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PROPERTIES OF TWO CALCIUM-ACTIVATED HYPERPOLARIZATIONS IN RAT HIPPOCAMPAL NEURONES

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SUMMARY

1. Intracellular recording from hippocampal CA1 pyramidal cells in the slice preparation was used to analyse the pharmacological sensitivity of action potential repolarization and the hyperpolarizations that follow the action potential. The Ca^{2+}-activated after-hyperpolarizations (a.h.p.s) could be divided into a fast a.h.p. with a time course of milliseconds, and a slow a.h.p. which lasted for a few seconds at a temperature of 30 °C.

2. The repolarization of the action potential is sensitive to the Ca^{2+} channel blocker Cd^{2+}. This effect is simultaneous with a block of the fast a.h.p. which follows immediately upon the repolarization of the action potential. The slow a.h.p. was also blocked by Cd^{2+}.

3. Low concentrations of the K^+ channel blocker, tetraethylammonium (TEA; 200–500 μM), block the fast a.h.p. and slow down action potential repolarization. The slow a.h.p. was not affected by low concentrations of TEA.

4. The action potential repolarization and the fast a.h.p. are also reversibly sensitive to charybdotoxin. This agent had no effect on the slow a.h.p.

5. When EGTA or BAPTA were added to the normal recording electrolyte ($KMeSO_4$), the generation of slow a.h.p.s was prevented. In addition, cells impaled with BAPTA-containing electrodes displayed broader action potentials and much reduced fast a.h.p.s compared to recordings made with electrodes containing $KMeSO_4$ alone or with EGTA.

6. The slow a.h.p. can be eliminated by noradrenaline, 8-bromocyclic AMP or carbachol. Under these conditions there are no effects on the fast a.h.p. or on action potential duration.

7. Block of the fast a.h.p. with TEA or CTX (charybdotoxin) is associated with an increased frequency of the first few action potentials during a depolarization. This is a quite distinct effect from the greatly increased number of action potentials which results from block of the slow a.h.p.

8. The results support a conclusion that the fast a.h.p. is generated by the TEA- and voltage-sensitive Ca^{2+}-activated K^+ current, I_C. This current is involved in spike repolarization and turns off upon the return to resting potential. Thus block of I_C has no effect on the slow a.h.p. which is caused by a separate membrane current.

INTRODUCTION

It is now axiomatic that repolarization of action potentials within many types of nerve fibres depends upon the activation of a voltage-dependent K^+ current (Hodgkin & Huxley, 1952; Frankenhaeuser, 1962). Evidence is now gathering that the repolarization of *somatic* action potentials is to some extent Ca^{2+} dependent; i.e. that all or part of the repolarizing K^+ current is both Ca^{2+} and voltage dependent (Adams, Constanti, Brown & Clark, 1982; MacDermott & Weight, 1982; Storm, 1985; Obaid & Salzberg, 1985; Rogawski, Dufy & Barker, 1985). A Ca^{2+}-activated K^+ current appears to underlie the slow a.h.p. in CA1 pyramidal neurones (Hotson & Prince, 1980; Gustafsson & Wigstrom, 1981; Madison & Nicoll, 1984; Lancaster & Wheal, 1984), but there are good indications that this current should be quite different from that which might be involved in action potential repolarization. Two distinct Ca^{2+}-activated K^+ currents have been described in rat muscle cells (Romey & Lazdunski, 1984), bull-frog sympathetic neurones (Pennefather, Lancaster, Adams & Nicoll, 1985) and *Aplysia* neurones (Deitmer & Eckert, 1985). One of the Ca^{2+}-activated K^+ currents in bull-frog neurones takes part in spike repolarization (I_C) and a second underlies the slow a.h.p. (Pennefather *et al.* 1985). This latter current ($I_{a.h.p.}$) is analogous to the current responsible for the slow a.h.p. in hippocampal pyramidal cells (Lancaster & Adams, 1986). Unfortunately, technical limitations resulting from the extended electrical structure of the pyramidal cell prohibit a kinetic analysis of the macroscopic currents which actually underlie the action potential. We have therefore used a pharmacological approach to separate the potential changes which occur during and after an action potential. Some of this material has appeared in abstract form (Lancaster, Madison & Nicoll, 1986).

METHODS

The methods used in this paper are similar to those used in other studies from this laboratory (Nicoll & Alger, 1981). Briefly, rat hippocampal slices, 400 μm thick, were cut and placed in a holding chamber for at least 1 h. A single slice was then transferred to the recording chamber and held between two nylon nets, submerged beneath a continuously superfusing medium that had been pre-gassed with 95% O_2, 5% CO_2. The composition of this medium was (mM): NaCl, 119; KCl, 2·5; $MgSO_4$, 1·3; $CaCl_2$, 2·5; NaH_2PO_4, 1·0; $NaHCO_3$, 26·2; and glucose, 11. The pH of the gassed medium was 7·4. A nominally zero-calcium medium was made by replacement of $CaCl_2$ with 10 mM-$MgCl_2$. The temperature of the medium was maintained between 29 and 31 °C. Drugs used in this study, which were obtained from Sigma Chemical Co. unless otherwise indicated, included: tetraethylammonium chloride (TEA), cadmium chloride, carbamylcholine chloride (carbachol), 8-bromoadenosine 3′,5′-monophosphate (8-bromocyclic AMP) and ethyleneglycol-bis-(β-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EGTA). The tetrapotassium salt of BAPTA (1,2-bis(*o*-aminophenoxy)ethane-*N,N,-N′,N′*-tetraacetic acid) was obtained from Molecular Probes Inc. Charybdotoxin (CTX) was generously supplied by Dr C. Miller, Brandeis University. Conventional intracellular recording techniques were used for most of the experiments reported here. Intracellular electrodes were pulled from 'omega-dot' glass capillary tubing (o.d. 1·2 mm, i.d. 0·6 mm; Glass Co. of America) and were filled with 2 M-potassium methylsulphate ($KMeSO_4$; ICN Pharmaceuticals; electrode resistance 100–140 MΩ). To load cells with either EGTA or BAPTA the recording electrode was filled with 2 M-$KMeSO_4$ plus either 0·2 M-EGTA (pH = 7) or 0·2 M-BAPTA (pH = 7). The intracellular concentration of EGTA and BAPTA is unknown, but an approximate concentration can be calculated based on the shift in the i.p.s.p. reversal potential caused by KCl-filled electrodes. The normal reversal potential is approximately −70 mV (Newberry & Nicoll,

1985) and can shift to about 0 mV (cf. Collingridge, Gage & Robertson, 1984) with a 3 M-KCl-filled electrode. This suggests that the intracellular concentration shifts from less than 10 mM to over 100 mM, which means that $[Cl^-]_i$ (intracellular Cl^- concentration) equilibrates to a value that is approximately 30-fold less than that of the electrode $[Cl^-]$. This dilution factor would give a concentration of about 7 mM-EGTA and BAPTA. This is an upper limit since these drugs are presumably less mobile than (Cl^-).

Results in this paper were recorded by a variety of methods. The slow a.h.p. was recorded on a chart recorder, while the action potentials were either recorded on an analog oscilloscope and photographed, or recorded on a digital oscilloscope (Nicolet 4094) and plotted with a digital plotter (Hewlett–Packard 7470A). These results are based on recordings from seventy-three pyramidal neurones with membrane potentials greater than −55 mV which were stable for at least 30 min.

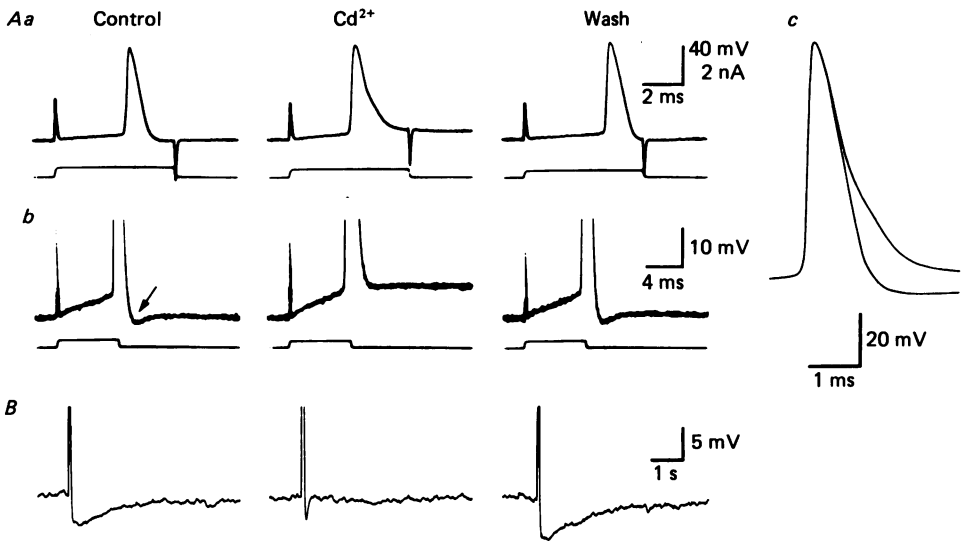

Fig. 1. Effects of Cd^{2+} on fast and slow a.h.p.s. *Aa*, low-gain records show reversible broadening of the action potential by 100 μM-Cd^{2+} applied for 12 min. This is invariably accompanied by loss of the fast a.h.p., shown by the arrow in the high-gain records of *Ab*. Right-hand traces (*c*) are superimposed control and Cd^{2+} traces. *B*, chart records show reversible block of the slow a.h.p. by 100 μM-Cd^{2+}. The remaining a.h.p. is the intermediate Ca^{2+}-independent component. All records from the same cell, membrane potential −63 mV.

RESULTS

The simplest method to assay any Ca^{2+} dependence of action potential repolarization is to add a Ca^{2+} channel blocker to the medium. In the experiment shown in Fig. 1, 100 μM-Cd^{2+} was used to block entry of Ca^{2+} through voltage-sensitive channels. Under these conditions the action potential duration was increased and this increase was confined to the lower two-thirds of the action potential. In addition the notch observed immediately upon repolarization (see arrow in Fig. 1 *Aa*) is eliminated. This notch is henceforth referred to as the fast a.h.p. after Storm (1985). Treatment with Cd^{2+} also blocks the slow a.h.p. which is activated by a short train of action potentials. Identical results were observed in a medium nominally free of Ca^{2+}. An intermediate, Ca^{2+}-independent a.h.p. is clearly seen in the chart record (Fig. 1 *B*)

obtained in the presence of Cd^{2+}. This paper is concerned with the distinction between the two Ca^{2+}-dependent after-potentials. An appropriate concentration of the K^+ channel blocker TEA could be used to discriminate between the fast and slow a.h.p. by a selective action on the fast a.h.p. (Fig. 2). This concentration of TEA (200 μM) reduced the fast a.h.p. with an attendant increase in spike duration. The train of five action potentials used to produce the slow a.h.p. is displayed at a high gain to

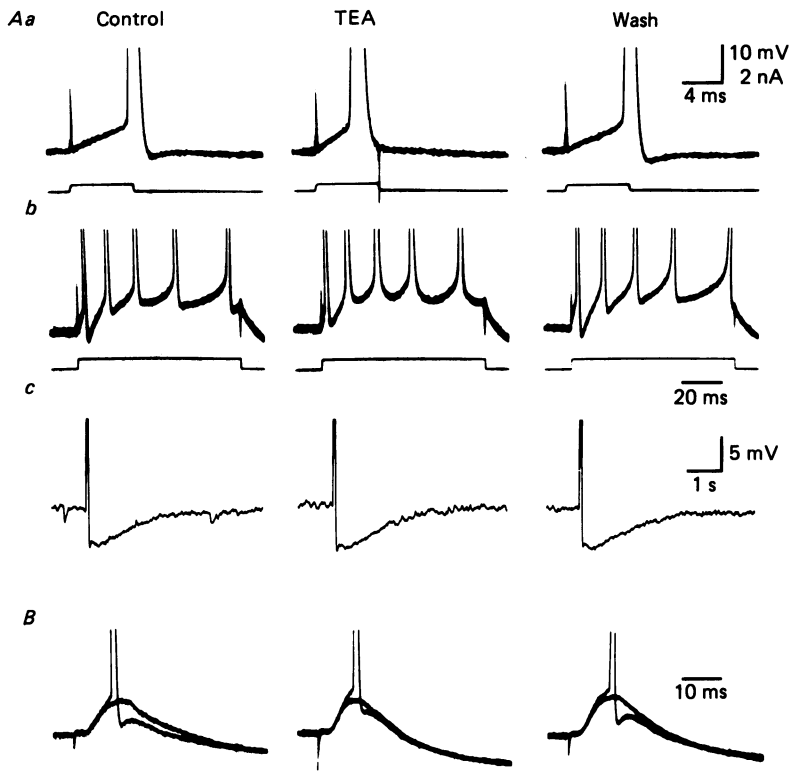

Fig. 2. Effects of TEA on the fast a.h.p. *Aa*, 200 μM-TEA applied for 8 min reversibly broadens the action potential with an attendant reduction of the fast a.h.p. *Ab*, the TEA-sensitive component contributes prominently to the interspike trajectory of the membrane potential. *Ac*, the slow a.h.p. is unaffected by 200 μM-TEA. *B*, a synaptically generated response contains a TEA-sensitive fast a.h.p. Records in *A* from one cell, membrane potential −57 mV. Records in *B* from a separate cell, membrane potential −73 mV. Voltage calibration for *B* as in *Aa*.

demonstrate the alteration in the interspike trajectory which is a function of blocking the fast a.h.p. Although TEA does not alter spike frequency adaptation, it does decrease the interspike interval (see below). The lower part of Fig. 2 is from a different cell to show how the fast a.h.p. contributes to the synaptically evoked response.

The use of TEA alone does not provide an unequivocal result because it can be argued that TEA was acting to block the delayed rectifier which repolarizes nerve fibre action potentials. If this were the case then TEA ought to have clear effects in the presence of Cd^{2+}, when any Ca^{2+}-dependent K^+ events have been abolished. Therefore, we compared the action of low concentrations of TEA ($\leqslant 500$ μM) in the

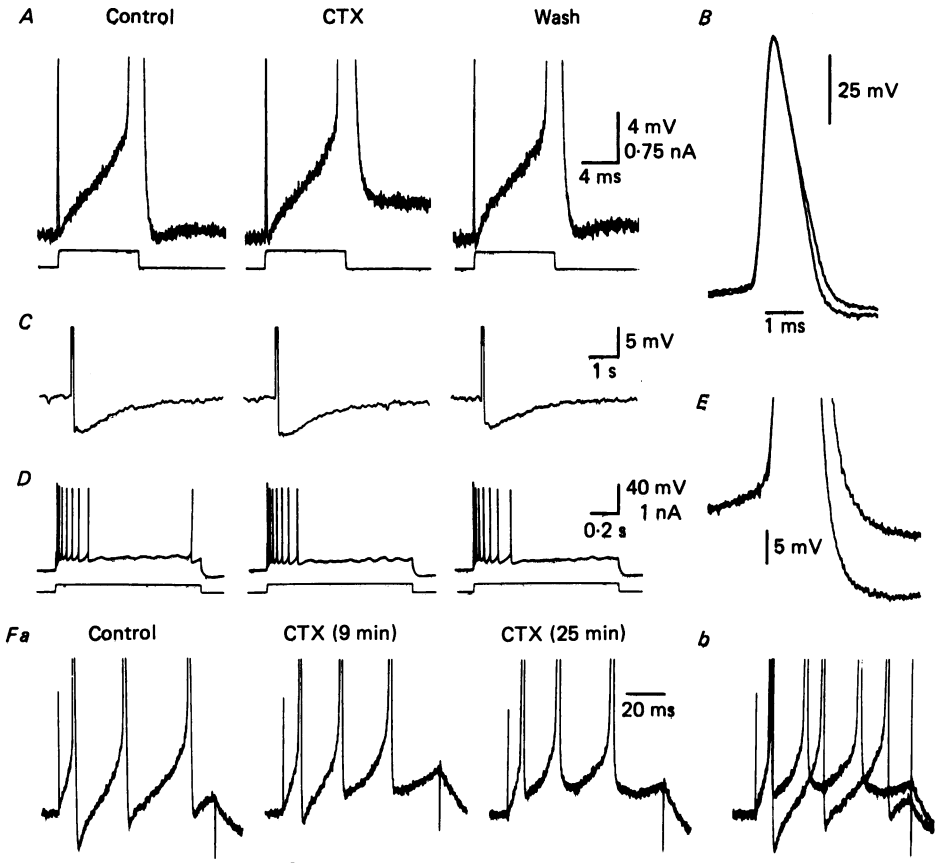

Fig. 3. Effects of charybdotoxin on the fast and slow a.h.p.s. *A*, a reversible block of the fast a.h.p, with an increase in spike duration is caused by 10 nM-charybdotoxin (CTX), applied for 25 min. *B*, the leading edge of the action potentials in control and charybdotoxin in *A* have been lined up to show the increase in spike width and loss of the fast a.h.p. This compound had no effect on the slow a.h.p. *C*, spike frequency adaptation (*D*) or on spike height (*B*). Membrane potential −73 mV. The effect of CTX was reversed with 45 min of washing in drug-free medium. *E*, effect of 25 nM-CTX applied for 25 min in another cell. The control and CTX-treated action potential are superimposed (time calibration same as in *B*). *Fa*, action potentials evoked by an 80 ms current injection. Gradual block of the fast a.h.p. by CTX has a similar effect on the interspike trajectory as TEA. *Fb*, superimposed control and 25 min CTX records. Note the decrease in the interval between action potentials after block of the fast a.h.p. Voltage calibration for *F* as in *E*. Membrane potential −60 mV.

absence and presence of Cd^{2+} in the same cell ($n = 3$). While the action of TEA at these low concentrations was clearly diminished in the presence of Cd^{2+}, it still produced a slight but distinct broadening of the action potential. It is therefore impossible to be certain that these low concentrations of TEA act solely on the Ca^{2+}-dependent K^+ current (see Discussion). In accord with this we found that much higher concentrations of TEA (10–20 mM) than were necessary to abolish the fast a.h.p. could indeed be demonstrated to cause a large, and reversible, increase in the width of the action potential in the presence of 200 μM-Cd^{2+}.

The sensitivity of the fast a.h.p. to submillimolar concentrations of external TEA is also a characteristic of the large-conductance, voltage-sensitive Ca^{2+}-activated K^+ current observed in many preparations (Inoue, Kitamura & Kuriyama, 1985; Hermann & Gorman, 1981*b*). Fortunately a further test for these channels, and thereby for the current underlying the fast a.h.p., has been provided by the recent discovery of a protein toxin termed charybdotoxin (CTX), which acts as a potent blocker of this channel (Miller, Moczydlowski, Latorre, & Phillips, 1985). Fig. 3 shows

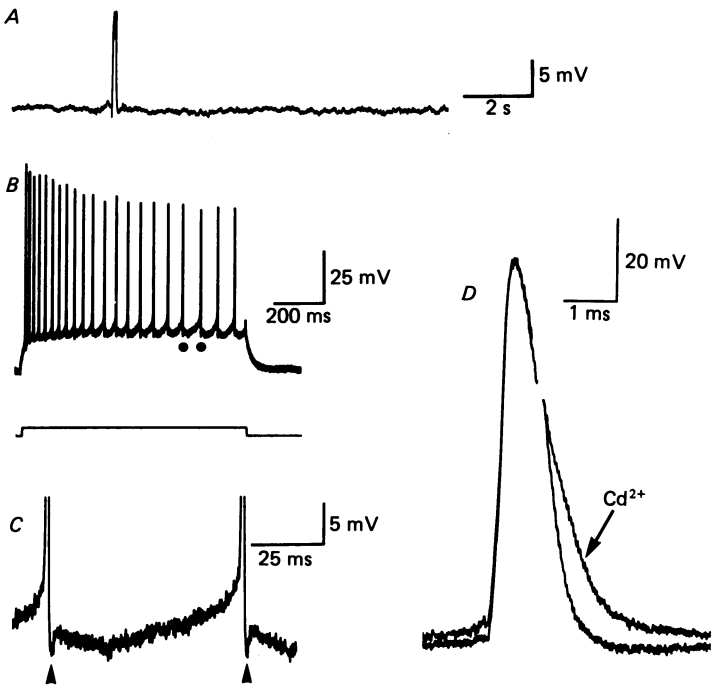

Fig. 4. Effects of EGTA on fast and slow a.h.p.s. Records in this Figure were obtained 10–35 min after penetrating a cell with an electrode which contained 200 mM-EGTA in addition to the normal electrolyte. *A*, an 80 ms current pulse evokes a train of action potentials, no slow a.h.p. follows the train. This is in contrast to normal cells (Figs. 1–4), which invariably display a slow a.h.p. following a train of action potentials. A corollary of this is a lack of spike frequency adaptation (*B*). The action potentials indicated (●) are shown at higher gain in *C*; each is clearly followed by a fast a.h.p. (arrowed). *D*, action potentials in a cell filled with EGTA can still be increased in width by addition of 100 μM-Cd^{2+} to the bathing medium. The capacitative artifact at the end of the current pulse, which occurs on the falling phase of the action potential, has been deleted. All records from the same cell, membrane potential −68 mV.

the result of the application of CTX to two cells (*A–D* and *E–F*). This compound (up to 25 nM, the highest concentration tested) appeared to reduce specifically the fast a.h.p. There was no effect on the slow a.h.p., spike frequency adaptation or the rise time and amplitude of the action potential. A consequence of the block of the fast a.h.p. was an increase in spike duration (see superimposed records in *B* and *E* for the two cells). This increase in duration was confined to the bottom two-thirds of the action potential, similar to the action of Cd^{2+}. These effects were reversible upon washing. The set of records in *F* illustrates the effect of CTX (25 nM) on the fast a.h.p.

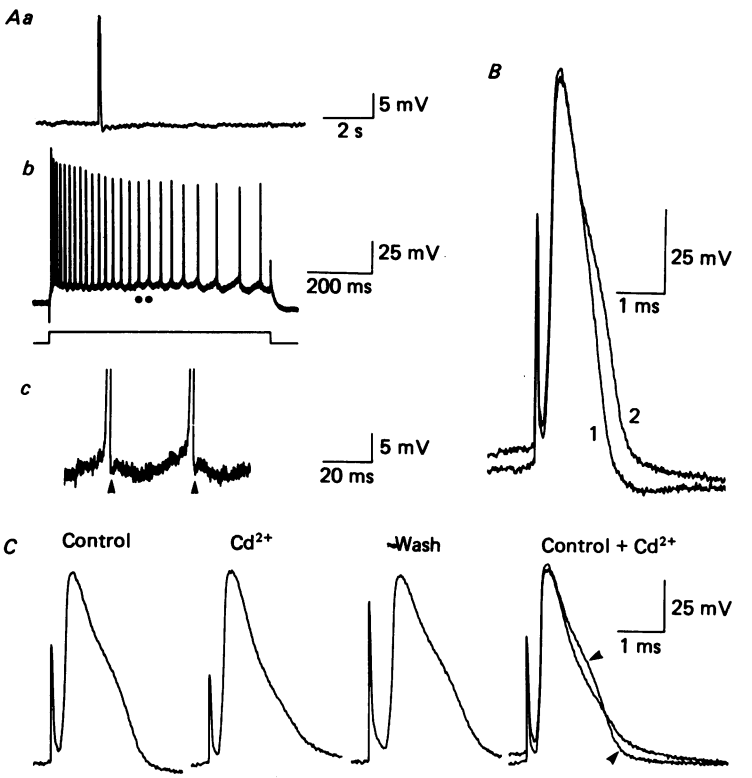

Fig. 5. Effect of BAPTA on fast and slow a.h.p.s. Records in this Figure were obtained with an electrode which contained 200 mM-BAPTA in addition to the normal electrolyte. *Aa*, an 80 ms current pulse which evokes a train of action potentials is not followed by a slow a.h.p. and the cell displays little spike frequency adaptation in recordings with a BAPTA-containing electrode. *Ab*, the action potentials indicated (●) are shown at a higher gain in *Ac*, and are followed by a fast a.h.p. (see arrow). The records in *A* were obtained shortly after impalement when the fast a.h.p. was still apparent. *B*, trace 1 shows an action potential shortly after impalement. Trace 2 shows an action potential 1 h later after development of the full effect of BAPTA. In six out of eleven cells recorded with BAPTA-containing electrodes a shoulder on the action potential was apparent immediately upon impalement. In the remaining five cells, this effect took from a few minutes to 1 h (as illustrated) to develop. *C*, 100 μM-Cd^{2+} reversibly abolishes the shoulder on the action potential. In the control + Cd^{2+} records, the arrowheads indicate the control response. All records taken from the same cell, membrane potential −70 mV.

during a longer depolarizing current pulse. As with TEA (Fig. 2), CTX reveals how the fast a.h.p. controls the early frequency of firing by repolarizing the membrane well below threshold following a single action potential.

It is well established that the use of EGTA-containing electrodes will block the Ca^{2+}-activated K^+ conductance which generates the slow a.h.p. (Schwartzkroin & Stafstrom, 1980; Lancaster & Wheal, 1984; Madison & Nicoll, 1984). We were rather surprised to discover that although this procedure blocked slow a.h.p.s and spike frequency accommodation (Fig. 4 *A* and *B*), action potentials were still followed by a distinct fast a.h.p. (arrows in Fig. 4*C*) in eight cells. The spike duration at one-third

peak amplitude of cells recorded with EGTA-filled micro-electrodes (1·10 ± 0·05 ms, $n = 5$) was not significantly different from control cells (1·09 ± 0·09 ms, $n = 15$). Furthermore, the addition of 100 μM-Cd^{2+} ($n = 2$) caused spike broadening and a block of the fast a.h.p. in a manner identical to that observed without EGTA.

The Ca^{2+} chelator BAPTA was designed to have certain advantages over EGTA. BAPTA is much faster at taking up and releasing Ca^{2+}, and has an affinity which is unaffected by pH changes (Tsien, 1980). In cells recorded from using BAPTA-containing electrodes ($n = 11$) the slow a.h.p. was blocked by the time that the impalement had stabilized (Fig. 5*A*). All of these cells displayed a shoulder on the repolarizing phase of the action potential, which is not a usual feature of CA1 neurones. In some cells (five of eleven) the shoulder on the action potential took

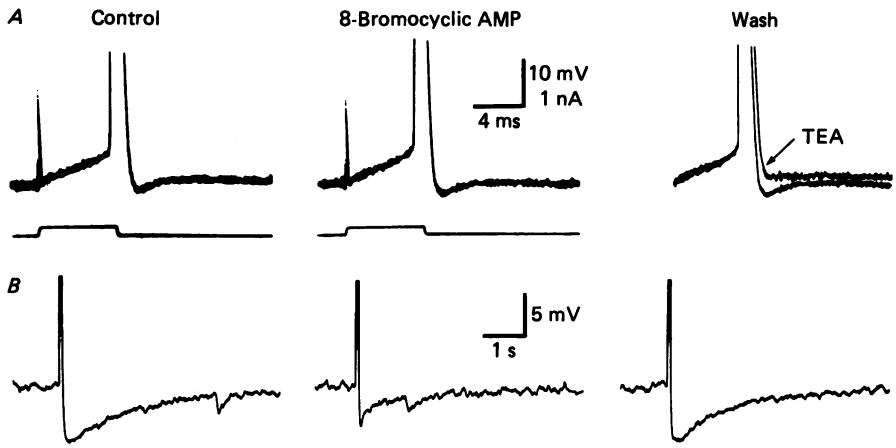

Fig. 6. Effect of 8-bromocyclic AMP on fast and slow a.h.p.s. *A*, the fast a.h.p. is resistant to 1 mM-8-bromocyclic AMP added to the bathing medium for 17 min, while the slow a.h.p. (*B*) is reversibly reduced. The superimposed records in 'wash' (*A*) show the fast a.h.p. recorded after washing the preparation for 25 min in drug-free solution and also the a.h.p. recorded after applying 500 μM-TEA for 12 min (arrow). All records from the same cell, membrane potential −63 mV.

some minutes to develop. In these examples (as in Fig. 5*B*) the spike broadening which developed with time was observed to be associated with a reduction in the fast a.h.p. The spike duration at one-third peak amplitude was 1·54 ± 0·16 ms (mean ± S.D., $n = 11$) for BAPTA cells and 1·09 ± 0·09 ms ($n = 15$) for a random sample of control cells. A Student's t test indicates a greater than 99·9 % chance of this difference being significant. The action of Cd^{2+} on BAPTA-filled cells was to decrease action potential duration; this is in contrast to the action of Cd^{2+} on control or EGTA-filled cells. When compared in the absence and presence of Cd^{2+}, BAPTA action potentials displayed a cross-over of the membrane potential towards the base of the spike (Fig. 5*C*) presumably due to block of residual fast a.h.p. by the Cd^{2+}.

The current which generates the slow a.h.p. is sensitive to various neurotransmitters which thereby attenuate spike frequency adaptation. Most notably, a rise in intracellular cyclic AMP and presumably, therefore, an activation of cyclic AMP-dependent protein kinase (Nestler, Walaas & Greengard, 1984) will block the slow

a.h.p. (Madison & Nicoll, 1986). A well-characterized physiological stimulus for this effect is noradrenergic activation of β_1-receptors (Madison & Nicoll, 1986). This action is mimicked by 8-bromocyclic AMP which is a membrane-permeant analogue of cyclic AMP. Both noradrenaline and 8-bromocyclic AMP were used as agents to help distinguish the mechanisms underlying the fast and slow a.h.p. The addition of either noradrenaline or 8-bromocyclic AMP to the medium caused the expected reduction of the slow a.h.p. (Fig. 6). However, the fast a.h.p. and spike repolarization were entirely insensitive to noradrenaline and 8-bromocyclic AMP. Although 8-bromocyclic AMP had no effect on the fast a.h.p., as expected the fast a.h.p. was reduced by the subsequent addition of 200 μM-TEA (Fig. 6*B*). While both the fast

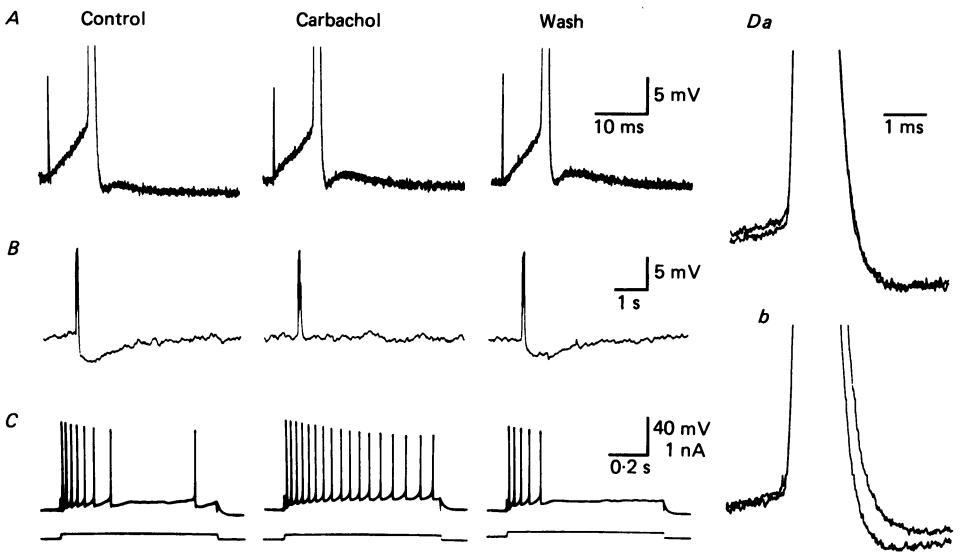

Fig. 7. Effect of carbachol on fast and slow a.h.p.s. *A*, carbachol (2 μM) applied for 23 min has no effect on the fast a.h.p. or on spike duration. *B*, the same application of carbachol reversibly abolishes the slow a.h.p. and prevents spike frequency accommodation (*C*). *Da*, action potentials on an expanded time base from control and carbachol in *A* to show that the fast a.h.p. is unchanged. *Db*, subsequent application of 200 μM-TEA broadens the spike and reduces the fast a.h.p. All records from the same cell, membrane potential −68 mV.

and slow a.h.p. are generated by Ca^{2+}-activated K^+ currents, activation of the cyclic AMP system acts specifically on the slow event.

The muscarinic action of acetylcholine in the hippocampus is, in part, similar to that of noradrenaline, i.e. a block of the slow a.h.p. with a concomitant reduction of spike frequency adaptation. However, the inability of muscarinic agonists to raise cyclic AMP levels (Hulme, Berrie, Birdsall & Burgen, 1981) indicates that acetylcholine and noradrenaline appear to act via distinct second messenger systems. The use of cholinergic agonists therefore provides an alternative approach to the separation of the fast and slow a.h.p. Since there are no nicotinic responses in hippocampal pyramidal cells (Cole & Nicoll, 1984) drugs such as carbachol produce only muscarinic responses. A concentration of carbachol (2 μM) which caused complete block of the

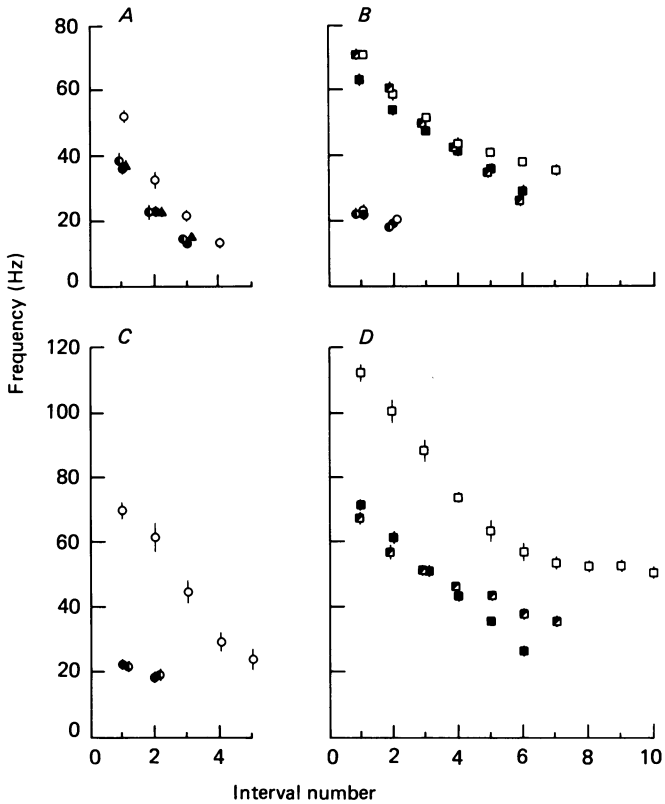

Fig. 8. Functional properties of fast and slow a.h.p.s. *A*, TEA, but not noradrenaline, increases the early spike frequency. After obtaining a control curve (●), 500 μM-TEA was applied for 13 min (○) and then was washed from the preparation for 5 min (◐). Subsequent addition of 4 μM-noradrenaline for 18 min (▲) which blocked the slow a.h.p. in this cell had no effect on the frequency. Points are mean ±S.D. from four observations. TEA significantly increases the frequency of the early action potentials in response to a 160 ms, 0·2 nA current pulse. *B*, effect of noradrenaline at two different stimulus strengths, 0·15 nA (circles) and 0·37 nA (squares). Control values (●, ■); 10 μM-noradrenaline applied for 15 min (○, □); wash for 16 min (◐, ◩). Noradrenaline has no effect on the spike frequency at the low stimulus strength. However, the frequency at the higher stimulus is increased after the fourth interval. (The change during the first two intervals is judged to be non-specific since the noradrenaline and wash values are superimposed.) *C*, same cell as *B*, stimulus strength 0·15 nA. Control (●), 100 μM-Cd^{2+} applied for 12 min (○), wash for 8 min (◐). *D*, same cell as *B* and *C*, stimulus strength 0·37 nA. Control (■), 100 μM-Cd^{2+} (□) and wash (◩). Values in *A* from one cell, membrane potential −68 mV. Values in *B*, *C* and *D* from a separate cell, membrane potential −61 mV.

slow a.h.p. and attenuated spike frequency adaptation had no effect on the fast a.h.p. or on action potential duration (Fig. 7), yet both of these parameters were affected by the subsequent application of 200 μM-TEA. Even tenfold higher concentrations of carbachol failed to affect spike duration significantly.

Apamin blocks the a.h.p. in cultured rat muscle cells (Romey & Lazdunski, 1984), in bull-frog sympathetic ganglion cells (Pennefather *et al.* 1985) and rat sympathetic neurones (Kawai & Watanabe, 1986). However, neither apamin from Sigma nor apamin known to be active on rat muscle cells in culture (a gift from M. Lazdunski)

had any action on the slow a.h.p. in hippocampus in concentrations of up to 1 μM applied for as long as 3 h.

We have described how the process responsible for the fast a.h.p. may influence the interspike interval under some conditions. This observation was pursued by using two strengths of depolarizing current; one which evoked a low frequency of firing during a 100–200 ms pulse and a second stronger stimulus where action potentials were clustered towards the beginning of the depolarization before the cessation of firing caused by the slow a.h.p. When the slow a.h.p. was blocked by noradrenaline, action potentials occur throughout the longer depolarization, so that the number of spikes to a given stimulus is increased (Madison & Nicoll, 1984). However, the early interspike intervals in response to a small depolarization are little affected by noradrenaline (Fig. 8*A* and *B*). At a time when the slow a.h.p. is abolished by noradrenaline, the same manipulations that block the fast a.h.p. (i.e. application of TEA or Cd^{2+}) will also increase the frequency of action potentials evoked by small depolarizations. Application of TEA alone tends to increase spike frequency but the number of action potentials is not greatly affected because the slow a.h.p. is undiminished (Fig. 8*A*). Cd^{2+} has the combined actions of TEA and noradrenaline by acting on the fast a.h.p., to cause increased spike frequency to all stimuli in addition to acting on the slow a.h.p. to increase the number of action potentials (Fig. 8*C* and *D*). The reduced interspike intervals were not correlated with increases in cell input resistance which might otherwise serve to explain changes of this nature.

DISCUSSION

The fast a.h.p. which immediately follows the repolarizing phase of the action potential in CA1 pyramidal cells is sensitive to Ca^{2+} channel blockers such as Cd^{2+} and to the K^+ channel blocker TEA. The elimination of the fast a.h.p. is invariably associated with an increase in action potential duration. A similar effect to Cd^{2+} and TEA on the fast a.h.p. can be achieved by the application of CTX. These results are therefore consistent with an interpretation that the Ca^{2+} dependence of spike repolarization is due to activation of the Ca^{2+}-activated K^+ current which has been termed I_C (Thompson, 1977; Adams *et al.* 1982). Macroscopic and microscopic currents which correspond to I_C have been described in hippocampal neurones (Brown & Griffith, 1983; Brett & Lancaster, 1985). This current or the single channels which underlie it have been extensively studied in other preparations where they are also blocked by similar concentrations of TEA (Galvan & Sedlmeir, 1984; Pennefather *et al.* 1985; Iwatsuki & Petersen, 1985; Inoue *et al.* 1985) and CTX (Miller *et al.* 1985).

Under conditions where the kinetics of this current can be studied (Barrett, Magleby & Pallotta, 1982; Wong, Lecar & Adler, 1982; Thomas, 1984; Lancaster & Pennefather, 1987) it is clear that I_C has a pronounced voltage sensitivity. Thus during an action potential there are two factors which combine to favour the activation of I_C. One is the entry of Ca^{2+} during the depolarization, and the second is the positive membrane potential. A corollary of this voltage sensitivity is that I_C deactivates quite rapidly as the membrane potential returns to rest. For this reason block of I_C (i.e. the fast a.h.p.) has no effect on the slow a.h.p. It is interesting that Cd^{2+}, TEA and CTX only affected the bottom two-thirds of the repolarizing phase

of the action potential. This suggests that there is a finite delay in expression of I_C during the action potential and that some other process is involved in the initial one-third of spike repolarization. In addition to its role in action potential repolarization, I_C also has an important role in controlling the interval between action potentials. However, because of the short duration of I_C at negative potentials, there is little summation of I_C during repetitive discharges and therefore I_C does not contribute appreciably to adaptation of action potential discharge.

A question remains as to the role of a purely voltage-dependent K^+ current in the action potential repolarization. For example, in sympathetic neurones of the bull-frog the delayed rectifier does not appear to be activated by an action potential (Lancaster & Pennefather, 1987). In the hippocampus, TEA (10 mM) added in the presence of 200 μM-Cd^{2+} causes a clear broadening of the action potential. However, this experiment is not completely satisfactory because it tells us only that the delayed rectifier can terminate the spike in the presence of Cd^{2+}. We must consider that this is probably an over-estimate of the normal role of the delayed rectifier because, in Cd^{2+}, the action potential is broader than normal and this prolonged excursion at depolarized potentials will enhance the degree to which the delayed rectifier will be activated.

Shortly after penetration with an EGTA-containing electrode, a slow a.h.p. could be generated but the spike duration and fast a.h.p. are unaltered regardless of the length of the impalement. Therefore, EGTA appears to be able to buffer $[Ca^{2+}]_i$ sufficiently to block generation of the slow a.h.p. but not the fast a.h.p. This could be explained if the current underlying the fast a.h.p. is more sensitive to $[Ca^{2+}]_i$ than that which underlies the slow a.h.p. Evidence from cultured muscle cells on TEA and voltage-sensitive currents *versus* apamin-sensitive, voltage-insensitive currents (which probably underlie the a.h.p.: Romey & Lazdunski, 1984; Blatz & Magleby, 1986) suggests that the reverse is the case, although based on apamin sensitivity the channels in muscle may be different from those on pyramidal cells. A more likely explanation, therefore, is that EGTA is relatively slow at buffering $[Ca^{2+}]_i$ and is unable to act on the time scale of the fast a.h.p. (Tsien, 1980; Neher & Marty, 1985). This conclusion is supported by the results of the BAPTA experiments. The EGTA derivative BAPTA (Tsien, 1980) has a greater ability to suppress transient rises of $[Ca^{2+}]_i$ (Neher & Marty, 1985). With BAPTA electrodes, the intracellular Ca^{2+} is presumably buffered sufficiently rapidly to prevent activation of much of the outward current, hence a Ca^{2+}-dependent hump in the action potential which would normally be prevented by the development of I_C. Thus with BAPTA, but not with EGTA, application of Cd^{2+} is able to decrease action potential duration. The Ca^{2+}-dependent hump observed in BAPTA-treated cells is very similar to the action potentials obtained in cultured dorsal root ganglion cell (Dichter & Fischbach, 1977) and superior sympathetic ganglion cells (Horn & McAfee, 1979).

Since the Ca^{2+} which enters during an action potential can activate I_C within 1–2 ms, this sets an upper limit on the distance which Ca^{2+} can diffuse to initiate this process. Given a diffusion coefficient of about $0{\cdot}8 \times 10^{-5}$ cm^2/s (Hille, 1984) then in 1 ms Ca^{2+} can diffuse over an area of 0·8 μm^2. This is an upper limit, since any buffering would reduce the distance over which Ca^{2+} can diffuse in a given time. Hence the I_C channel is likely to be very close to the site of Ca^{2+} entry. This contrasts markedly with the long delay before the peak of the $I_{a.h.p.}$ conductance (Lancaster & Adams, 1986). The rising phase of the $I_{a.h.p.}$ may arise from $I_{a.h.p.}$ channels localized further

away from the sites of Ca^{2+} entry than I_C channels. In this scheme, the time to peak would be due to a more generalized rise and redistribution of intracellular Ca^{2+}. However, the temperature dependence of the time to peak (Lancaster & Adams, 1986) suggests that diffusion alone is not a sufficient explanation; kinetics of Ca^{2+} buffering may also be involved or there may be co-operativity in channel opening.

The results with CTX and TEA indicate that the fast and slow a.h.p.s can be separated from each other. In the case of I_C, the channels are susceptible to low concentrations of external TEA which is known to enter the channel and act as a blocking ion (Vergara, Moczydlowski & Latorre, 1984). As a means of further separating the fast and slow a.h.p.s, second messenger systems provide a physical and conceptual alternative to the use of external blockers. β-Adrenergic receptor activation has been found to block the slow a.h.p. via the second messenger cyclic AMP (Madison & Nicoll, 1986). Muscarinic receptor activation also blocks the slow a.h.p. (Benardo & Prince, 1982; Cole & Nicoll, 1984). The second messenger for this action is not entirely certain, but is clearly not cyclic AMP. The lack of effect of noradrenaline and acetylcholine on spike repolarization and the fast a.h.p., indicates that Ca^{2+} entry during the action potential is not reduced by these transmitters and suggests that the blockade of the slow a.h.p. by noradrenaline and acetylcholine occurs at some step after the rise in intracellular Ca^{2+}, most likely at the $I_{a.h.p.}$ channel itself. Although the present results do not exclude the possibility that the block of the slow a.h.p. occurs as a result of a slow sequestering of intracellular Ca^{2+}, analogous to the action of EGTA, I_C evoked by 1 s depolarizing voltage-clamp steps is also resistant to the action of noradrenaline (Lancaster & Adams, 1986) and acetylcholine (Madison, Lancaster & Nicoll, 1987). The fast and slow a.h.p. are therefore distinguishable by modifications presumably made at the intracellular surface of the channel. These findings taken together with the results with TEA and CTX, both of which act primarily from the external surface at the concentrations used (Vergara *et al.* 1984; Miller *et al.* 1985), strongly suggest that quite separate channels underlie the two Ca^{2+}-dependent a.h.p.s. Ca^{2+}-activated K^+ channels with a much smaller conductance than the TEA and voltage-sensitive channel have been found in mammalian skeletal muscle (Blatz & Magleby, 1986). Interestingly, this small channel in muscle and also a Ca^{2+}-activated K^+ current in clonal pituitary cells (Ritchie, 1985) are sensitive to apamin. This is a feature shared with the bull-frog a.h.p. current and with a slow a.h.p. in rat sympathetic neurones (Kawai & Watanabe, 1986), but not with the hippocampal equivalent.

The transient outward current (A-current) appears to repolarize action potentials in rat sympathetic neurones (Belluzzi, Sacchi & Wanke, 1985) and the existence of A-current in hippocampal pyramidal cells has been established (Gustafsson, Galvan, Grafe & Wigstrom, 1982). However, the involvement of A-current in pyramidal cell action potentials is difficult to establish unambiguously, since 4-aminopyridine (the most selective blocker available) may affect other currents (Yeh, Oxford, Wu & Narahashi, 1976; Hermann & Gorman, 1981*a*) even at concentrations as low as 10 μM (Meves & Pichon, 1977).

An inhibition of A-current, together with increased action potential duration and a block of spike frequency adaptation has been reported to be a consequence of muscarinic action in cultured hippocampal neurones (Nakajima, Nakajima, Leonard & Yamaguchi, 1986). Since we observed a block of spike frequency adaptation and

of the slow a.h.p. without any effects on the action potential, it seems that the effects on the slow a.h.p. can be dissociated from any actions on the A-current.

Even less well understood than A-current is a Ca^{2+}-dependent, transient, outward current described in CA3 pyramidal cells (Zbicz & Weight, 1985) and bull-frog ganglion cells (MacDermott & Weight, 1982), where it was claimed that it may take part in action potential repolarization. Independent evidence that the underlying Ca^{2+} currents were not themselves transient (Carbone & Lux, 1984; Bean, 1985) is lacking at this stage. In bull-frog ganglion cells, the Ca^{2+} dependence of the action potential repolarization is due to the activation of a K^+ conductance which shows no inactivation over several tens of milliseconds (Lancaster & Pennefather, 1987). There is no such direct evidence for hippocampal cells. But if the spike repolarization is partly due to I_C as judged by the TEA and CTX susceptibility, one implicitly assumes common features with I_C in other systems where it is observed to be a non-inactivating current (Thomas, 1984; Lancaster & Pennefather, 1987).

We have benefited in this respect from the use of CTX which is active on the large-conductance Ca^{2+}-activated K^+ channels from mammalian skeletal muscle (Miller *et al.* 1985) and optical measurements indicate a prolongation of action potentials and a decreased spike undershoot in frog neurohypophysis (Obaid & Salzberg, 1985). The concentrations we have found to be active in the hippocampus are comparable to the results obtained in *Aplysia* neurones (Hermann, 1985). In both of these other systems CTX appeared to be specific at the concentrations used, an observation which is echoed by the results presented here. Similar results of CTX on the fast a.h.p. in hippocampal pyramidal cells have been observed by Storm (1986).

If the prime role of I_C then is to repolarize the action potential, what are the physiological sequelae of activating this current? Both TEA and CTX application serve to illustrate that the fast a.h.p. generated by spikes contributes to the early frequency within a train of action potentials. This effect occurs without any reduction of the slow a.h.p. The fast a.h.p. repolarizes the membrane sufficiently below threshold to offset temporarily any tonic depolarizing influence. The initial firing frequency is therefore regulated more by the fast a.h.p. than the slow a.h.p.; this is especially so with low-intensity depolarizing stimuli. Conversely, the slow a.h.p. can be abolished with little effect on the response to low-intensity stimuli. The functional distinction of the two Ca^{2+}-dependent processes during adaptation lies in their relative importance during low- and high-intensity stimulation.

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