an involvement of presynaptic mechanisms in LTP_m. In the following experiments, paired stimuli were delivered to the Schaffer collateral fibres with an interpulse interval of 50 ms to elicit PPP. Each pair of stimuli was delivered at a frequency of 0·0167 Hz for the duration of the experiment. The slopes of both EPSPs of the pair were measured and plotted (Fig. 2*B*). Next, the ratio of the second of the pair of EPSP slopes to that of the first was

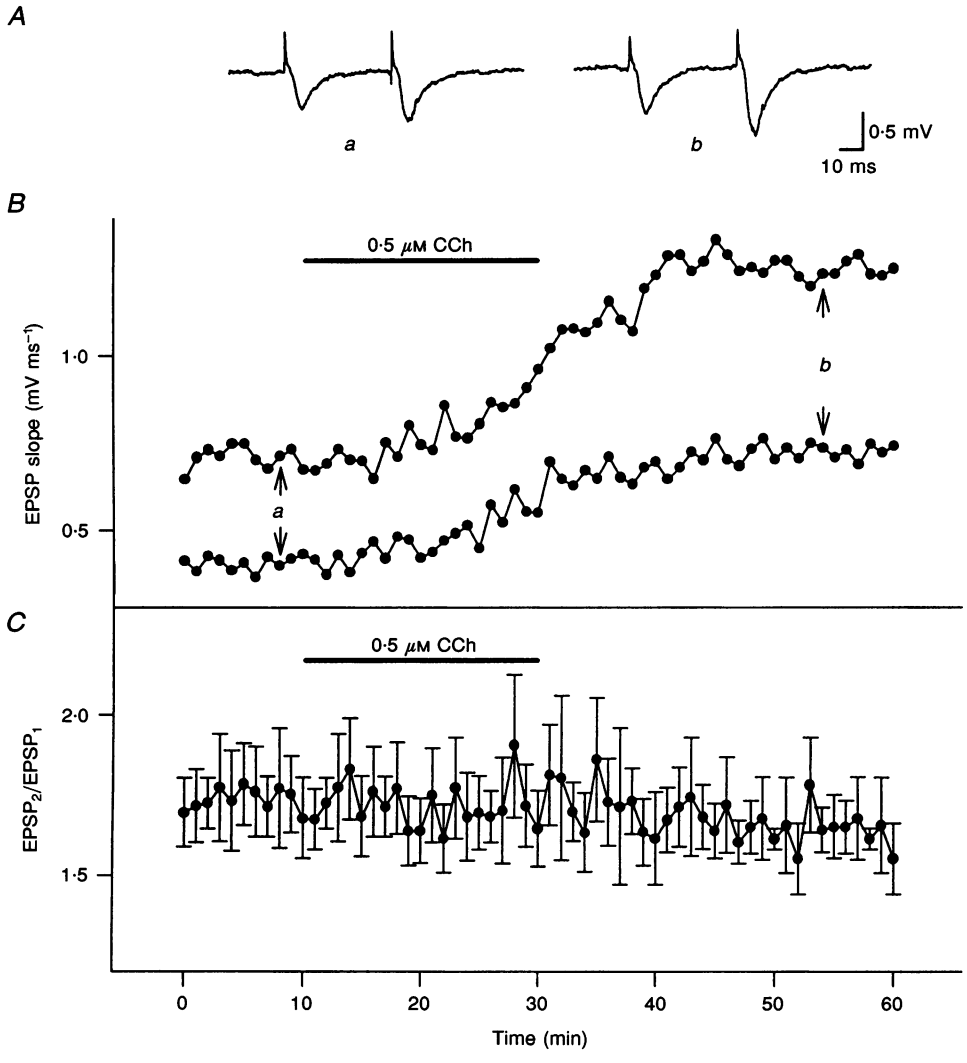

Figure 2. Paired-pulse potentiation (PPP) of the EPSP slope does not change during or after induction of LTP_m

A, sample traces from two pairs of EPSPs evoked by a pair of stimuli with an interstimulus interval of 50 ms. The traces were taken from a representative experiment in which LTP_m was induced (see *B*, below). The first pair (*a*) was recorded before the addition of CCh (0·5 μM) to the bath and the second pair (*b*) after washout of CCh. Note that after washout, both EPSPs of the pair were potentiated. *B*, EPSP slope data from a single slice in which paired stimuli (50 ms interval) were delivered at a frequency of 0·0167 Hz while LTP_m was induced. The upper trace represents data from the second EPSP of the pair. During and after the application of CCh both EPSPs of the pair were potentiated, as above. *a* and *b* refer to time points at which the traces in *A* were sampled. *C*, summary of four experiments performed according to the protocol used in *B*. Paired stimuli (50 ms interval) were delivered as LTP_m was induced. The ratio of the second EPSP slope ($EPSP_2$) of the pair to that of the first ($EPSP_1$) was determined, and the ratios were averaged and plotted. Throughout the duration of the experiments in which LTP_m was induced, there was no change in $EPSP_2/EPSP_1$.

determined, averaged over all slices ($n = 4$), and plotted (Fig. 2*C*). Under conditions which induce LTP_m, the slopes of both of the EPSPs in the pair increased in parallel and, thus, there was no change in PPP as determined by the ratio of the slopes of the EPSP pair ($EPSP_2/EPSP_1$).

AMPA receptor-mediated currents are potentiated by 0·5 μM CCh. We have previously shown that ACh potentiates the intracellular inward current response to NMDA (Markram & Segal, 1990). On the other hand, we have recently shown that LTP_m is independent of the NMDA receptor (Auerbach & Segal, 1994). Therefore, we studied the relation between CCh effects on NMDA- and AMPA-induced inward currents during prolonged superfusion of the slice with 0·5 μM CCh. We recorded intracellularly from voltage-clamped CA1 pyramidal cells while iontophoretically applying NMDA or AMPA in stratum radiatum close to the recorded cell. The drugs were ejected from the iontophoretic pipette by current pulses applied once per minute, alternating between AMPA and NMDA. Upon addition of CCh to the superfusion medium, the cellular response to NMDA immediately increased above

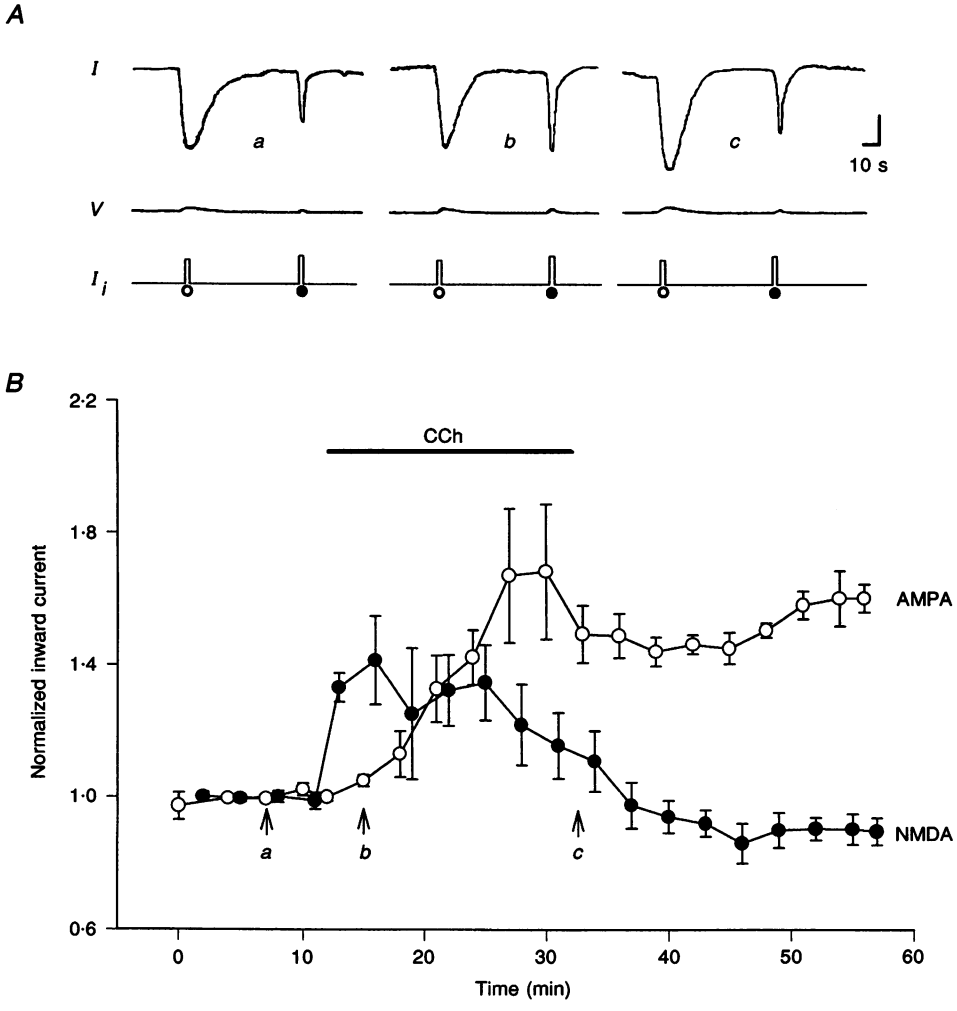

Figure 3. Changes in intracellular inward currents in response to iontophoretically applied AMPA and NMDA brought about by superfusion with CCh

AMPA and NMDA were alternately applied at 1 min intervals via iontophoresis near the voltage-clamped cell. *A*, samples of inward current taken before (*a*), during (*b*) and after (*c*) superfusion with 0·5 μM CCh. *I*, cellular current response; *V*, voltage; I_i, iontophoretic current for drug ejection. Scale bar, 0·25 nA for top trace, 5 mV for middle trace, and 10 nA for bottom trace. Symbols below the lower trace represent application of AMPA (○) and NMDA (●). *B*, summary of six cells recorded in the protocol described above. Inward current was recorded and normalized according to baseline values. As 0·5 μM CCh was added to the superfusion medium, the cellular response to NMDA rose almost immediately and then declined slowly to reach a steady value of 0·904 ± 0·046 of baseline. At the same time, the cellular response to AMPA gradually increased, reaching a peak approximately 15 min after the start of CCh application. The AMPA responses reached a stable plateau at 1·542 ± 0·079 of baseline.

baseline, reached a maximum within a few minutes, and then proceeded to decline. The response reached a stable level of 0·904 ± 0·046 of baseline values after washout of CCh. The cellular response to AMPA, on the other hand, increased gradually, reaching a stable potentiated level of 1·542 ± 0·079 of baseline (Fig. 3; $n = 6$ cells). Both the fast onset potentiation of cellular response to NMDA and the gradually developing potentiation of AMPA responses were blocked by 10 μM atropine added to the superfusion medium ($n = 4$ cells, data not shown).

Muscarinic depression is accompanied by an increase in PPP

Application of higher CCh concentrations (≥5·0 μM) to a submerged hippocampal slice produces a short latency depression which recovers upon washout of the drug. An indication of the pre- or postsynaptic locus of action of CCh can be derived from PPP during CCh-induced depression of the EPSP. Paired stimuli were delivered to the Schaffer collateral fibres with an interpulse interval of 50 ms to elicit PPP, as described above. Upon addition of 5·0 μM CCh to

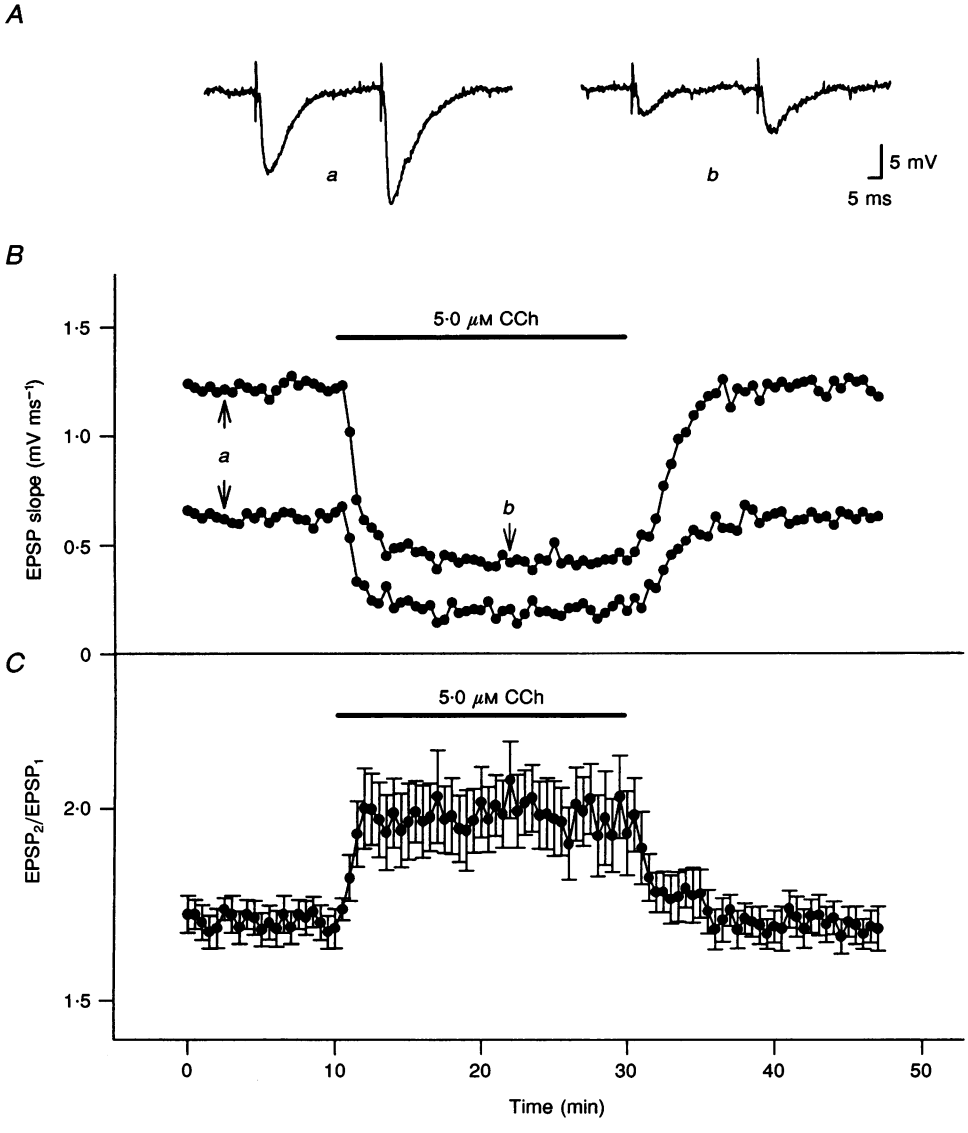

Figure 4. Paired-pulse potentiation increases upon addition of 5·0 μM CCh

A, traces from two pairs of EPSPs evoked at an interstimulus interval of 50 ms. The traces were taken from a single experiment. The first pair (*a*) was recorded during acquisition of baseline and the second pair (*b*) during superfusion with 5·0 μM CCh, as indicated in *B*. *B*, EPSP slope data from a single slice in which paired stimuli (50 ms interval) were delivered at a frequency of 0·0167 Hz. The upper trace represents data from the second EPSP of the pair. During the application of 5·0 μM CCh both EPSPs of the pair were depressed. *C*, summary of nine experiments performed according to protocol used in *B*. The ratio of the second EPSP slope ($EPSP_2$) of the pair to that of the first ($EPSP_1$) was determined. The ratios were averaged and plotted. An increase in PPP was seen upon addition of CCh to the medium. This increase returned to baseline values following washout.

Table 1. Summary of the pharmacological effects of cholinergic muscarinic ligands

Receptor	Agent	Compound	Concentration (n) (μM)	Effect
All	Agonist	CCh	0·25–0·75 (21)	Potentiation
			5–10 (15)	Depression
All	Antagonist	Atropine	10 (8)	Block of potentiation and depression
M1	Agonist	McN-343	0·5–10 (11)	None
	Agonist	AF-267	10 (2)	None
	Antagonist	Pirenzepine	0·25 (9)	None
M2	Agonist	Oxotremorine	0·1–0·5 (15)	Potentiation
			1–5 (5)	Depression
	Antagonist	Methoctramine	0·25 (4)	Block of potentiation
			0·25 (4)	No effect on depression
	Antagonist	AFDX-116	0·3 (4)	Block of potentiation
M3	Antagonist	4-DAMP	1 (8)	Block of depression
			1 (4)	No effect on potentiation
M4	Antagonist	Dicyclomine	1 (11)	None

Agonists were tested for the ability to induce either LTP_m or cholinergic depression resulting from a 20 min superfusion of the agent over the slice. Antagonists were tested for the ability to block LTP_m induced by superfusion of CCh (0·5 μM, 20 min) or to block cholinergic depression of the EPSP slope induced by 5·0 μM CCh.

the medium, there was a rapid depression of both EPSPs of the pair (Fig. 4*A* and *B*). However, in relative terms the second EPSP slope decreased less than the first as revealed by the increase in the ratio $EPSP_2/EPSP_1$ upon introduction of CCh (Fig. 4*C*). Upon washout of CCh, both EPSP slopes of the pair returned to baseline values, as did the ratio $EPSP_2/EPSP_1$. This change in PPP is consistent with a presynaptic locus of CCh action in depression of the EPSP.

Muscarinic M2 and M3 receptors mediate the potentiation and depression effects of CCh, respectively

To study the muscarinic receptors involved in the biphasic concentration-dependent effects of CCh on the EPSP, we tested a series of specific agonists and antagonists of the different muscarinic receptors. Each agonist was tested for its ability to induce either LTP_m or cholinergic depression during a 20 min superfusion period. Each antagonist was applied before and during exposure of the slice to CCh and tested for its ability to block either cholinergic depression resulting from superfusion of 5·0 μM CCh or to block LTP_m resulting from a 20 min superfusion of 0·5 μM CCh. The results are summarized in Table 1. The concentrations of the drugs used were near their K_D values, as reported elsewhere (see Discussion).

Pirenzepine (0·25 μM), a specific M1 antagonist, failed to block either cholinergic depression or LTP_m. Of the two M1 agonists used, neither 10 μM AF-267 ($n = 2$) nor McN-343 at concentrations ranging from 0·5 to 10·0 μM ($n = 11$) had any effect on the EPSP. In addition, the M4 antagonist dicyclomine at 1·0 μM failed to block either the cholinergic depression or LTP_m. Oxotremorine (Oxo), a muscarinic M2 receptor agonist, mimicked CCh in its biphasic concentration effects on EPSP slope (Fig. 5*A* and *B*). The higher concentrations of Oxo tested (>0·5 μM) caused a reversible depression of the EPSP ($n = 5$), whereas lower concentrations of Oxo (0·1–0·5 μM) induced a gradually developing, long lasting potentiation of the EPSP slope ($n = 15$). A still lower concentration (0·025 μM) had no observable effect ($n = 4$). The dose–response curve for Oxo-induced effects on the EPSP slope was constructed for a time point shortly following Oxo washout (Fig. 5*B*). A major difference between CCh- and Oxo-induced effects was their effective concentration ranges. It is likely that Oxo induced LTP_m at concentrations where it is a specific agonist for the M2 receptor, while it produced the depression at higher concentrations where it is no longer specific. To substantiate further our finding that LTP_m is mediated by an M2 muscarinic receptor, we tested two specific M2 antagonists for their ability to block LTP_m. Application of 0·25 μM methoctramine together with 0·25 μM Oxo was sufficient to block LTP_m (Fig. 5*C*; $n = 4$). Similarly, 0·3 μM AFDX-116 applied together with 0·25 μM Oxo blocked LTP_m ($n = 4$; data not shown).

None of the specific muscarinic receptor antagonists tested above was able to block the cholinergic depression induced by high concentrations of CCh or Oxo. We found that application of 1·0 μM 4-DAMP, a specific M3 antagonist, was sufficient to block the depression of the EPSP slope caused by 5·0 μM CCh (Fig. 6*A*). Figure 6 shows that 5·0 μM CCh caused a depression of the EPSP slope to 0·523 $\pm$ 0·047 of baseline values ($n = 10$). However, when 1·0 μM 4-DAMP was added before and together with 5·0 μM CCh the depression was blocked and the EPSP did not vary significantly from baseline values for the duration of the experiment ($n = 8$).

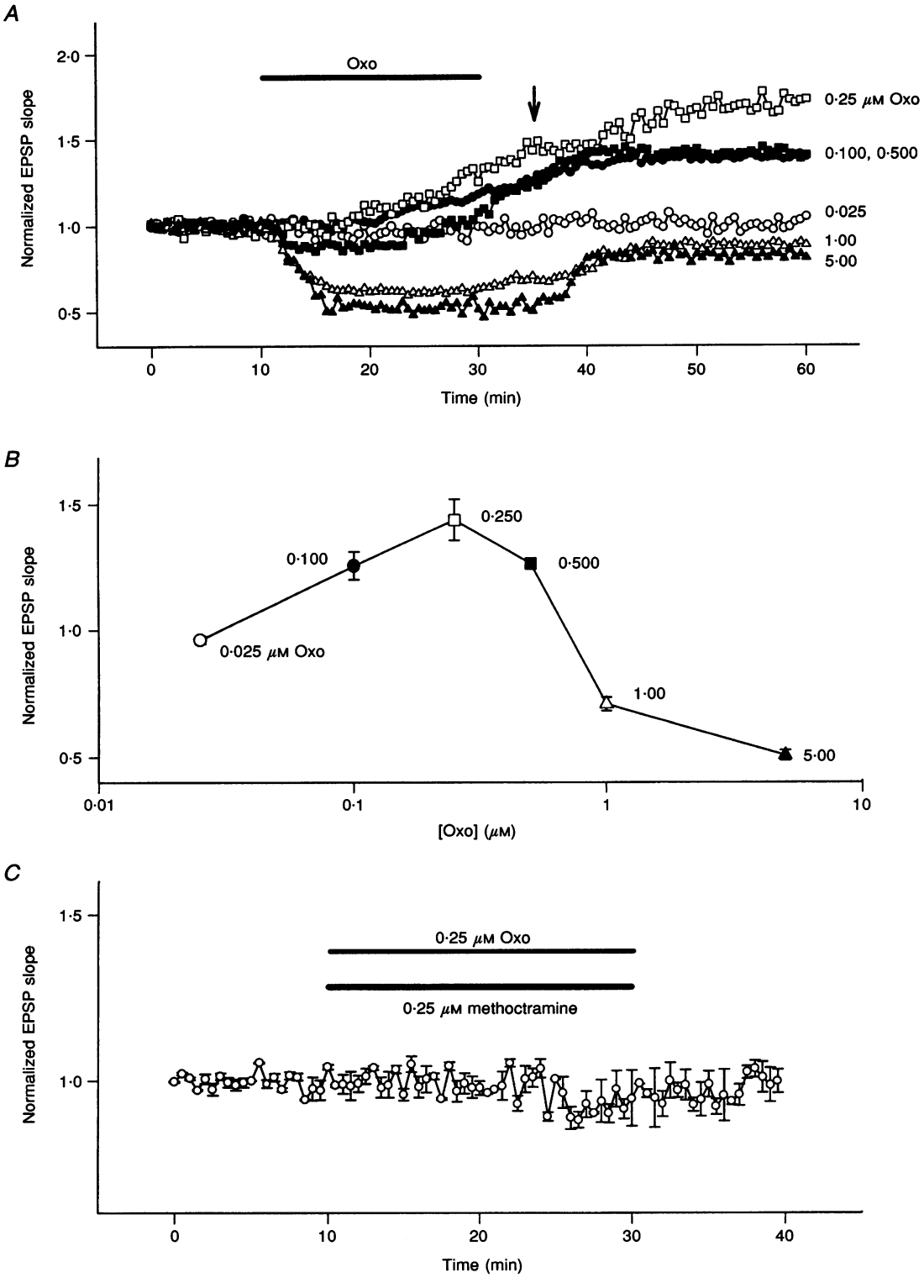

Figure 5. Biphasic effect of the M2 muscarinic agonist oxotremorine (Oxo) on EPSP slope

A and *B*, concentrations of Oxo: ○, 0·025 μM, $n = 4$; ●, 0·100 μM, $n = 6$; □, 0·250 μM, $n = 6$; ■, 0·500 μM, $n = 3$; △, 1·00 μM, $n = 3$; ▲, 5·00 μM, $n = 2$ (n is the number of slices corresponding to each curve in *A*). *A*, high concentrations (1·0 and 5·0 μM) of Oxo depressed the EPSP slope while low concentrations (0·1–0·5 μM) potentiated it. An even lower concentration (0·025 μM) of Oxo had no effect on the EPSP. The greatest amount of potentiation was induced by 0·25 μM Oxo (1·699 ± 0·066, $n = 6$). The arrow pertains to part *B*. For clarity, error bars are not shown here. *B*, dose–response relation for effects of Oxo on the EPSP is shown for the one time point indicated by the arrow in *A*. *C*, methoctramine (0·25 μM), a specific M2 muscarinic antagonist, was added to slices together with 0·25 μM Oxo ($n = 4$). The antagonist was sufficient to completely block the potentiation normally caused by this concentration of Oxo.

Role of inhibition in generation of LTP_m

One question remained, however: why, once the depression of the EPSP was blocked by 4-DAMP, did LTP_m not surface? One possibility is that CCh acts at an additional locus to prevent LTP_m, e.g. the inhibitory interneuron. It has been shown that high concentrations of CCh (10–50 μM) applied to the hippocampal slice increase spontaneous IPSPs recorded in area CA1, indicating a CCh-induced increase in GABAergic neuronal activity (Pitler & Alger, 1992). We therefore blocked inhibition using the $GABA_A$ receptor antagonist picrotoxin and monitored the effects of high CCh concentrations on the EPSP slope. To prevent hyperexcitability in slices used in the picrotoxin experiments, a cut was made transecting the CA1–CA3

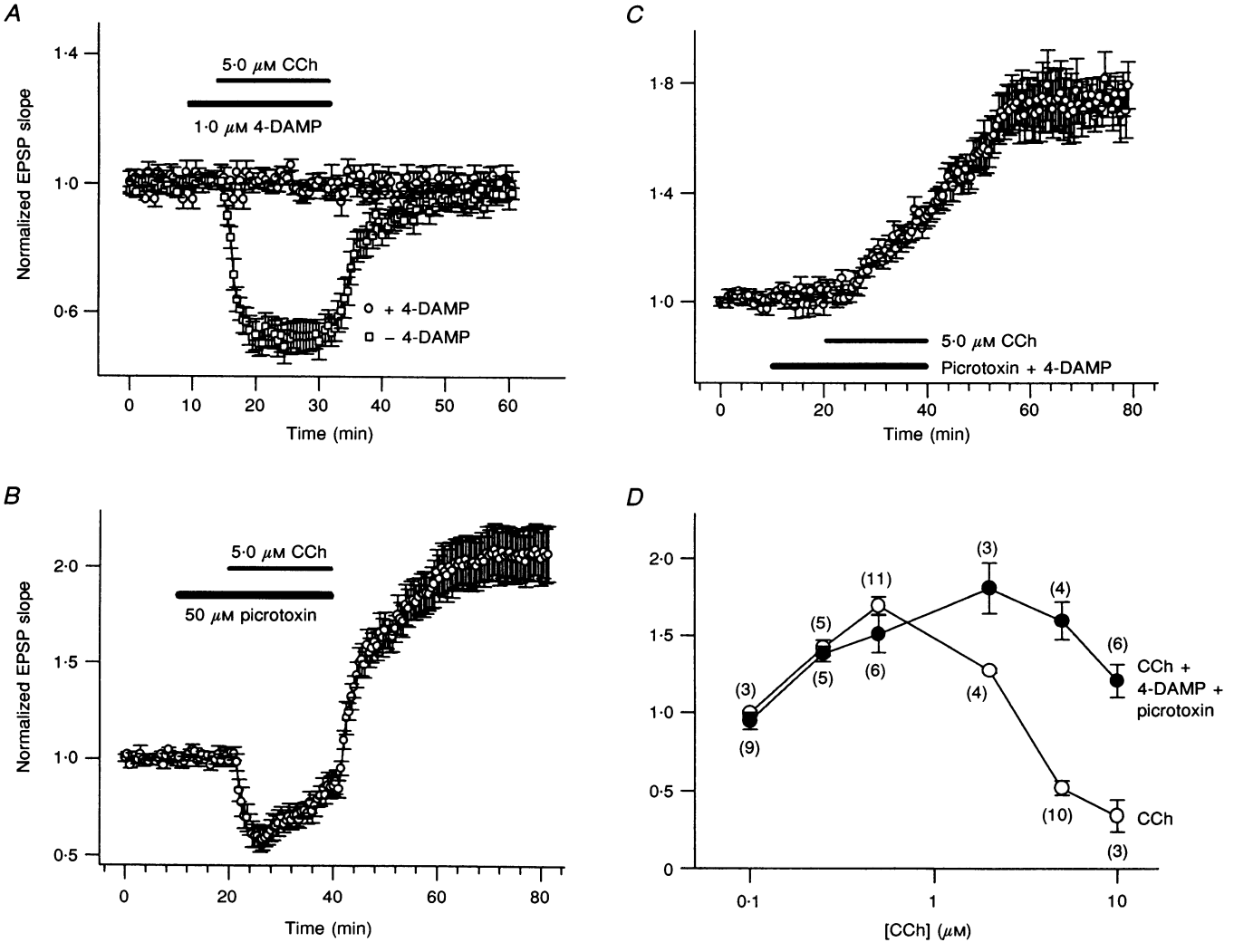

Figure 6. Depression of the EPSP induced by high concentrations of CCh is mediated by the M3 muscarinic receptor

A, CCh (5·0 μM) added to slices caused a depression of the EPSP slope to levels 0·523 ± 0·047 of the baseline value (□; $n = 10$). Addition of the M3 muscarinic antagonist 4-DAMP (1·0 μM) to the superfusion medium starting 5 min before 5·0 μM CCh blocked the depression of the EPSP (○; $n = 8$). Note that 4-DAMP does not affect baseline response. *B*, the $GABA_A$ receptor antagonist picrotoxin (50 μM) was added 10 min before 5·0 μM CCh ($n = 7$). Under these conditions, picrotoxin had no effect on baseline response, nor did the drug affect the depression caused by CCh (responses went down to 0·587 ± 0·058 of the baseline EPSP slope). However, following washout of CCh, LTP_m became evident and reached potentiation of 2·059 ± 0·134 of baseline. *C*, picrotoxin (50 μM) together with 1·0 μM 4-DAMP added 10 min before and together with 5·0 μM CCh blocked the cholinergic depression of the EPSP slope and revealed an underlying LTP_m (potentiation of 1·709 ± 0·090; $n = 6$). Picrotoxin with 4-DAMP had no effect on baseline response as seen during the first 10 min of application. *D*, dose–response curve constructed from experiments in which increasing concentrations of CCh were superfused over a slice for 20 min in the presence (●) or absence (○) of 50 μM picrotoxin and 1·0 μM 4-DAMP. The numbers in parentheses refer to the number of slices used for each concentration.

connections. In addition, the ACSF used for superfusion contained 4·0 mM $CaCl_2$ and 4·0 mM $MgSO_4$. Application of 50 μM picrotoxin to a submerged slice for 10 min under these conditions had no effect on the baseline EPSP. In addition, picrotoxin did not affect LTP_m produced by 0·5 μM CCh (see Fig. 8). Picrotoxin had no immediate effect on the suppressive action of 5·0 μM CCh (Fig. 6*B*); however, it did reveal a latent LTP_m, which was expressed upon washout of CCh. In the presence of picrotoxin and 4-DAMP (which by itself also did not affect LTP_m) 5·0 μM CCh now produced LTP_m (1·709 ± 0·090; $n = 6$; Fig. 6*C*) without the typical depression of the EPSP slope. Note that picrotoxin together with 4-DAMP applied for 10 min before CCh application had no observable effect on the baseline response (Fig. 6*C*). A dose–response curve was constructed from experiments in which increasing CCh concentrations were added to slices in the presence of picrotoxin and 4-DAMP. For this curve, a time point immediately following the start of drug washout was selected. Comparison of this curve with the dose–response relationship of control slices under the same conditions revealed a change in the descending phase only, i.e. at high concentrations of CCh,

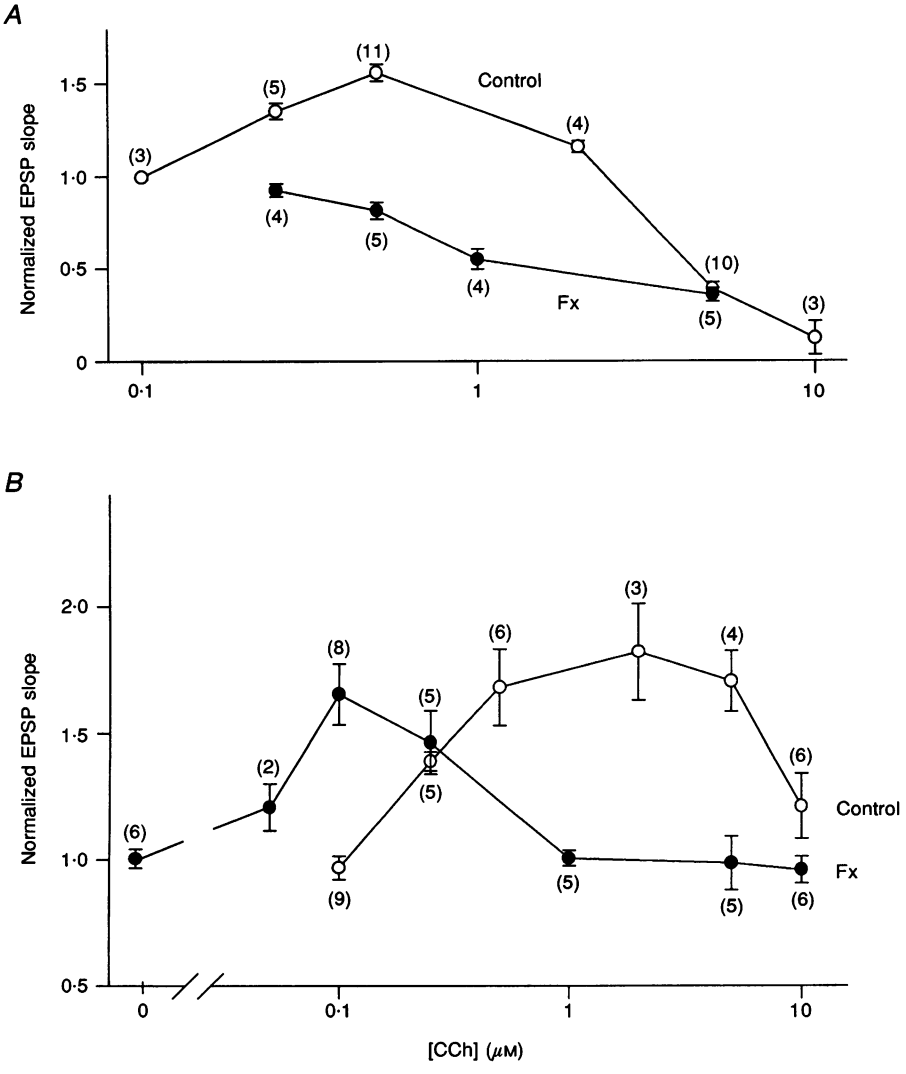

Figure 7. Muscarinic supersensitivity is seen in fimbria–fornix-transected rats

A, dose–response curves comparing slices from normal animals (○) with those from animals lesioned in the fimbria–fornix (Fx; ●), for a time point 15 min after the start of CCh application to the slice. The control group showed peak potentiation at 0·5 μM CCh (1·555 ± 0·045) and peak depression at 10 μM CCh (0·122 ± 0·089). The EPSP was monotonically depressed to an increasing degree with increasing concentrations of CCh for the fimbria–fornix-lesioned group. *B*, dose–response curves for the effects of CCh on slices from fimbria–fornix-transected animals (●) and normal animals (○), in the presence of 50 μM picrotoxin and 1·0 μM 4-DAMP. For both *A* and *B* the numbers in parentheses refer to the number of slices tested for each concentration.

LTP_m is induced in the presence of picrotoxin and 4-DAMP (Fig. 6*D*).

Spatial dependence of LTP_m: stratum radiatum *versus* stratum oriens

Binding of [^{3}H]oxotremorine-M in the hippocampus indicates that the M2 muscarinic receptor has a higher density in the stratum oriens than in the stratum radiatum of CA1 (Vilaro, Wiederhold, Palacios & Mengod, 1992). One can therefore expect that if LTP_m were mediated by an M2 muscarinic receptor, CCh will evoke a greater LTP_m in stratum oriens than in stratum radiatum. To test this, EPSPs were recorded in response to stimulation of stratum radiatum and stratum oriens at an alternating rate of 0·033 Hz. In these experiments increasing concentrations of CCh were added in the presence of 50 μM picrotoxin and 1·0 μM 4-DAMP. At the threshold concentration tested (0·1 μM) there was a significant potentiating effect of CCh in stratum oriens (1·150 $\pm$ 0·044) but not in stratum radiatum (0·970 $\pm$ 0·044; $n = 9$; paired t test, $P < 0·01$). There was no significant difference between the responses to stimulation of st. oriens and st. radiatum at higher CCh concentrations tested (data not shown).

Cholinergic denervation: effects on LTP_m and the cholinergic depression of EPSPs

The hippocampus receives a major cholinergic input from the medial septum via the fimbria–fornix (Lewis & Shute, 1967). Lesioning of this pathway causes degeneration of the cholinergic fibres (Mellgren & Srebo, 1973) and results in cholinergic receptor supersensitivity (Benson, Blitzer, Haroutunian & Landau, 1989). There are M2 receptors on cholinergic terminals which control release of acetylcholine (Doods *et al.* 1993*a*); therefore, if these receptors are the ones activated to produce LTP_m, one can expect disappearance of LTP_m in slices taken from fimbria–fornix-transected rats.

Slices from fimbria–fornix-transected animals expressed a marked increase in M3 receptor-mediated response (Fig. 7*A*). That is, at each CCh concentration tested, starting from 0·25 μM, a reversible suppression of the EPSP slope was seen. This occurred together with an apparent abolition of the M2-mediated potentiation. One exception was seen using 0·5 μM CCh, a concentration which caused an initial depression followed by a gradually developing, low level potentiation (1·317 $\pm$ 0·113; $n = 5$). This is in contrast to a

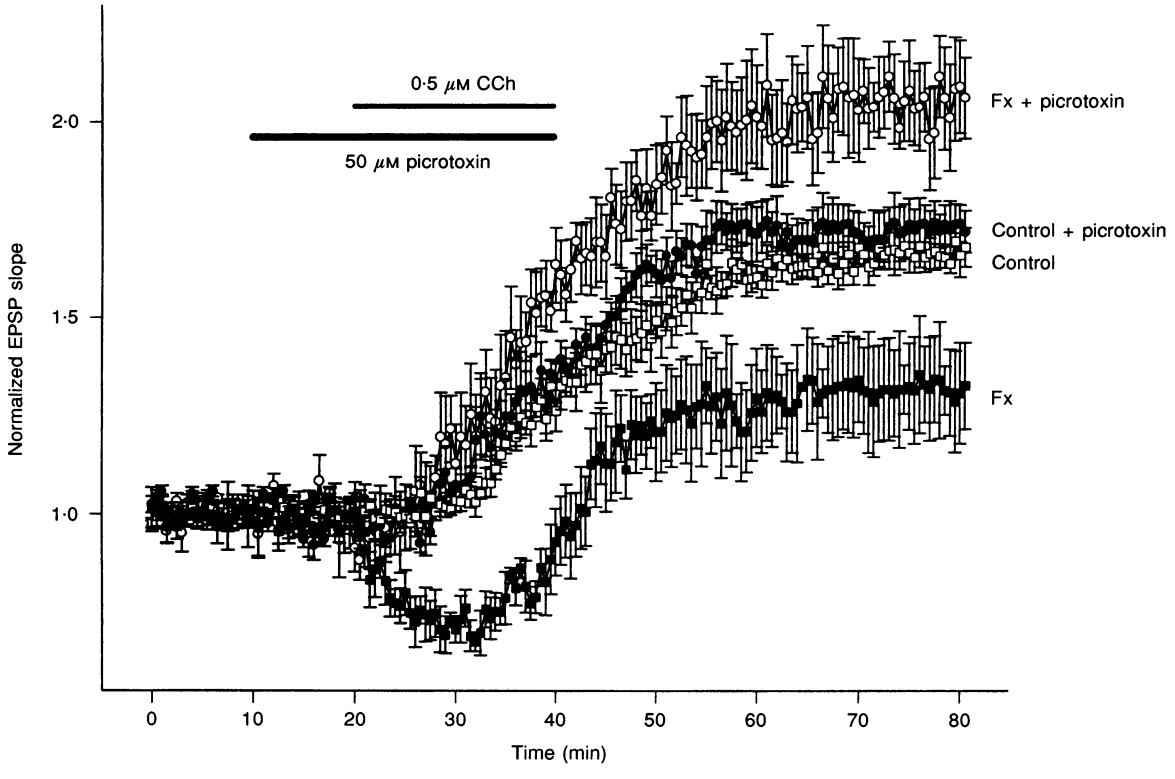

Figure 8. Effects of 0·5 μM CCh on EPSP slope depend on presence of picrotoxin in slices taken from fimbria–fornix-transected rats, but not in normal slices

In slices from fimbria–fornix-transected rats (Fx), 0·5 μM CCh produced a transient depression of the EPSP slope followed by low level potentiation (1·317 $\pm$ 0·113; $n = 5$; ■). When 50 μM picrotoxin was present in the bath, the transient depression was blocked and LTP_m was enhanced to a level above that attained in normal slices (2·045 $\pm$ 0·113; $n = 4$; ○). In slices from control animals, 0·5 μM CCh together with 50 μM picrotoxin produced typical LTP_m (1·714 $\pm$ 0·059, $n = 4$; ●), which was not significantly different from control, normal slices (1·665 $\pm$ 0·041, $n = 6$; □).

potentiation of 1·698 ± 0·057 ($n = 6$) induced by the same concentration of CCh in slices from untreated animals (see Fig. 1*B*). The dose–response curves of control slices and slices from fimbria–fornix-transected animals, constructed for a time point 15 min into CCh application, illustrate the enhanced M3-mediated response of the latter group.

Picrotoxin added to slices from fimbria–fornix-transected animals blocked the transient depression caused by 0·5 μM CCh and enhanced the potentiation induced by this concentration of CCh (2·045 ± 0·113; $n = 4$; Fig. 8, top trace). This is a larger potentiation than that induced by 0·5 μM CCh in normal control slices with (1·714 ± 0·059, $n = 4$) or without (1·665 ± 0·041; $n = 6$) picrotoxin (Fig. 8, two middle traces) and than the potentiation induced by 0·5 μM CCh in slices from fimbria–fornix-transected animals in the absence of picrotoxin (1·317 ± 0·113; $n = 5$; Fig. 8, bottom trace).

LTP_m was studied in isolation in control slices and slices from fimbria–fornix-transected animals, all treated with 50 μM picrotoxin and 1·0 μM 4-DAMP. Comparison of the dose–response curves plotted for the two groups showed an apparent shift of the lesion group to the left (Fig. 7*B*). The fimbria–fornix-lesioned group reached peak potentiation at 0·1 μM CCh (1·652 ± 0·120; $n = 8$), whereas the control group peaked at 2·0 μM CCh (1·819 ± 0·190; $n = 3$). In addition, the fimbria–fornix-lesioned group returned to baseline at 1·0 μM CCh ($n = 5$) indicating the failure of 4-DAMP to block the M3-mediated depression. In contrast, the control group only began to approach baseline levels at 10 μM CCh (1·214 ± 0·129 of baseline; $n = 6$). This shift of the fimbria–fornix curve relative to the control indicates an overall supersensitivity in all the muscarinic subtype receptors involved in LTP_m and the muscarinic depression.

DISCUSSION

We have previously reported that LTP_m can be induced in hippocampal slices by prolonged superfusion of low concentrations of CCh (Auerbach & Segal, 1994). This potentiation and tetanus-induced LTP are similar in several respects, including a shared requirement for a rise in intracellular calcium and activation of endogenous protein kinases. In addition, subthreshold cholinergic stimulation acts synergistically with subthreshold tetanic stimulation to cause robust LTP. LTP_m appears to be postsynaptic, as indicated by the present experiments in which there was no apparent change in presynaptic parameters (fibre volley and paired-pulse potentiation) during the development of LTP_m. In addition, the increase in cellular responsivity to AMPA with prolonged superfusion of the slice with 0·5 μM CCh is consistent with a postsynaptic locus of LTP_m. Recently, a hypothesis has been put forth postulating a postsynaptic mechanism for tetanic LTP which involves phosphorylation-induced potentiation of AMPA receptor responses (for review see Nicoll & Malenka, 1995).

In the brain, the association of a selective muscarinic action with a specific receptor subtype is difficult and sometimes controversial (Dutar & Nicoll, 1988; Sheridan & Sutor, 1990). The M2 receptor in the brain, specifically in the hippocampus and cortex, is at least partly located on presynaptic cholinergic nerve terminals where it is responsible for regulating ACh release (Doods *et al.* 1993*a*). This is supported by findings in which blockade of these putative presynaptic M2 receptors in behaving rats caused an increase in endogenous ACh release in the hippocampus and improved the performance of memory impaired rats in the water maze (Quirion *et al.* 1995). However, there is also a substantial proportion of M2 receptors in the hippocampus not associated with cholinergic fibres, as indicated by their survival in fimbria–fornix-transected hippocampi which lack cholinergic innervation (Levey, Edmunds, Koliatsos, Wiley & Heilman, 1995). Moreover, M2 receptor immunoreactivity has been visualized on postsynaptic spines as well as in presynaptic boutons, both associated with putative non-cholinergic synapses (Mrzljak, Levey & Goldman-Rakic, 1993) indicating an important role for the M2 receptor in the regulation of excitatory neurotransmission. Our present data indicate that the M2 receptor involved in LTP_m is most likely to be located postsynaptically on pyramidal neurons.

For each of the M2 and M3 antagonists, a range of concentrations was tested. The concentration stated above as the effective concentration used was the lowest concentration which completely blocked LTP_m or muscarinic depression. Comparison of the antagonist concentrations we used with those cited in the literature reveal the following: in one competitive binding assay 0·25 μM methoctramine was sufficient to displace >80% of [^{3}H]-labelled *N*-methylscopolamine ([^{3}H]NMS) bound to M2 receptors, whereas it displaced ~20% of [^{3}H]NMS bound to M1 and M4 receptors and <5% bound to M3 receptors (Doods, Willim, Boddeke & Entzeroth, 1993*b*). In another work, 0·25 μM methoctramine was sufficient to displace ~90% of [^{3}H]NMS bound to M2 receptors, ~50% bound to M1 and M4 receptors and <5% bound to M3 receptors (Waelbroeck *et al.* 1990). The relative affinity profile of AFDX-116 for the M1–M4 receptors is very similar to that of methoctramine, only slightly lower; e.g. for the M2 receptor, methoctramine has a K_D of 13 nm and AFDX-116 has a K_D of 50 nm (Waelbroeck *et al.* 1990). The M3 antagonist 4-DAMP has the following affinity profile: M3 ⩾ M1 > M2 (Thomas, Hsu, Griffin, Hunter, Luong & Ehlert, 1992), with pK_i values given for the various muscarinic receptors as follows: M1, 9·05 ± 0·01; M2, 8·08 ± 0·04; M3, 9·33 ± 0·12; and M4, 8·55 ± 0·04 (Doods *et al.* 1993*b*). Although at a concentration of 1·0 μM, 4-DAMP probably affects the M1 receptor as well as the M3 receptor, we ruled out M1 effects because of the failure of pirenzipine to block either LTP_m or muscarinic depression and the failure of AF-267 and McN-343 to induce these effects.

The interactions between the different muscarinic receptor subtypes is complex. The M3 receptor has been shown to be presynaptic on cholinergic terminals as well as postsynaptic (Marchi & Raiteri, 1989) indicating a role for the M3 receptor in regulation of transmitter release. Postysynaptic localization in the hippocampus places the M3 receptor on pyramidal cell bodies and dendrites as well as on interneurons (Levey *et al.* 1995). Tentative conclusions have been reached from various comparative binding studies which indicate the following rank order of potency of CCh at the muscarinic receptor subtypes: M2 ≈ M4 > M3 > M1 (Bujo *et al.* 1988; McKinney, Miller, Gibson, Nickelson & Aksoy, 1991). According to this scheme, a reduction of ACh release caused by activation of presynaptic M2 or M3 receptors will restrict cholinergic action to high affinity postsynaptic M2 receptors. This will enhance reactivity of the hippocampus to afferent stimulation – resulting in LTP_m. Enhanced ACh release, caused by blockade of M2 receptors located presynaptically on cholinergic fibres, for instance, will result in activation of the lower affinity M3 receptors which will act to reduce reactivity of the hippocampus to afferent stimulation, i.e. cholinergic depression. The M1 muscarinic receptor, having the lowest affinity, will be activated only when much higher amounts of ACh are released, and result in a blockade of potassium channels on pyramidal cells and interneurons, enhancing the firing of both groups of neurons and their reactivity to an apparent reduced input into the hippocampus.

In fimbria–fornix-transected animals, this cascade of potencies appears to have been broken by the elimination of the cholinergic terminals and the subsequent receptor supersensitivity. In slices from fimbria–fornix-transected brains, a lower concentration of CCh was sufficient to suppress the EPSP slope, and LTP_m was masked again. Only removal of the suppression with the M3 antagonist 4-DAMP together with picrotoxin uncovered a robust, larger than normal LTP_m, indicating a possible supersensitivity of the M2 receptor.

In slices from normal animals, although the depression of the EPSP slope caused by 5·0 μM CCh was blocked by application of 1·0 μM 4-DAMP, LTP_m was unmasked only upon addition of picrotoxin to the bath. A possible explanation for this is that high CCh concentrations potentiate evoked responses of both pyramidal cells and interneurons to the same degree such that, once the M3-mediated depression is blocked, no significant variation from baseline values is observed. Upon addition of picrotoxin, the enhanced interneuronal activity is blocked and LTP_m is seen. GABAergic interneurons are involved in the effects of CCh on pyramidal cells. Pitler & Alger (1992) have shown that high concentrations of CCh potentiate GABAergic neuronal activity. Following this line of reasoning, the putative muscarinic receptor on interneurons should have lower affinity for CCh than the M2 receptor on pyramidal neurons which mediates LTP_m, as low concentrations of CCh alone induce LTP_m. Partial support for this hypothesis is provided by immunohistochemical studies showing the existence of both M2 and M3 receptors on GABAergic interneurons (Mrzljak *et al.* 1993; Levey *et al.* 1995).

The regional distribution of the muscarinic receptor subtypes in the hippocampus complicates the interactions between them even further: the low affinity M1 receptors are dense in stratum radiatum of CA1 whereas the M2 receptors are more abundant in stratum oriens/alveus. There are fewer cholinergic fibres terminating in stratum radiatum than in stratum oriens (Lewis & Schute, 1967; Milner, Loy & Amaral, 1983). Thus, the likelihood of activating the M1 receptors in normal hippocampal operation is small indeed. On the other hand, M2 receptors should be activated more readily. Indeed, LTP_m is slightly easier to evoke in stratum oriens than in radiatum. The functional significance of the M1 receptors in stratum radiatum remains unclear.

The differential effect of CCh on cellular reactivity to NMDA and AMPA is consistent with both our previous observations on the fast potentiating action of CCh on reactivity to NMDA (Markram & Segal, 1990) and the lack of involvement of the NMDA receptor in LTP_m. The differential time course of onset, as well as the duration of CCh effects, may indicate that two different second messenger mechanisms are involved in the action of CCh on NMDA and AMPA. Both appear to involve a rise in intracellular calcium, but the transient nature of the enhancement of NMDA reactivity (probably mediated by activation of the IP_3 cascade), as well as its long lasting suppression, indicate that it is controlled in a more complex manner than the potentiation of the AMPA response. The latter is probably mediated by activation of a protein kinase (Auerbach & Segal, 1994), the nature of which remains to be determined.

Finally, the long-term effects of CCh in the hippocampus, namely enhancement of the reactivity of hippocampal neurons to afferent stimulation, in a process similar to that of the well characterized tetanic LTP, bridges the gap between the known involvement of ACh in cognitive processes associated with the hippocampus and the action of ACh on hippocampal neural circuitry.

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