Temporal specificity of muscarinic synaptic modulation of the Ca^{2+}-dependent K^+ current (I_{sAHP}) in rat hippocampal neurones

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1. We examined synaptic modulation of the Ca^{2+}-dependent K^+ current (I_{sAHP}), which underlies the slow after-hyperpolarization (sAHP) in hippocampal CA1 neurones of rat brain slices. I_{sAHP} was evoked in whole-cell voltage-clamp mode by depolarizing pulses, and synaptic afferents to CA1 neurones were stimulated electrically with a paired-pulse protocol.

2. Afferent stimulation delivered 200–1500 ms prior to the depolarizing pulse produced a profound reduction of I_{sAHP} by 58%, but not other Ca^{2+}-dependent outward currents that preceded I_{sAHP}. Perfusion of slices with atropine significantly attenuated the synaptic reduction of I_{sAHP}, indicating an event mediated largely by muscarinic receptor activation. When delivered < 400 ms after the depolarizing pulse, similar synaptic stimuli produced no substantial reduction in I_{sAHP}, even in neurones where the duration of I_{sAHP} was prolonged to 8–10 s either by lowering the recording temperature or by intracellular application of a calcium chelator.

3. To examine the effect of cholinergic stimulation on the depolarization-activated Ca^{2+} influx, high-threshold voltage-activated Ca^{2+} currents were recorded in the conventional or perforated whole-cell mode. Perfusion of slices with 5–10 μM carbachol for 5–10 min caused no substantial decrease in these Ca^{2+} currents, suggesting that the synaptic reduction of I_{sAHP} is unlikely to be due to a blockade of depolarization-induced Ca^{2+} influx which triggers the generation of I_{sAHP}.

4. The present data demonstrate that afferent stimulation reduces I_{sAHP} only if it occurs prior to the depolarization-induced Ca^{2+} influx. We propose that modulation of inactive sAHP channels by muscarinic stimulation may decrease their sensitivity to the influx of Ca^{2+}, whereas sAHP channels activated by Ca^{2+} may compete with the receptor-coupled modulation thus rendering the sAHP channels unresponsive to cholinergic afferent stimulation.

In hippocampal neurones and neurones of other brain areas, an elevation of $[Ca^{2+}]_i$, resulting from repetitive discharges or depolarization, triggers a slow after-hyperpolarization (sAHP) and the underlying outward current (I_{sAHP}: Brown & Griffith, 1983; Lancaster & Adams, 1986; see review by Storm, 1990). sAHP/I_{sAHP} is an important participant in regulating the rate of neuronal discharges, and it is well known that stimulations of a variety of neurotransmitter receptors can decrease sAHP/I_{sAHP}, which decreases spike firing adaptation (Cole & Nicoll, 1983; Madison & Nicoll, 1986; Baskys, Bernstein, Barolet & Carlen, 1990; Charpak, Gähwiler, Do & Knöpfel, 1990; Müller, Petrozzino, Griffith, Danho & Connor, 1992; Pedarzani & Storm, 1993; Abdul-Ghani, Valiante, Carlen & Pennefather, 1996). Interestingly, the reduction of sAHP/I_{sAHP} following receptor stimulation is not associated with a blockade of the corresponding Ca^{2+} signal when examined by simultaneous intracellular recordings and fluorescent Ca^{2+} imaging (Knöpfel, Vranesic, Gähwiler & Brown, 1990; Müller & Connor, 1991). These studies suggest that the mechanism underlying receptor-mediated reduction of sAHP/I_{sAHP} occurs downstream from the depolarization-induced Ca^{2+} signal. In excised-patch recordings and noise analysis of whole-cell currents, single-channel behaviour underlying I_{sAHP} exhibits fast kinetics in response to applied Ca^{2+} (Lancaster, Nicoll & Perkel, 1991; Sah & Isaacson, 1995). A relatively rapid, sAHP-like response was also induced following photolytic release of caged Ca^{2+} into the cytoplasm (Lancaster & Zucker, 1984). Collectively, these data suggest that sAHP/I_{sAHP} is activated directly by elevated $[Ca^{2+}]_i$ rather than coupled through other intermediate events. Therefore, it is reasonable to propose that sAHP/I_{sAHP} reduction might

result from a loss of sensitivity of sAHP channels to Ca^{2+} due to receptor-mediated modulation. Because sAHP channels are highly sensitive to an elevation of $[Ca^{2+}]_i$ in the range of 50–200 nM (Knöpfel *et al.* 1990; Müller & Connor, 1991) and the whole-cell I_{sAHP} currents last for a few seconds following depolarizing stimulation, it is of interest to know whether these channels can be modulated once activated by raised intracellular Ca^{2+}.

To address this issue, we examined the effect of cholinergic synaptic stimulation on I_{sAHP} in CA1 hippocampal neurones of rat brain slices. Previous studies have demonstrated that hippocampal neurones receive dense cholinergic innervation, which largely originates from the medial septum (Lewis & Shute, 1967), and that muscarinic cholinergic stimulation of hippocampal neurones is readily produced in brain slices following electrical stimulation of cholinergic afferent fibres (Cole & Nicol, 1983, 1984; Madison, Lancaster & Nicoll, 1987; Zhang, Weiner & Carlen, 1992; see also review by Krnjević, 1993). Because the sAHP/I_{sAHP} in hippocampal neurones is highly sensitive to muscarinic stimulation (Bernardo & Prince, 1982; Madison *et al.* 1987; Knöpfel *et al.* 1990; Dodd, Dingledine & Kelly, 1991; Müller *et al.* 1992; Sah & Isaacson, 1995; Zhang, Pennefather, Velumian, Tymianski, Charlton & Carlen, 1995), the reduction of I_{sAHP} by synaptically released acetylcholine was considered to be a convenient protocol through which the temporal relation between I_{sAHP} generation and receptor-mediated processes could be determined. We demonstrate here that stimulation of cholinergic afferent fibres produces a selective reduction of I_{sAHP} only if the stimulation precedes the depolarization that triggers I_{sAHP}.

METHODS

Experimental procedures for slice preparation, afferent stimulation and the whole-cell recordings of the sAHP/I_{sAHP} have been described previously (Zhang *et al.* 1994, 1995). Briefly, male Wistar rats (25–35 days old) were anaesthetized with halothane and decapitated. Transverse brain slices (400 μm) were obtained using a Vibratome and maintained at room temperature (22–23 °C) in artificial cerebrospinal fluid (ACSF) oxygenated with 95% O_2–5% CO_2. All experiments and animal handling were performed according to Canadian Animal Care Protocols and approved by the Animal Care Committee of the University of Toronto and Toronto Hospital. The composition of the ACSF was (mM): NaCl, 125; KCl, 2·5; NaH_2PO_4, 1·25; $MgSO_4$, 2; $CaCl_2$, 2; $NaHCO_3$, 25; and glucose, 10. To block synaptic currents mediated by activation of glutamate ionotropic receptors and $GABA_A$ receptors, we added the following drugs to the ACSF: 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM; Research Biochemicals International (RBI)), D-2-amino-5-phosphonopentanoic acid (AP5, 50 μM; RBI), and bicuculline methiodide (10 μM; RBI). In some experiments, kynurenic acid (1·5–2 mM; RBI) was used in place of CNQX and AP5. To study voltage-activated Ca^{2+} currents, 20 mM tetraethylammonium chloride (TEA; Sigma), 2 mM 4-aminopyridine (4-AP; Sigma), 5 mM CsCl and 0·5 μM tetrodotoxin (TTX; Sigma) were added to the ACSF. The concentration of NaCl was decreased equimolarly.

Recordings were made from submerged slices at a temperature of 33–34 °C, except when indicated. Humidified, warmed 95% O_2–5% CO_2 was also applied over the solution submerging the slice to maintain a warm, oxygen-enriched local environment. Electrical stimulation of afferents was accomplished by using a bipolar tungsten electrode and the electrode tips were separated by about 0·5 mm. The stimulating electrode was placed in stratum radiatum close to the recording site. Stimulation consisted of paired pulses (duration of 0·2–0·5 ms) with an interpulse interval of 5–10 ms. Constant current output was delivered through a stimulating isolation unit (Grass S88). Afferent stimulation was applied 500–1500 ms prior to or following the depolarizing command used to activate I_{sAHP}. For testing the effect of synaptic stimulation on I_{sAHP}, eserine (1 μM), a cholinesterase inhibitor, was applied throughout the recording period.

We performed whole-cell recordings of I_{sAHP} in CA1 pyramidal neurones, using a basic patch pipette solution that contained: 150 mM potassium methylsulphate, 2 mM Hepes, 2 mM K-ATP and 50 μM K-EGTA. When required, 1 mM BAPTA and 0·1 mM $CaCl_2$ were added to the internal solution. The recording patch pipettes were pulled from thin-walled borosilicate glass tubes (TW150F-4; World Precision Instruments) using a two-stage Narishige puller (Tokyo, Japan). The recording pipettes had a resistance of 3–4 MΩ when filled with the internal solution. The series resistance after forming the whole-cell recordings was usually less than 15 MΩ. In our experimental conditions, the evoked I_{sAHP} was relatively stable after more than 30 min of whole-cell dialysis (Zhang *et al.* 1994).

Signals were recorded using an Axoclamp-2A amplifier (Axon Instruments) in discontinuous single-electrode voltage-clamp (SEVC) mode. The sampling rate of SEVC was 3–6 kHz, and the single pole low-pass filter was set at 1 kHz. Data were digitized via a 12-bit A/D interface (TL-1; Axon Instruments), and then stored and analysed using pCLAMP software (version 5.5; Axon Instruments). I_{sAHP} currents were evoked by constant positive voltage pulses (40–50 mV, 200–300 ms) from the holding potentials of −50 to −60 mV. The holding potentials were −45 to −50 mV in BAPTA-dialysed neurones because of the depolarized membrane potentials (Zhang *et al.* 1995). Using this protocol, the currents evoked during the commands were often not fully clamped owing to gain and space-clamp limitations. However, the voltage control during the I_{sAHP} signal that develops after the end of the depolarization can be well maintained (see Constanti & Sim, 1987; Lancaster & Adams, 1986; Sah & McLachlan, 1991). The amplitude of I_{sAHP} was measured at 500 ms after the end of the depolarizing pulses at 33 °C. When recorded at room temperature (22–23 °C), the measurements were taken at 3000 ms after the pulses because of the slow decay of I_{sAHP} at this temperature. Changes in I_{sAHP} after stimulation were expressed as a percentage of the control amplitude.

To study Ca^{2+} currents in the conventional whole-cell mode, potassium methylsulphate was replaced with caesium methanesulphonate (Zhang *et al.* 1994) in the basic patch pipette solution. For recording Ca^{2+} currents in perforated whole-cell mode (Horn & Marty, 1988; Bley & Tsien, 1990; Köhr & Mody, 1991; Zhang, Valiante & Carlen, 1993), we used a patch pipette solution which contained: 30 mM CsCl, 120 mM caesium methanesulphonate, 5 mM Hepes, 100 μM Cs-EGTA and 50–100 μg amphotericin B (Sigma) (Rae, Cooper, Kates & Watsky, 1991). In some experiments, 10 mM 2(triethylamino)-*N*-(2,6-dimethylphenyl)-acetamide (QX-314; Alomone Laboratories, Jerusalem, Israel) was added to the patch pipette solution to suppress the voltage-gated Na^+ current from the intracellular side. Amphotericin B was initially dissolved in DMSO (2 mg (100 μl)$^{-1}$) as a stock solution and

stored at −80 °C in a deep freezer until use. Then the amphotericin B stock was appropriately diluted in the patch pipette solution and this solution was kept in a 4 °C refrigerator for not more than 2 h. Because of the difficulty in seal formation in the presence of amphotericin B in the patch pipette solution (Rae *et al.* 1991), the patch pipette tip was filled with a solution containing: 150 mM potassium gluconate, 2 mM Hepes and 100 μM EGTA, and then backfilled with the amphotericin B-containing solution. The final content of the amphotericin B in the patch pipette solution was about 50–100 μg ml^{-1}. With this arrangement, 3–10 GΩ seal formation was readily achieved, and the whole-cell recordings were performed 20–40 min after the formation of the tight seal. The whole-cell series resistance was 40–70 MΩ. All internal solutions had a pH of 7·25 adjusted with KOH or CsOH and an osmolality of 280 ± 10 mosmol kg^{-1}.

Calcium currents were evoked every 30 s by constant voltage pulses of +40 to +55 mV (300–500 ms) from a holding potential of −50 to −60 mV. To examine the voltage dependence of the Ca^{2+} currents, neurones were clamped at −80 mV and incremental depolarizing pulses of +10 to +100 mV were given in 10 mV steps. In some experiments, intracellular application of QX-314 rather than external application of TTX was used to suppress the voltage-gated Na^+ current, and neurones were voltage clamped at −30 mV to inactivate the residual Na^+ currents. Because of space-clamp limitations, the voltage during the fast component of the current onset was not fully controlled. Thus, the measurements of Ca^{2+} currents were taken at 50–100 ms after the beginning of the depolarizing pulses.

Data included in this study were collected from CA1 neurones that had initial resting potentials more negative than −55 mV and overshooting action potentials. Means ± S.E.M. are presented throughout the text. Internal and external solutions were made using deionized sterile water (resistivity 18·2 MΩ cm^{-1}) from a Milli-Q UV-Plus system (Millipore). Chemicals for internal solutions were purchased from Fluka (New York) except for potassium methylsulphate, which was obtained from ICN (New York).

RESULTS

Selective reduction of I_{sAHP} following stimulation of cholinergic afferent fibres

I_{sAHP} was evoked in the presence of 20 μM CNQX and 50 μM AP5 or 1·5–2 mM kynurenic acid, which were used to block polysynaptic transmission mediated by ionotropic glutamate receptors. Recorded under these conditions, the outward tail current following the depolarizing voltage pulse showed two components, i.e. an early component that decayed in 100–200 ms, and a sustained portion that lasted for a few seconds (Fig. 1*A*). The early component, referred to as I_{mAHP}, is largely generated by a Ca^{2+}-dependent potassium current ($I_{K(Ca)}$) and a slowly inactivating $I_{K(M)}$ (Storm, 1990; Alger, Sim & Brown, 1994). We refer to the slow component of the outward tail current as I_{sAHP}. To stimulate afferents in the CA1 region and to minimize the stimulation-induced large inward current (Cole & Nicoll 1983, 1984; Madison *et al.* 1987; Zhang *et al.* 1992; see also Krnjević, 1993), a paired-pulse protocol (see Methods) rather than a high frequency tetanus was used to stimulate afferents in CA1 stratum radiatum in the presence of 1 μM eserine, a cholinesterase inhibitor. The paired stimuli, when applied from 400 to 1500 ms prior to the depolarizing pulse, produced a profound reduction of the following I_{sAHP}, leaving I_{mAHP} relatively intact (Fig. 1*A*). No consistent change in holding current was observed following the synaptic stimulation. In a total of sixty-four measurements collected from twenty-three neurones, I_{sAHP} reduction following the stimulation ranged from 23 to 91%, with a mean reduction of 57·6 ± 3·5%, as measured at 500 ms after the end of the depolarizing pulse. I_{sAHP} reduction following the stimulation recovered in about 30–60 s (Fig. 1*A* and *C*), and it was reproducible in the same neurones by similar stimulation in about 80% of neurones examined (Fig. 1*B* and *C*). Blockade of $GABA_A$-mediated IPSCs with 10 μM bicuculline was without effect on I_{sAHP} reduction (Fig. 1*B*), suggesting an effect independent of synaptically evoked Cl^- conductances.

To explore the pharmacology of the synaptic reduction of I_{sAHP}, we examined the effect of afferent stimulation in the presence of the muscarinic receptor antagonist, atropine. Perfusion of slices with 1 μM atropine (in the presence of 1 μM eserine) either blocked or attenuated the synaptic reduction of I_{sAHP} (Fig. 2). The overall decrease of I_{sAHP} amplitude in the presence of atropine was only 26·0 ± 5·0% (44 measurements in 18 neurones), which was significantly smaller than that obtained in the absence of atropine (57·6 ± 3·5%, 64 measurements from 23 neurones; $P < 0·05$, Student's non-paired *t* test). Similar effects were also achieved by perfusion of slices with 10 μM atropine. In a set of five neurones examined, I_{sAHP} reduction following afferent stimulation was 67 ± 13% before and 22 ± 8% after perfusion of 10 μM atropine. A selective depression of I_{sAHP} amplitude (by 78·3 ± 5·5%, 8 neurones) was also observed following perfusion of slices with 2·5–5 μM carbachol for 1–2 min. These results suggest that the reduction of I_{sAHP} produced by afferent stimulation is largely, but not exclusively, mediated through activation of muscarinic receptors.

I_{sAHP} was not sensitive to synaptic stimulation applied after the depolarizing pulse

The reduction of I_{sAHP} produced by prior afferent stimulation recovered rapidly and was highly reproducible in an individual neurone. We next examined whether synaptic activation delivered after the depolarizing pulses could reduce I_{sAHP}. We predicted that the post-depolarization stimuli would not alter the following I_{sAHP} substantially if only the inactive sAHP channels were sensitive to this type of modulation. In five neurones, afferent stimulation applied before the depolarizing pulse produced a decrease in the following I_{sAHP} of 61·3 ± 3·7%, while similar stimuli, when applied at 100–400 ms after the end of the depolarizing pulse, caused only a small decrease in I_{sAHP} of 9·8 ± 3·8% in the same neurones (Fig. 3).

Because I_{sAHP} reduction is mediated by receptor-coupled second messenger cascades (Müller *et al.* 1992; Pedarzani & Storm, 1993), which may require a certain time to develop

(Levitan, 1994), one might argue that the minor change in I_{sAHP} following post-depolarization stimulation may simply be due to an insufficient latency between the stimuli and the generation of I_{sAHP}. To test this possibility, we recorded I_{sAHP} at room temperature (22–23 °C) in an attempt to slow the energy-dependent Ca^{2+} extrusion, hence prolonging I_{sAHP} decay, and examined the effects of the afferent stimulation on the prolonged I_{sAHP}. Since we have previously shown that muscarinic stimulation at room temperature causes profound enhancement of a voltage-gated potassium current in CA1 hippocampal neurones of rat brain slices (Zhang *et al.* 1992), we expected that I_{sAHP} would be similarly modulated at room temperature following stimulation of cholinergic afferent fibres. In five neurones examined at room temperature, I_{sAHP} lasted for more than 10 s, with a half-decay time of 3·7 ± 0·7 s. Afferent stimulation applied at 200–300 ms prior to the depolarizing pulse reduced the prolonged I_{sAHP} currents by 47 ± 10% ($n = 5$), when measured 3000 ms after the end of depolarizing pulses. Similar stimulation caused no significant decrease (9·4 ± 5%) in I_{sAHP} when applied at 200–400 ms after the end of depolarizing pulses (Fig. 4). The onset and recovery of I_{sAHP} reduction by pre-depolarization stimuli was not noticeably different between neurones recorded at 22–23 °C and at 33–34 °C. The mean reduction of I_{sAHP} at 22–23 °C appeared to be smaller than that observed at 33–34 °C (47 ± 10 *vs.* 61·3 ± 3·7%, see above), probably due to a relatively low turnover rate of G protein-coupled intracellular cascades at room temperature. However, the temperature-related difference did not reach statistical significance at the $P < 0·05$ level.

We also recorded prolonged I_{sAHP} at 33–34 °C by dialysing CA1 neurones with a patch pipette solution containing 1 mM BAPTA and 0·1 mM Ca^{2+}. The prolonged I_{sAHP} produced by BAPTA is probably due to redistribution of

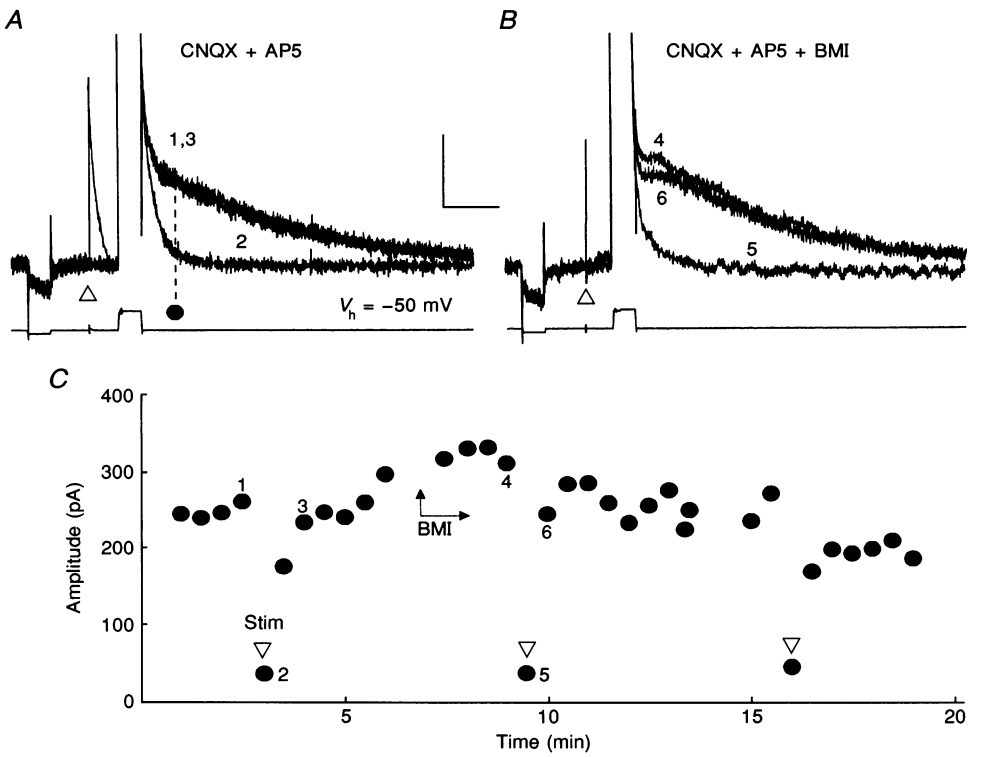

Figure 1. Synaptic reduction of I_{sAHP} in a hippocampal CA1 neurone

I_{sAHP} currents were evoked every 30 s by constant +40 mV, 400 ms depolarizing pulses from a holding potential of −50 mV. The amplitude of I_{sAHP} was measured at 500 ms after the end of the depolarizing pulse (dashed line in *A*) and was plotted *vs.* time in *C*. The recordings began about 5 min after breaking through the membrane. Numbered data points represent I_{sAHP} evoked before (1, 4), immediately following (2, 5) and 30–60 s after (3, 6) the synaptic stimulation, and the corresponding records are superimposed in *A* and *B*. Note that I_{sAHP}, but not I_{mAHP}, is greatly decreased following the stimuli and it took over 30 s before full recovery of I_{sAHP}. The electrical stimulation of stratum radiatum consisted of two identical current pulses (0·1 ms duration, 500 μA, interpulse interval of 10 ms) and the stimuli were delivered 600 ms before the depolarizing pulse (open triangles). CNQX (20 μM), AP5 (50 μM) and eserine (1 μM) were applied throughout the recording period. In *B*, the synaptic reduction of I_{sAHP} remained after adding 10 μM bicuculline methiodide (BMI) to the perfusate. The starting time of BMI application is indicated in *C* by filled arrows. Calibration bar: 200 pA, 200 mV and 1 s.

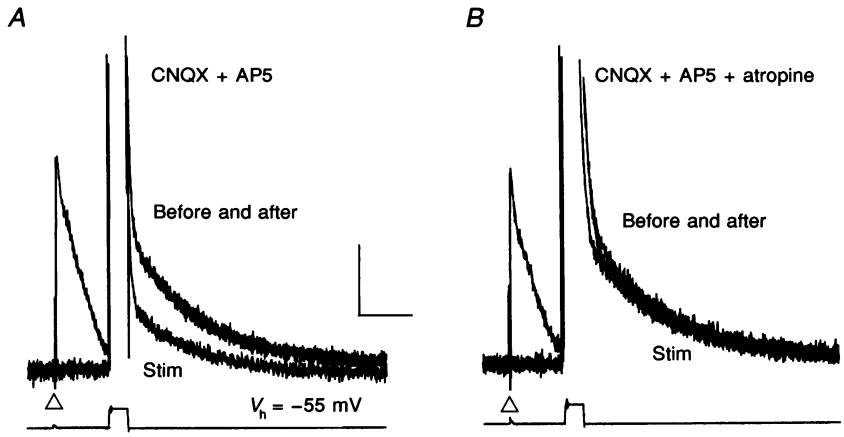

Figure 2. Muscarinic synaptic reduction of I_{sAHP} in a hippocampal CA1 neurone

A and *B*, I_{sAHP} currents were evoked every 30 s by constant +50 mV depolarizing pulses from a holding potential of −55 mV. CNQX (20 μM), AP5 (50 μM) and eserine (1 μM) were bath applied throughout the recording period. Superimposed currents were taken before, immediately following and 30 s after the synaptic stimulation. Two sets of records were taken about 8 min apart. Note in *B* that I_{sAHP} reduction following the stimulation was abolished 4 min after adding 1 μM atropine to the perfusate. Calibration: 200 pA, 200 mV and 1 s.

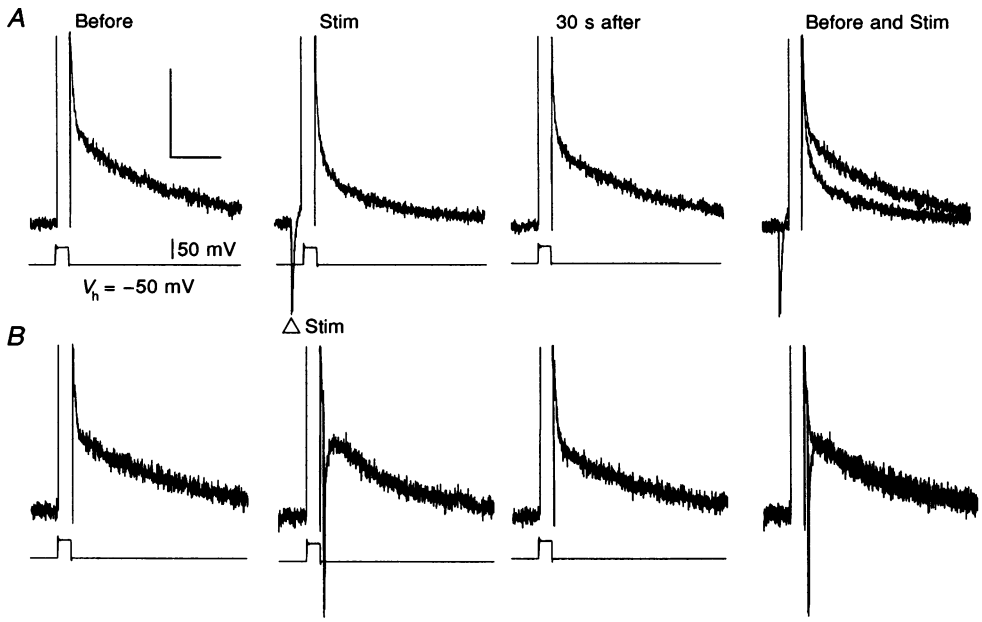

Figure 3. I_{sAHP} is reduced by synaptic stimuli applied before, but not after, the depolarizing pulse

All records were collected from a CA1 neurone in the presence of 2 mM kynurenic acid, 10 μM bicuculline and 1 μM eserine. I_{sAHP} currents were evoked every 30 s by constant depolarizing pulses of +50 mV from a holding potential of −50 mV. Traces from left to right are records which were taken before, immediately following and 30 s after stimulation. The records collected before and immediately following stimulation are superimposed at the far right. The two sets of records were taken ~5 min apart. Note in *A*, synaptic stimuli applied about 200 ms before the depolarizing pulse depressed the following I_{sAHP}. In *B*, after I_{sAHP} recovered from the first stimulation, similar stimuli applied 100 ms after the depolarizing pulse caused only a negligible decrease in the following I_{sAHP}. Calibration: 300 pA, 200 mV and 1 s.

intracellular Ca^{2+} owing to the presence of an excessive calcium buffer with high affinity and fast kinetics, and the prolonged I_{sAHP} is also sensitive to muscarinic stimulation (Schwindt, Spain & Crill, 1992; Zhang *et al.* 1995). In BAPTA-loaded neurones, I_{mAHP} was diminished and I_{sAHP} exhibited a long decay time that was nearly doubled when compared with neurones dialysed with the basic solution (Zhang *et al.* 1995; see Fig. 5 also). When given at about 200–500 ms before the depolarizing pulse, synaptic stimuli produced a large reduction of the prolonged I_{sAHP} by 66·5 ± 5·1% when measured 3000 ms after the end of the depolarizing pulse (13 measurements in 5 neurones; Fig. 5*A*). Similar stimulation, when applied during (Fig. 5*B*) or at 100–400 ms after the depolarizing pulse (Fig. 5*C*), caused only a small decrease (8·2 ± 4·3%) of the prolonged I_{sAHP}, respectively (Fig. 5*D*). The reduced I_{sAHP} took 2–3 min to recover following the stimulation, such that subsequently evoked I_{sAHP} currents at 30–60 s later were still reduced (Fig. 5). It is not clear at present why, in the presence of intracellular BAPTA, I_{sAHP} recovers slowly following stimulation. However, these observations do imply that synaptic stimuli are effective in reducing I_{sAHP} with the constraint that the stimuli precede the generation of I_{sAHP} (Fig. 5*D*).

Voltage-activated Ca^{2+} currents were not decreased by carbachol

To test the effect of cholinergic stimulation on the depolarization-induced Ca^{2+} influx, we first recorded voltage-activated Ca^{2+} currents in the conventional whole-cell recording mode, and slices were perfused with the ACSF containing TTX and K^+ channel blockers (see Methods). When dialysed with Cs^+-containing internal solution, CA1 neurones showed large inward currents (1–2 nA) evoked by depolarizing pulses of 50–60 mV from a holding potential of −50 mV. These Ca^{2+} currents were relatively stable when evoked every 30 s and no significant decrease in the amplitude of the Ca^{2+} currents (change by −12·8 ± 7·5%, 7 cells) was observed at the end of bath application of carbachol (5 μM for 5 min). However, a decrease of Ca^{2+} currents by −28·8 ± 6·7% was observed during carbachol washout over 5–8 min.

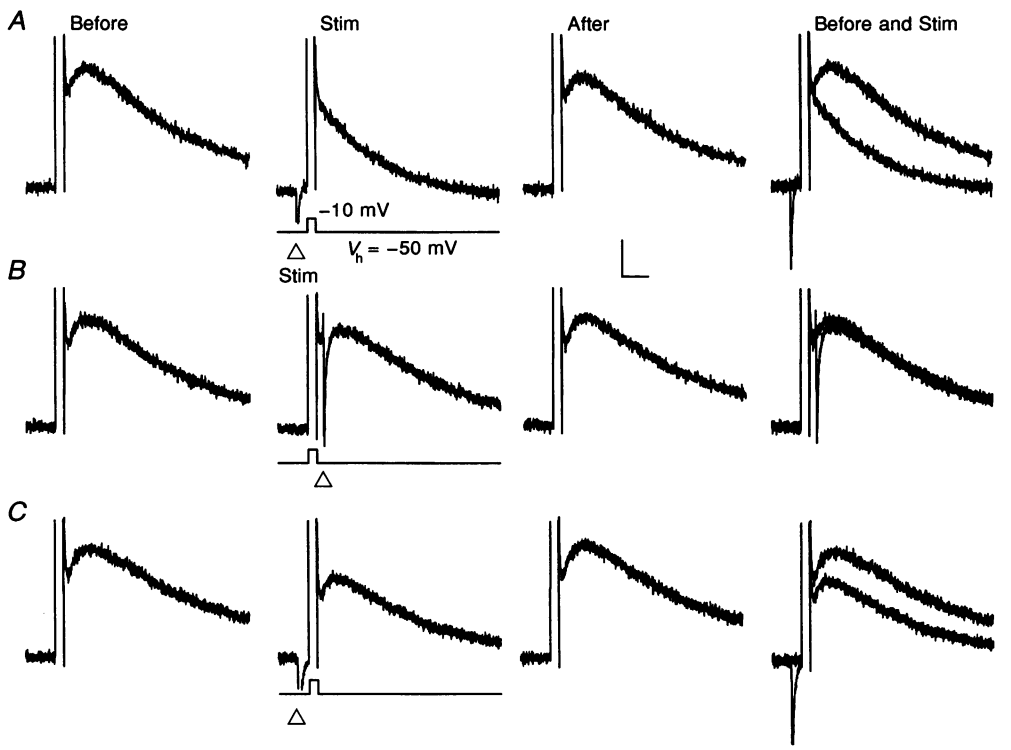

Figure 4. Effects of synaptic stimulation on I_{sAHP} recorded at room temperature (22–23 °C)

All records were taken from a CA1 neurone in the presence of 20 μM CNQX, 10 μM bicuculline and 1 μM eserine. I_{sAHP} currents were evoked every 30 s by depolarizing pulses of 40 mV from a holding potential of −50 mV. Records from left to right were taken before, during and after synaptic stimulation (arrows), and the extent of I_{sAHP} depression was illustrated at the far right by superimposing the records taken before and during stimulation. *A*, synaptic stimuli induced a profound reduction of I_{sAHP} when applied before the depolarizing pulse. *B*, 4 min after the first synaptic stimulation, similar stimuli did not alter I_{sAHP} when applied after the depolarizing pulse. Note that I_{sAHP} lasted for > 6 s following the post-depolarization stimulation. *C*, 4 min after the second set of recordings, similar stimuli when applied before the depolarizing pulse again decreased I_{sAHP}. Calibration: 100 pA and 1 s.

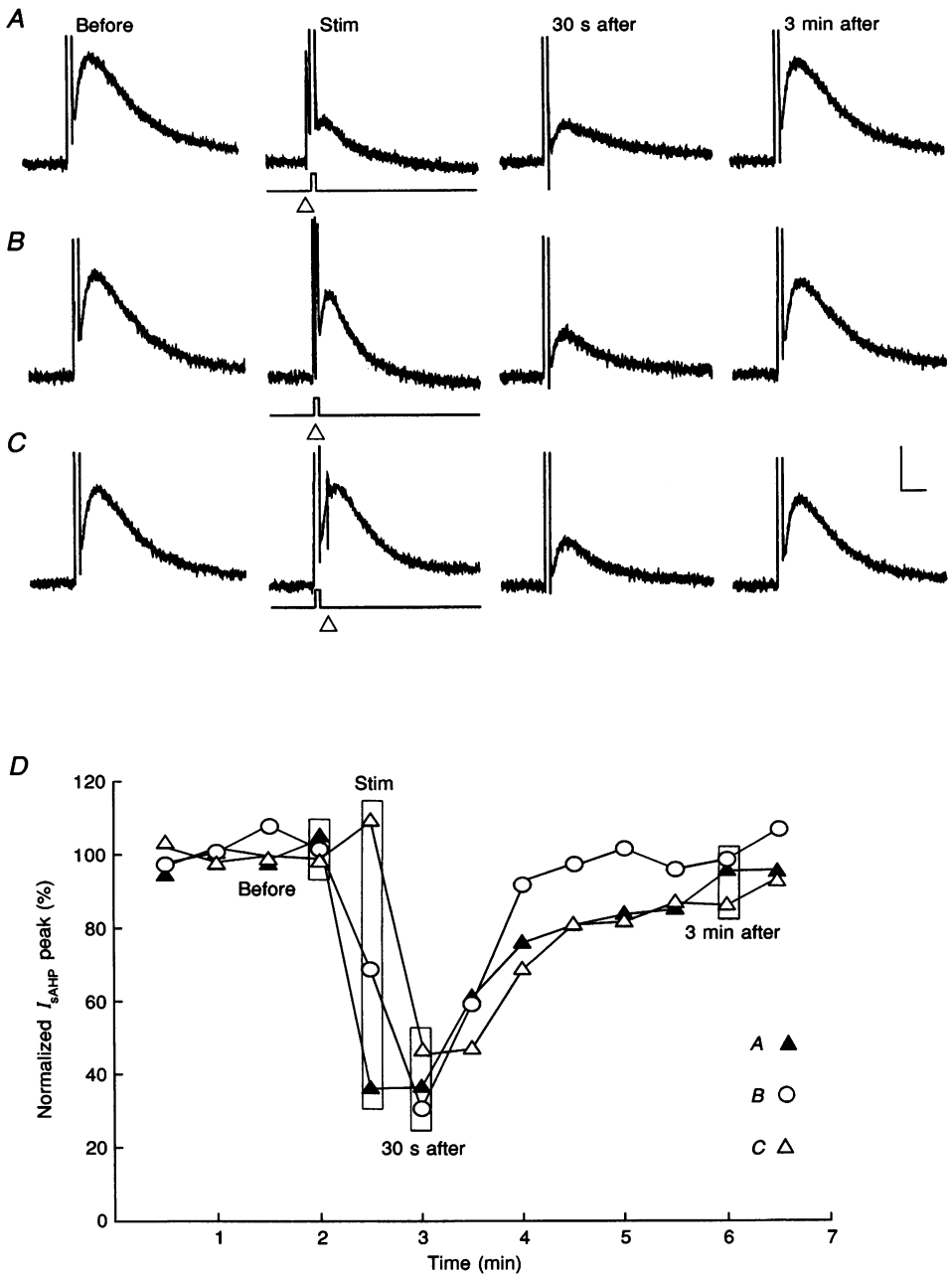

Figure 5. Effects of synaptic stimuli on BAPTA-potentiated I_{sAHP}

All records were taken from a CA1 neurone dialysed with a patch pipette solution containing 1 mM BAPTA and 0·1 mM Ca^{2+}. I_{sAHP} currents were evoked every 30 s from holding potentials of −48 mV. Illustrations from left to right are: I_{sAHP} evoked 30 s before synaptic stimulation (open triangles); I_{sAHP} recorded immediately following the stimulation; I_{sAHP} recorded at 30 s after, and I_{sAHP} recorded 3 min after. Each set of recordings was taken ~5 min apart, and the synaptic stimuli were applied at 300 ms before (*A*), during (*B*) and 400 ms after (*C*) the depolarizing pulses. *D*, the percentage of the peak I_{sAHP} following the synaptic stimulation was plotted against time. For each set of recordings, the mean value of four measurements before the synaptic stimulation was taken as 100%. Note that synaptic stimuli applied before the depolarizing pulse induced a profound reduction of the following I_{sAHP} and I_{sAHP} evoked subsequently at 30 s after (*A* and ▲ in *D*). However, similar stimuli applied during the depolarizing pulse caused a partial reduction of the following I_{sAHP} and a great reduction of the subsequently evoked I_{sAHP} (*B* and ○ in *D*). Similar stimuli applied shortly after the depolarizing pulse did not substantially decrease the following I_{sAHP}, but did decrease I_{sAHP} evoked 30 and 60 s later (*C* and △ in *D*). Calibration in *A*–*C*: 200 pA and 1 s.

To investigate whether the delayed decrease of the Ca^{2+} currents resulted from current run-down following prolonged whole-cell dialysis, we performed perforated whole-cell recordings of Ca^{2+} currents in CA1 neurones (Horn & Marty, 1988; Bley & Tsien, 1990; Köhr & Mody 1992; Zhang, Valiante & Carlen, 1993; see Methods also). Stable Ca^{2+} currents were repeatedly evoked by constant depolarizing stimulation from a holding potential of −50 mV. In five neurones examined, bath application of 10 μM carbachol for 5–6 min caused no substantial decrease in the Ca^{2+} currents. The changes in Ca^{2+} current amplitude were −8·5 ± 5·2 and −10·4 ± 5·9%, as measured at the end of the carbachol application and 5 min after washing out carbachol, respectively (Fig. 6*A* and *B*). We also examined the voltage

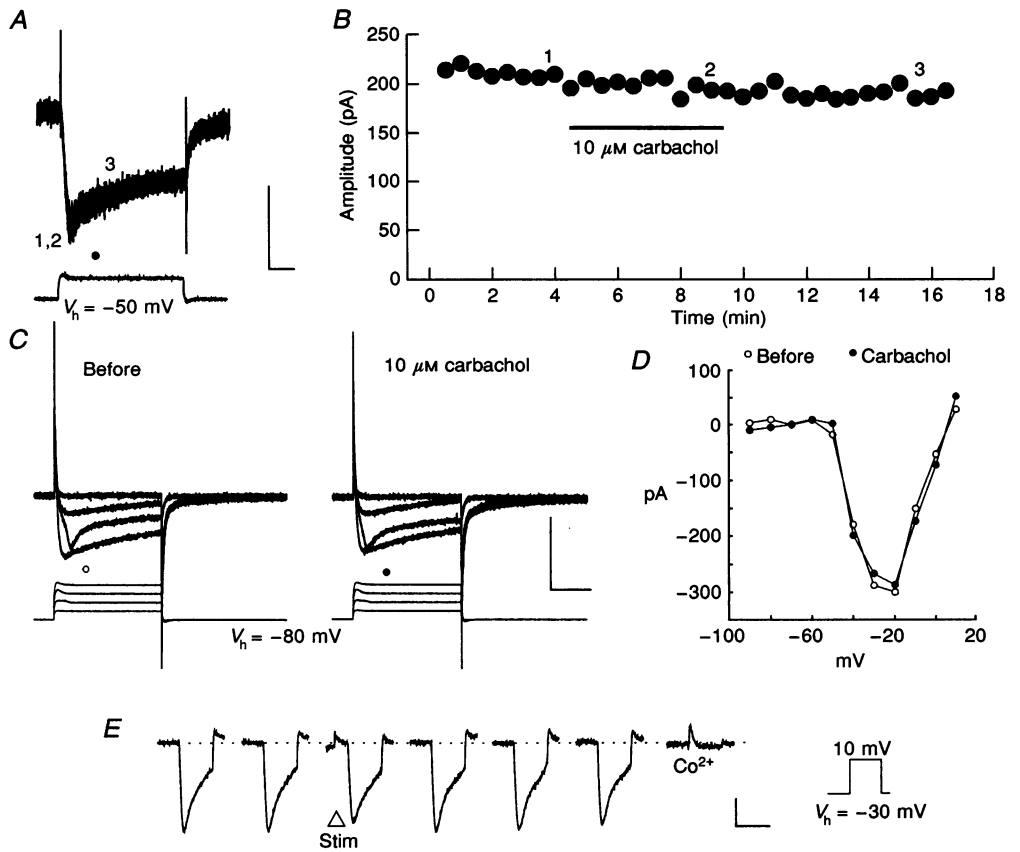

Figure 6. Carbachol application caused no substantial decrease in voltage-activated Ca^{2+} currents recorded in the perforated whole-cell mode

A and *B*, Ca^{2+}currents were evoked from a CA1 neurone every 30 s by constant +50 mV voltage pulses from a holding potential of −50 mV. The current amplitude *vs.* time was plotted in *B* and the measurements were made at 80 ms after the onset of the depolarizing pulses (● in *A*). The numbered data points in the plot represent the currents recorded before and after application of 10 μM carbachol, and the corresponding records were leak subtracted and superimposed in *A*. Note that the Ca^{2+} currents are relatively stable before and after carbachol application. *C* and *D*, Ca^{2+} currents were recorded from another CA1 neurone before and 10 min after application of 10 μM carbachol. The neurone was held at −80 mV and inward currents were evoked by voltage pulses more positive than −50 mV. The amplitude of the Ca^{2+} currents was measured at 80 ms after the onset of the depolarizing pulses (●), and the *I–V* relation of the Ca^{2+} currents was plotted in *D*. Leak-subtracted currents were superimposed in *C* and they were evoked by depolarizing voltage pulses to −60, −40, −20 and 0 mV, respectively. Note the similar *I–V* relation for Ca^{2+} currents recorded before and after carbachol application. *E*, leak-subtracted inward currents were collected from a CA1 neurone in the perforated whole-cell recording mode; the patch pipette solution contained Cs^+ and 10 mM QX-314 (see Methods). The neurone was clamped at −30 mV in an attempt to inactivate residual Na^+ currents, and inward currents were induced by constant depolarizing pulses to +10 mV. The records were collected at about 70 min after forming the cell-attached recording, and 2 mM kynurenic acid, 10 μM bicuculline and 1 μM eserine were bath applied throughout the recording period. Note that only the early peak of the inward current was decreased following the afferent stimulation, and that these currents were blocked by adding 2 mM $CoCl_2$ in the ACSF. Calibrations: 200 pA, 200 mV and 50 ms in *A*; 350 pA, 200 mV and 100 ms in *C*; and 250 pA and 100 ms in *E*.

dependence of the Ca^{2+} currents by varying the voltages of depolarizing pulses from a holding potential of −80 mV. As shown in Fig. 6*C* and *D*, inward currents were elicited by step voltages more positive than −40 mV and were sustained over the duration of the depolarizing stimulation, suggesting that they belong to the high-threshold, voltage-activated Ca^{2+} current families. In three of three neurones examined, application of 10 μM carbachol for 10–14 min caused no obvious change in the *I–V* relation of these currents (Fig. 6*C* and *D*).

We also recorded depolarization-induced inward currents in perforated whole-cell mode by using the patch pipette solution containing Cs^+ and 10 mM QX-314 (see Methods). QX-314 molecules may pass through the perforated membrane and suppress the Na^+ currents from the intracellular site (Hille, 1992). If so, we may be able to record the Ca^{2+} currents and assess the effects of synaptic stimulation on these currents. It was difficult to fully block the Na^+ current by intracellular dialysis of QX-314 in the perforated mode, probably because of limited passage of QX-314 molecules through the perforated membrane. Successful recordings were achieved in only three neurones in which Co^{2+}-sensitive inward currents were recorded at 50–65 min after making the cell-attached recording. In eight measurements made from these three neurones, afferent stimuli did not substantially decrease the depolarization-induced inward currents (Fig. 6*C*), and the amplitudes of these currents were 615 ± 115 pA before and 575 ± 107 pA immediately following the stimulation.

We recognize that in the slice preparation the Ca^{2+} currents may not be accurately measured from the somatic point recording because of space-clamp limitations. Nevertheless, these experiments provide supporting evidence that Ca^{2+} currents are not decreased following carbachol application at concentrations sufficient to fully block I_{sAHP} (Madison *et al.* 1987; see Results also). These results are consistent with previous observations that the sAHP/I_{sAHP} reduction by muscarinic stimulation is not associated with the blockade of the corresponding intracellular Ca^{2+} signals (Knöpfel *et al.* 1990; Müller & Connor, 1991).

DISCUSSION

The present data demonstrate that afferent stimulation produces a profound reduction of Ca^{2+}-dependent I_{sAHP} in hippocampal CA1 neurones, while I_{mAHP} is minimally affected. The synaptic reduction of I_{sAHP}: (1) exhibits fast onset and recovery; (2) is largely prevented by muscarinic receptor blockade; and (3) can be mimicked by external application of the cholinergic agonist carbachol, at low micromolar concentrations. These results suggest that in physiological conditions *in vivo*, cholinergic synapses and related intracellular messengers might be located in proximity to Ca^{2+}-dependent sAHP channels, and may have strong influence on neuronal discharges primarily through modulation of I_{sAHP}. Reduction of I_{sAHP} following synaptic stimulation can also serve as a useful experimental protocol to study the modulation of ion channels by native muscarinic cholinergic synapses.

It should be pointed out that the present experiments were carried out in brain slices obtained from young rats (25–35 days old), and that I_{sAHP} reduction was consistently induced by stimulation of stratum radiatum near the recording site (see Methods), but the extent of I_{sAHP} reduction varied (see Results). We have noted that in slices obtained from rats ⩾ 2 months old, I_{sAHP} of CA1 neurones was only minimally affected by stratum radiatum stimulation (data not shown). Using chopped brain slices from mature rats, Cole & Nicoll (1984) reported that a muscarinic EPSP is readily induced following electrical stimulation of stratum oriens, whereas stratum radiatum stimulation is relatively ineffective in inducing the slow EPSP. Previous studies using anatomical and immunohistochemical methods have shown that hippocampal neurones are heavily innervated by septal cholinergic fibres (Lewis & Shute, 1967) and immunoactivity for muscarinic receptors is densely distributed over the apical and basal dendritic field of the CA1 region (Levey, Edmunds, Koliatsos, Wiley & Heilman, 1995). A dendritic component of the sAHP/I_{sAHP} has also been demonstrated recently in CA1 neurones of rat brain slices (Andreasen & Lambert, 1995). Since perfusion of brain slices with low micromolar cholinergic agonists effectively depresses the I_{sAHP} of CA1 neurones in adult rat brain slices (Madison *et al.* 1987; Zhang *et al.* 1995), the varied effects of afferent stimulation on I_{sAHP} and other ionic events may be largely determined by the degree of preserved functional cholinergic innervation following slicing procedures. We are currently testing different slicing methods to improve the efficiency of afferent stimulation in mature rat brain slices.

In rat hippocampal CA1 neurones, I_{sAHP} is modulated by stimulation of multiple neurotransmitter receptors and related intracellular messengers, including 5-HT receptors (Pedarzani & Storm, 1993), β-adrenergic receptors (Madison & Nicoll, 1986), muscarinic receptors (Müller *et al.* 1992) and metabotropic glutamate receptors (Baskys *et al.* 1990; Charpak *et al.* 1990; Watkins & Collingridge 1994; Abdul-Ghani *et al.* 1996). Although I_{sAHP} reduction following afferent stimulation is largely attenuated by the muscarinic receptor blocker atropine (Cole & Nicoll, 1984; Madison *et al.* 1987; Zhang *et al.* 1995; see also review by Krnjević, 1993), the atropine-insensitive portion of I_{sAHP} reduction is probably mediated by other neurotransmitter systems, particularly metabotropic glutamate receptors. Future experiments are required to fully characterize the pharmacology of atropine-insensitive synaptic reduction of I_{sAHP}.

In hippocampal CA1 neurones, several ionic events are altered following stimulation of cholinergic afferents besides the reduction of I_{sAHP}, including: (1) blockade of the leak conductance which leads to the slow muscarinic EPSP; (2) reduction of the voltage-activated $I_{K(M)}$; (3) reduction of

the voltage- and Ca^{2+}-dependent $I_{K(Ca)}$; and (4) enhancement of the Ca^{2+}-independent, voltage-activated potassium current (Cole & Nicoll, 1983, 1984; Madison *et al.* 1987; Zhang *et al.* 1992). $I_{K(Ca)}$ contributes little to I_{sAHP} because of its fast kinetics (Brown & Griffith, 1983; Alger *et al.* 1994). Decrease of the muscarinic leak conductance is unlikely to have participated in I_{sAHP} reduction in the present experiments since the paired stimuli caused no consistent change in the holding current. It has been shown that I_{sAHP} is about ten times more sensitive to cholinergic stimulation than $I_{K(M)}$, such that IC_{50} values are 0·3 and 5 μM for carbachol blockade of I_{sAHP} and $I_{K(M)}$, respectively (Madison *et al.* 1987). Thus, it is conceivable that the moderate afferent stimulation used in the present experiments primarily induces modulation of sAHP channels, and the stimulation might have minimal, if any, effect on $I_{K(M)}$.

A key question is why afferent stimulation reduced I_{sAHP} only when delivered before, but not after, the depolarizing pulse which induces Ca^{2+} influx and the generation of I_{sAHP}. There are several possible explanations. Firstly, it is unlikely that pre-depolarization synaptic stimuli reduce I_{sAHP} by blocking the depolarization-induced Ca^{2+} currents because I_{mAHP}, which is at least partly generated by a Ca^{2+}-dependent I_C-type current, is minimally affected (Storm, 1990; Alger *et al.* 1994). Moreover, the afferent stimuli or carbachol applications at concentrations sufficient to reduce I_{sAHP} did not substantially block depolarization-induced Ca^{2+} currents (Fig. 6). These results are consistent with previous reports that the receptor-mediated reduction of I_{sAHP} is not associated with a decrease in corresponding intracelluar Ca^{2+} signals (Knöpfel *et al.* 1990; Müller & Connor, 1991). Thus, the inability to reduce I_{sAHP} by the post-depolarization stimuli cannot be explained by a lack of their actions on the Ca^{2+} influx. Secondly, the failure to decrease I_{sAHP} by post-depolarization stimulation cannot be attributed to an insufficient latency between the stimulation and the generation of I_{sAHP}. The latency necessary to cause reduction was relatively short, i.e. I_{sAHP} reduction was clearly seen at 1000–2000 ms following pre-depolarization afferent stimuli (Figs 4*A* and 5*A*), and some I_{sAHP} reduction was even noted following afferent stimuli delivered during the depolarizing pulse (Fig. 5*B*). I_{sAHP} showed no substantial decrease for its duration of up to 5 s following post-depolarization stimuli (Figs 4*B* and 5*C*), strongly suggesting that although the time following the post-depolarization afferent stimulation was sufficiently long to permit I_{sAHP} reduction, another process is required to explain the data. Thirdly, although the post-depolarization afferent stimuli failed to alter the following I_{sAHP}, their actions on the I_{sAHP} evoked over the next 30–60 s were well maintained (Fig. 5). This suggests that the afferent stimuli turned on the second messenger cascades at least for that period of time and that they became effective in reducing I_{sAHP} once the $[Ca^{2+}]_i$ returned to basal levels and sAHP channels were in an inactive state. From the present results we suggest that I_{sAHP} is altered by neurotransmitter receptor-mediated events only before its activation by an increase in intracellular Ca^{2+}. We propose that modulation of the inactive sAHP channels by kinase-dependent processes (Baskys *et al.* 1990; Müller *et al.* 1992; Pedarzani & Storm, 1993; Abdul-Ghani *et al.* 1996) may somehow decrease their sensitivity to the influx of Ca^{2+}, thereby leading to a low activity state (Sah & Isaacson, 1995) when these channels are exposed to Ca^{2+} (Levitan, 1994). The modulation may take place on the Ca^{2+} binding sites of sAHP channels or Ca^{2+} binding steps during channel activation, and occupation of these sites by Ca^{2+} may prevent any further modulation, thereby making activated channels unresponsive to the consequences of synaptic stimulation. It remains to be determined in future studies whether this phenomenon can be generalized to other neurotransmitter systems.

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