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Intracellular Fluoride Alters the Kinetic Properties of Calcium Currents Facilitating the Investigation of Synaptic Events in Hippocampal Neurons

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We have attempted to suppress voltage-dependent conductances in hippocampal neurons by introducing various intracellular agents. Voltage-clamp studies were carried out using acutely dissociated hippocampal neurons from adult guinea pigs. Synaptic events were examined using intracellular recordings in the slice preparation. Sodium conductance was suppressed when the quaternary lidocaine derivative QX 314 was introduced intracellularly. Potassium conductances were blocked by intracellular cesium or Tris. We also found that the anion fluoride could affect calcium conductance by an intracellular action. When anions other than fluoride were used for intracellular recordings, the voltage-dependent calcium current inactivated slowly and showed persistent activation at membrane potentials between −40 and −10 mV. In contrast, when fluoride was present intracellularly, the inactivation kinetics of the calcium current were accelerated and the persistent component of the current was largely suppressed. Intracellular recordings in the hippocampal slice showed that when electrodes contained cesium, QX 314, and fluoride, the spiking and nonlinear responses of the neuronal membrane to depolarization were blocked. In these conditions the time course and voltage-dependence of EPSPs could be examined in detail without complications due to voltage-dependent currents of the postsynaptic cell.

The activity of nerve cells is defined by the interaction of intrinsic neuronal properties with spontaneously occurring synaptic inputs. In the CA2–3 region of the hippocampus the principal neurons have complex intrinsically generated firing patterns and, in addition to receiving afferent synaptic inputs, are interconnected by a network of recurrent synapses. We have shown that the synaptic depolarization resulting from unitary EPSPs mediated by recurrent connections may initiate active subthreshold responses in a postsynaptic cell (Miles and Wong, 1986). The activation of these ionic conductances prolongs the time course of the resulting EPSP and complicates assessment of synaptic properties such as electrotonic location, statistics of transmitter release, and voltage dependence.

The present study was undertaken to determine whether active responses of a postsynaptic neuron could be suppressed to facilitate studies on synaptic events. In order to retain functional synaptic transmission without influencing axonal conduction or presynaptic mechanisms, it was necessary to use blocking agents effective from inside the neuronal membrane. The quaternary lidocaine derivative QX 314 is such an agent which has been used to suppress voltage-dependent Na^+ currents (Connors and Prince, 1982; Frazier et al., 1970). Similarly, intracellular cesium and tetraethyl ammonium ions can suppress K^+ currents when introduced intracellularly (Armstrong and Binstock, 1965; Tillotson, 1979).

Regenerative depolarizing responses in hippocampal cells result from inward currents carried by calcium as well as sodium (Brown and Griffith, 1983; Johnston et al., 1980). Agents that suppress Ca^{2+} currents in hippocampal cells through an intracellular action have not yet been described. However, it has been shown that intracellular fluoride blocks the gating and activation of voltage-dependent calcium current in snail neurons (Kostyuk and Krishtal, 1977). This observation led us to examine the effect of intracellular fluoride on hippocampal neurons.

Calcium-dependent responses were characterized in voltage-clamp studies on acutely isolated hippocampal neurons and the action of fluoride on inward current carried by Ca^{2+} was assessed. We found that intracellular fluoride ions accelerated the inactivation of the Ca^{2+} current and suppressed a persistent component activated at membrane potentials between −40 and −10 mV. Electrodes containing fluoride were also used to introduce the ion into neurons recorded from hippocampal slices. In these cells, intracellular fluoride suppressed Ca^{2+}-dependent regenerative depolarizations. Thus, when electrodes containing cesium fluoride, QX 314, and EGTA were used, nonlinear responses of the neuronal membrane were minimized. This facilitated an examination of some properties of the recurrent EPSPs.

Materials and Methods

Experiments were carried out using slices and acutely dissociated cells obtained from the hippocampus of adult guinea pigs weighing 200–400 g.

Preparation of isolated cells

The procedure for obtaining isolated cells from adult guinea pig hippocampus is similar to that described previously (Numann and Wong, 1984), with recent modifications (Kay and Wong, 1986) that increase the cell yield and quality. Transverse hippocampal slices 650 μm thick were prepared and dissected into segments of 1 mm long along the pyramidal cell layer. The tissue blocks were then placed inside a vial containing 10 ml of a HEPES-buffered solution with 6–8 mg of the enzyme trypsin (Sigma Type XI). Solution within the vial was maintained at 32°C under an atmosphere of 5% CO_2 in O_2. The composition of the solution was (in mM): NaCl, 130; KCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; *d*-glucose, 25; HEPES, 20. The tissue was stirred continuously with a magnetic stir bar. After 90 min, the solution in the vial was replaced with one without enzyme. The contents of the vial were continually stirred and allowed to come to room temperature.

Neurons were then prepared as needed by removing 2 tissue pieces

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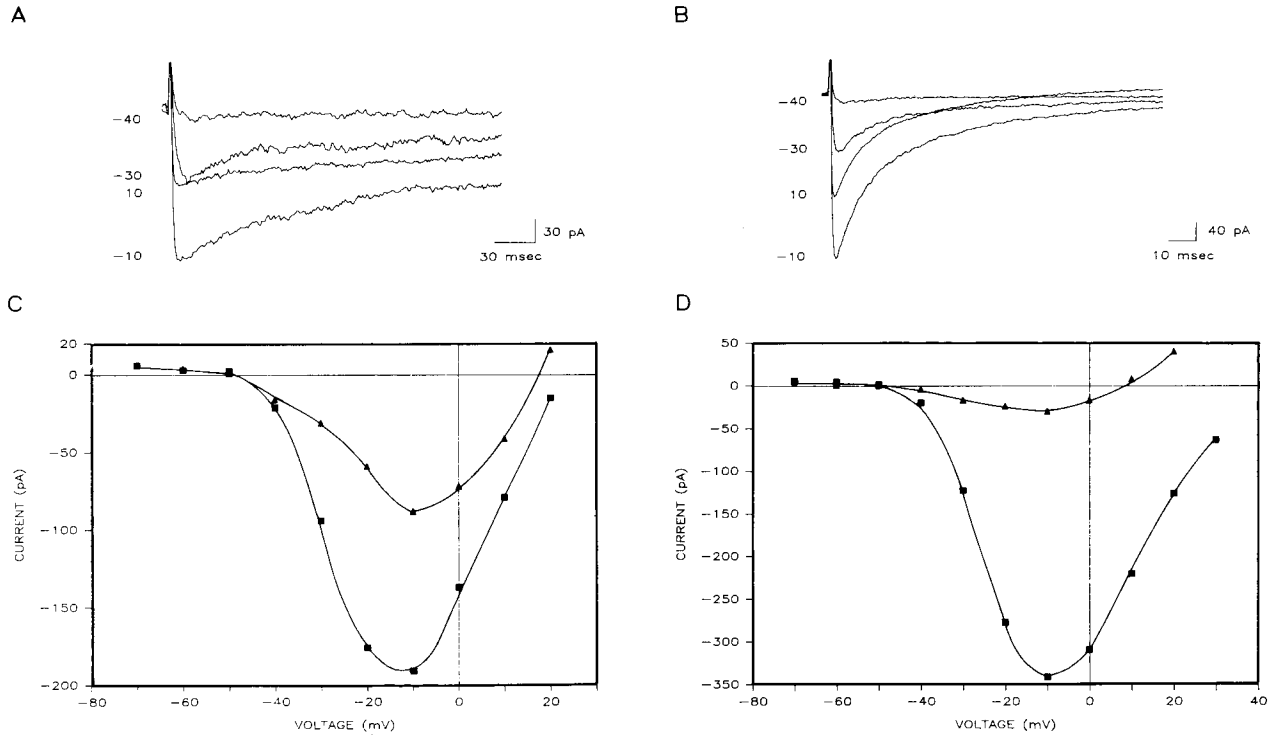

Figure 1. Intracellular fluoride alters the time course of calcium-dependent currents recorded in isolated hippocampal neurons. Whole-cell currents evoked by voltage steps from a holding potential of −80 mV to the potential shown, with electrodes containing Tris-phosphate (*A*) and cesium fluoride (*B*). Note difference in time calibration. Responses were obtained in the presence of 5 mM extracellular Ca^{2+} and were not corrected for leakage current. *C* and *D*, Current–voltage relations obtained from cells of *A* and *B*, respectively. *Squares* show peak current, and *triangles* show persistent current, measured at 250 msec for Tris-phosphate (*C*) and at 150 msec for Cs fluoride (*D*).

from the chamber and triturating them with fire-polished Pasteur pipettes having tip diameters close to 1 and 0.5 mm. Cells isolated in this way were allowed to settle in a tissue culture dish for about 15 min before electrophysiological recordings were attempted.

Electrophysiological recordings from isolated neurons

Recordings from isolated cells were made using electrodes pulled from WPI glass on a modified Kopf puller. Electrodes were filled with solutions containing either (in mM): (1) Trizma phosphate, 110; Trizma base, 28; EGTA, 11; $MgCl_2$, 2; $CaCl_2$, 0.5; or (2) CsF, 110; HEPES, 10; EGTA, 11; $MgCl_2$, 2; $CaCl_2$, 1. The pH of solutions used to fill recording electrodes was adjusted to 7.4, and their resistance was 3–5 MΩ. Cells were perfused with an extracellular solution containing (in mM): NaCl, 120; $MgCl_2$, 2; $CaCl_2$, 1–5; CsCl, 5; HEPES, 10; tetraethyl ammonium bromide (TEA), 20; and *d*-glucose, 25. Tetrodotoxin (TTX) was added (3 μM) to block Na currents.

A procedure similar to that described by Hamill et al. (1981) was used to obtain recordings of whole-cell currents with a patch-clamp amplifier (List). Current records were filtered with a low-pass filter at a cutoff frequency of 1 kHz. No compensation was applied for the series resistance of the electrode. This introduces errors ranging from 0.5 to 5 mV in membrane potential control for membrane currents between 0.1 and 1 nA. The series resistance also limits the rate of response of membrane potential to a change in command potential. For the range of membrane capacitance measured for hippocampal cells (25–75 pF) and an average electrode resistance of 5 MΩ, the time constant of the transmembrane voltage change following a step depolarizing command pulse was between 0.12 to 0.38 msec. Data were stored on a Racal tape recorder or on-line on an IBM PC, with data acquisition through a 12 bit A/D converter (Tekmar).

Preparation of hippocampal slices

Transverse hippocampal slices 400 μm thick were prepared with a Vibratome (Miles and Wong, 1984). They were then transferred to a recording chamber where they were supported on nylon mesh and exposed to a warm, moist 5% CO_2 in O_2 atmosphere and perfused with solution with the following composition (in mM): NaCl, 124; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1.6; $NaHCO_3$, 26; and *d*-glucose, 10. The pH of solution sampled from the chamber was 7.4, and its temperature was maintained at 37°C. In some experiments, picrotoxin (10^{-5} M) was added to suppress synaptic inhibition mediated by GABA.

Recordings from hippocampal slices

Electrodes used to record from neurons in hippocampal slices were pulled from fiber-filled glass capillaries. They were filled with one of the following solutions: (1) Cs acetate, 1 M; EGTA, 0.03 M; QX 314 (Astra Pharmaceuticals), 0.05 M; or (2) Cs fluoride, 0.3–0.5 M; EGTA, 0.03 M; QX 314, 0.05 M. The pH of the solutions was adjusted to 7.2 by adding KOH, and electrodes were beveled to a final resistance of 40–90 MΩ. Good recordings could be made only with fluoride-containing electrodes when EGTA was present. EGTA may act to prevent precipitation of CaF_2 at the electrode tip. The application of hyperpolarizing current immediately following cell penetration did not improve recordings made with these electrodes.

Intracellular potentials were amplified using a high input impedance amplifier, with the capacity for current injection through the recording electrode using an active bridge circuit (WPI M707). Bipolar tungsten electrodes of tip diameter 20 μm and separation 200 μm were used to stimulate fiber pathways with 0.1 msec electrical pulses. Recurrent synapses between CA2 and CA3 cells were selectively activated using a technique fully described elsewhere (Miles and Wong, 1986). Briefly, CA2 cells were activated antidromically by stimulating their axon collaterals in the stratum oriens of the CA1 field, and the resulting recurrent postsynaptic potentials were recorded in CA3 neurons. A cut was made between CA2 and CA3 extending from the edge of the slice to the distal edge of the mossy fiber layer to sever afferent pathways running in the stratum oriens of CA2–3. During an experiment, electrical signals were displayed on a digital oscilloscope (Nicolet 4562) and recorded with an FM tape recorder (Vetter). Permanent records were made with a chart recorder (Gould 2400).

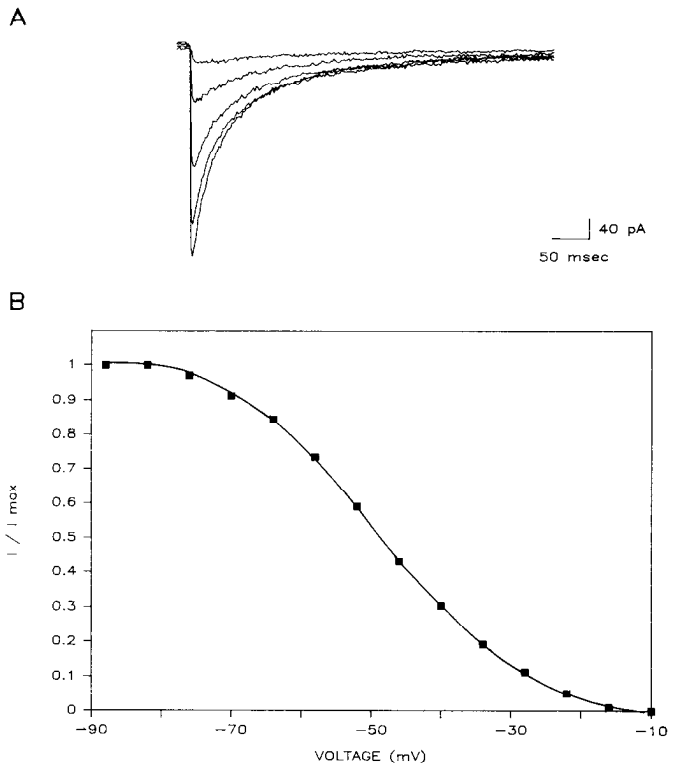

Figure 2. Steady-state inactivation of the calcium current in a cell perfused with fluoride. A conditioning membrane potential step in the range −90 to −10 mV was applied for 1 sec from a holding potential of −100 mV, and a test potential of −10 mV was then applied to generate the whole-cell currents shown in *A*. External Ca^{2+} was 5 mM. Prepulse potentials were, in order of decreasing current: −82, −64, −52, −40, and −28 mV. *B*, Normalized peak current plotted against prepulse potential.

Results

Effects of fluoride on the calcium current

Calcium currents of 2 hippocampal cells are shown in Figure 1. Traces shown in Figure 1*A* were obtained with a recording electrode containing Tris-phosphate and the traces in Figure 1*B* with an electrode containing Cs fluoride. Whole-cell calcium currents were measured in the presence of 3 μM TTX, to block Na currents, and 20 mM TEA and 5 mM Cs^+, to eliminate outward currents. The effectiveness of these blockers was confirmed by the absence of significant outward currents (less than 10 pA in the range −100 to 20 mV) when 2 mM cobalt was present in the extracellular solution. A contribution from a TTX-resistant Na^+ current (Kostyuk et al., 1981) was excluded by showing that the currents persisted with unaltered time course and amplitude when choline was substituted iso-osmotically for extracellular Na^+. Inward currents recorded in these conditions thus seem to have been carried solely by calcium ions.

The time course of the Ca-dependent inward current was significantly affected by the presence of intracellular fluoride. When Tris-phosphate was used, the mean time to peak of the inward current was 17.9 ± 4.9 msec (n = 8 cells) when a depolarizing pulse to −10 mV was applied from a holding potential of −80 mV. The current decayed slowly during the pulse; a reduction of 30% during a pulse of duration 250 msec was typical (Fig. 1*A*). The rate of inactivation depended on the voltage step and was most pronounced when larger currents were elicited. Calcium currents of similar time course were also recorded with electrodes containing Cs gluconate and Cs acetate. The current observed under these conditions was labile and tended to decline with time (see Fedulova et al., 1985; Forscher and Oxford, 1985). Disappearance of Ca^{2+} current was retarded by the addition of 2 mM ATP to the solution in the recording electrode. In the presence of ATP, currents were maintained for over 60 min.

The time course of inactivation of calcium currents recorded with electrodes containing Cs fluoride was significantly faster (Fig. 1*B*). Following a depolarizing pulse from −80 to −10 mV, the mean time to peak of the inward current was 3.8 ± 0.9 msec (n = 10 cells), and the current inactivated with an average half-time of 30 msec. However, fluoride appeared not to affect the threshold for activation of calcium currents. The threshold was −49.1 ± 6.7 mV (n = 10 cells) in the presence of fluoride, and the threshold was 45.4 ± 7.4 mV (n = 8 cells) when Tris-phosphate-containing electrodes were used. The peak current recorded with Cs fluoride electrodes was 432 ± 93 pA (n = 10), and the peak current observed with Tris-phosphate electrodes was 502 ± 186 pA (n = 8).

The "steady-state" inactivation of the calcium current in the presence of fluoride was examined by application of conditioning voltage prepulses in the range −90 to −10 mV for 1 sec followed by a test pulse to −10 mV. Figure 2*A* shows the residual inward current activated by such test pulses in one cell. The peak current declined monotonically with increasingly depolarized conditioning pulses. Inactivation curves were derived from these data (Fig. 2*B*). In 4 cells, half-maximal inactivation occurred at a mean potential of −53 ± 8 mV.

These results suggest that calcium currents in hippocampal

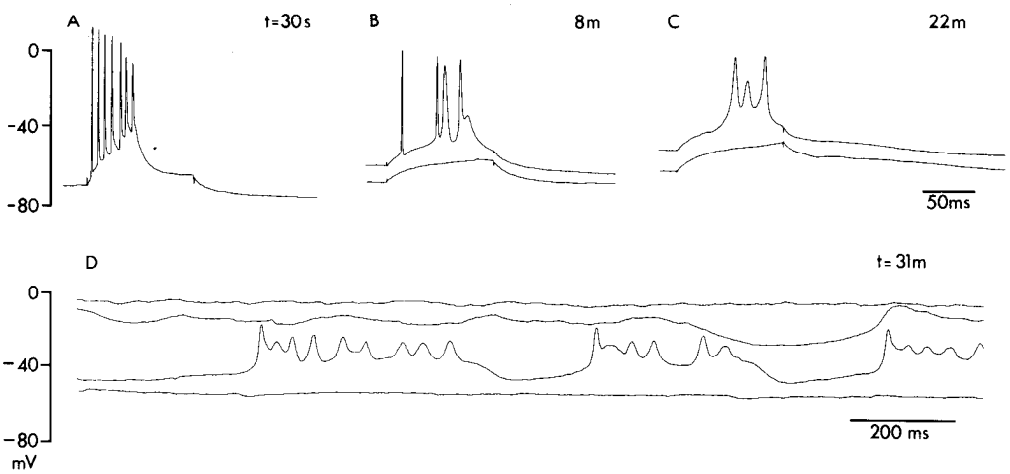

Figure 3. Intracellular recordings from a CA3 neuron in a hippocampal slice made with an electrode containing 2 M Cs acetate, 0.05 M QX 314, and 0.03 M EGTA. *A–C*, Responses to depolarizing current injections of constant amplitude and 100 msec duration at various times following penetration. Maintained depolarizing current was applied in *B* and *C* in order to determine the threshold for active responses. After 22 min, only broadened action potentials could be evoked, and they were succeeded by an after-depolarization. *D*, Spontaneous activity recorded at various levels of depolarization after 31 min. Slow membrane potential oscillations occurred between −50 and −10 mV.

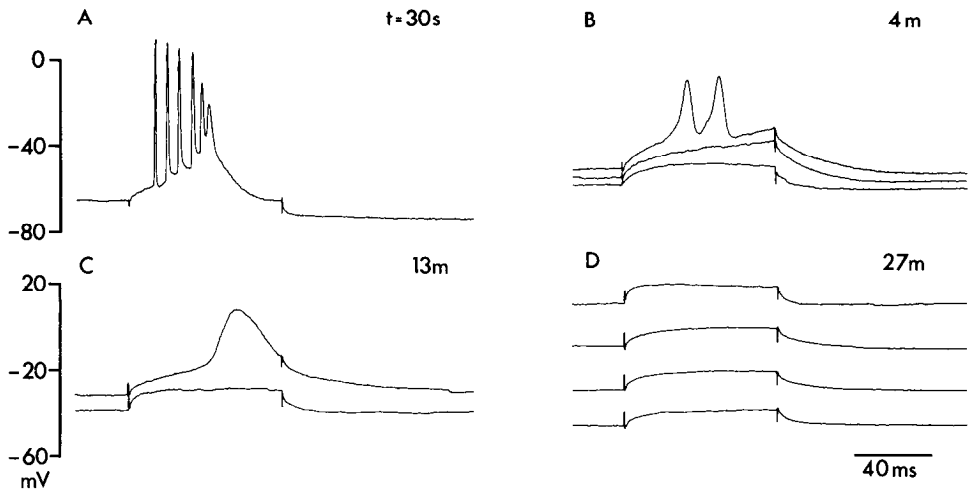

Figure 4. Intracellular recordings made from a CA3 cell with an electrode containing 0.5 M Cs fluoride, 0.05 M QX 314, and 0.03 M EGTA. *A–D,* Responses to depolarizing current injections of constant intensity and duration at various times following penetration. Membrane potential was varied by maintained current injection. After 4 min, only broadened action potentials were evoked (*B*), and no regenerative responses could be elicited after 27 min (*D*).

neurons are not totally blocked by intracellular fluoride, in contrast to a previous observation that fluoride completely suppressed the Ca^{2+} current of snail neurons (Kostyuk and Krishtal, 1977). However, a persistent component of the current was suppressed. It seemed possible that intracellular fluoride might be used to block slow Ca-dependent responses of hippocampal neurons in the slice preparation especially in a depolarized potential range (above −40 mV). The next section describes experiments to test this possibility.

Effect of fluoride on regenerative activity in neurons of the slice

A higher concentration of all agents was required to affect the membrane properties of neurons in the slice. Furthermore, a recording time of 15–30 min was needed, presumably for intracellular equilibration, before complete effects were achieved. In contrast, the effects of agents introduced into isolated cells via suction pipettes appeared to reach a steady state within 3 min. Both the higher resistance of electrodes used in slice recordings and the simplified morphology of isolated cells seem likely to contribute to this difference.

Regenerative activity dependent on Ca^{2+} was first characterized in recordings made from 13 cells with electrodes containing Cs acetate (2 M), QX 314 (50 mM), and EGTA (30 mM). Figure 3 shows a sequence of records made from a CA3 neuron with such an electrode. Immediately on penetration the membrane potential was −68 mV. When depolarized to threshold, the cell fired a burst of 3–8 action potentials riding on a slow depolarization that was followed by an afterhyperpolarization lasting up to 2 sec (Fig. 3*A*). The first change in firing pattern was that the afterhyperpolarization following a burst was suppressed. As the recording was maintained, rapidly rising action potentials could no longer be elicited from resting potential. However, broadened action potentials (duration, 5 msec) were still evoked and were succeeded by a sustained depolarization rather than a hyperpolarization (Fig. 3*C*). Neuronal activity typically observed in recordings of duration longer than 20 min is shown in Figure 3*D*. At membrane potentials more hyperpolarized than −50 mV or more depolarized than −10 mV, no regenerative activity was observed. Between these potentials, rhythmic membrane oscillations consisting of plateau-like depolarizations followed by slow repolarizations were observed. These plateau potentials seem likely to have been sustained by the calcium currents observed in experiments on isolated cells.

In an attempt to suppress activity mediated by persistent Ca currents, recordings were made from 11 cells with electrodes containing QX 314 and EGTA but with 0.3–0.5 M CsF substituted for Cs acetate. Figure 4, *A–D,* shows a sequence of changes in membrane properties during a recording. The initial membrane potential was −62 mV, and the threshold for burst firing was −55 mV (Fig. 4*A*). During recordings with CsF-containing electrodes, both the duration and threshold of action potentials gradually increased (Fig. 4, *B, C*). After 25 min regenerative activity could no longer be elicited in this cell (Fig. 4*D*). These observations suggest that intracellular fluoride can effectively suppress slow membrane oscillations that occur at potentials between −50 and −10 mV. This action of fluoride apparently results from its effect on the persistent calcium current shown in Figure 1*A*. Input resistances measured at depolarized membrane potentials were in the range 8–17 MΩ ($n = 9$ cells). These values are lower than those measured at resting potential in CA3 cells recorded with electrodes containing K-acetate (Wong and Prince, 1981). The incomplete suppression, by Cs^+, of K^+ conductances, which would be activated at depolarized potentials, might tend to reduce input resistance.

EPSPs recorded in the presence of intracellular fluoride

In the presence of picrotoxin, the CA3 neuronal population discharges synchronously. Intracellular events reflect a complex interaction between the intrinsic bursting capability of pyramidal cells (Wong and Prince, 1981) and powerful synaptic actions mediated by recurrent excitatory synapses between them (Miles and Wong, 1983). The form of summed EPSPs impinging on a neuron from many simultaneously active presynaptic cells is masked by voltage-dependent responses triggered by the synaptic depolarization.

We examined some properties of summed EPSPs associated with synchronous discharges by making recordings with electrodes containing CsF, QX 314, and EGTA, so that active responses were largely suppressed. Figure 5*A* shows responses to selective stimulation of recurrent excitatory synapses (Miles and Wong, 1986), obtained 25 min after penetration of a CA3 cell. The resulting EPSPs were succeeded at longer latency by the synchronized discharge of the CA3 neuronal population, as indicated by the records of the extracellular field potential. The amplitude of summed EPSPs occurring simultaneously with the field discharge was dependent on membrane potential (Fig. 5*A*). In 7 cells the reversal potential for the synaptic event varied between −2 and 9 mV. Depolarizing pulses were applied at each potential to show that active responses were not evoked and to ensure that potential was accurately measured. Figure 5*B* shows the relationship between peak amplitude of summed EPSP and membrane potential. The reversal potential of the synchronized event in this cell was found by linear regression to be 5 mV. The somatic conductance change due to the summed EPSP was derived from the slope of the relationship and a mean value of 12 MΩ for the neuronal input resistance (Ginsborg, 1973). In

this cell, the mean value for neuronal input resistance over the range −40 to +40 mV was 12 MΩ. The peak conductance change for summed EPSP in this cell was 258 nS, and in 6 other cells values between 78 and 203 nS were calculated.

Discussion

This study has shown that intracellular fluoride alters the time course of calcium current recorded from isolated hippocampal neurons. The rate of inactivation of the current was accelerated, and no persistent component was observed. Furthermore, conditioning depolarizations to levels more depolarized than −40 mV for 1 sec resulted in substantial inactivation of the remaining current. These actions of intracellular fluoride may account for its effects on neurons in the hippocampal slice. The absence of slow membrane oscillations at potentials in a depolarizing range from −40 mV allowed several properties of EPSPs to be examined systematically.

Intracellular fluoride has been reported to suppress completely the calcium current of molluscan neurons by an action on gating mechanisms (Kostyuk and Krishtal, 1977). The actions of fluoride described here appear not to conform to these observations. If hippocampal neurons possess a single calcium current, it would appear that fluoride acted to accelerate both its activation and inactivation kinetics. Pharmacological agents have been shown to modify the inactivation properties of several types of voltage-dependent channels: For instance, batrachotoxin action on Na^+ channels (Narahashi et al., 1971) and effects of TEA on K^+ channels (Armstrong, 1969).

However, there is now evidence at the single-channel level that more than one type of calcium channel may exist in some mammalian neurons (Carbone and Lux, 1984; Nowycky et al., 1985). In dorsal root ganglion cells, 3 types of channels have been distinguished on the basis of differences in their threshold and inactivation properties. Some aspects of calcium currents recorded in fluoride resemble those of the "N" channel described by Nowycky et al. (1985). Inactivation was only completely removed at potentials more negative than −80 mV, and strong depolarizations were needed to activate the current. It is possible that 2 types of calcium channel may exist in hippocampal cells; one that inactivates slowly, as described in a number of previous studies (Brown and Griffith, 1983; Johnston et al., 1980) and one that inactivates faster (Halliwell, 1983). Intracellular fluoride might act to suppress a persistent channel while a rapidly inactivating channel remained unaffected. However, this mode of action cannot easily account for the observation that the peak current of cells recorded with fluoride (432 ± 93 pA) was of similar magnitude to that in cells recorded with Tris-phosphate electrodes (502 ± 186 pA). It is likely that fluoride increases the numbers of a calcium channel that was minimally activated under normal conditions. Further support for this hypothesis comes from the observation that the time to peak of currents recorded with intracellular fluoride was consistently faster than those recorded with Tris-phosphate electrodes (Fig. 1, *A, B*). Such a mechanism has been invoked to explain the enhancement of calcium current by norepinephrine in heart muscle (Bean et al., 1984).

Although we have not clarified the exact mechanisms of fluoride action, it apparently blocks a persistent calcium current, leaving a component that is largely inactivated at potentials more positive than −40 mV. Results obtained from neurons in the slice confirm the voltage-clamp observations on isolated neurons. Excitatory synaptic events may therefore be examined in this depolarized potential range, close to their reversal potential and in the apparent absence of regenerative neuronal responses. Following the injection of fluoride and QX 314, the shape of EPSPs should depend only on the location of the synaptic terminals and the cable properties of the postsynaptic cell.

There was no indication that EPSPs were significantly affected by the use of intracellular fluoride. Reversal potentials for summed EPSPs associated with synchronized events were close to 0 mV, similar to those determined in the absence of fluoride (Johnston and Brown, 1984). In addition, values for the somatic conductance changes associated with these events approximate those derived from voltage-clamp studies on CA3 neurons (Johnston and Brown, 1984). The intracellular application of fluoride may therefore be a useful technique in the investigation of some aspects of excitatory synaptic transmission.

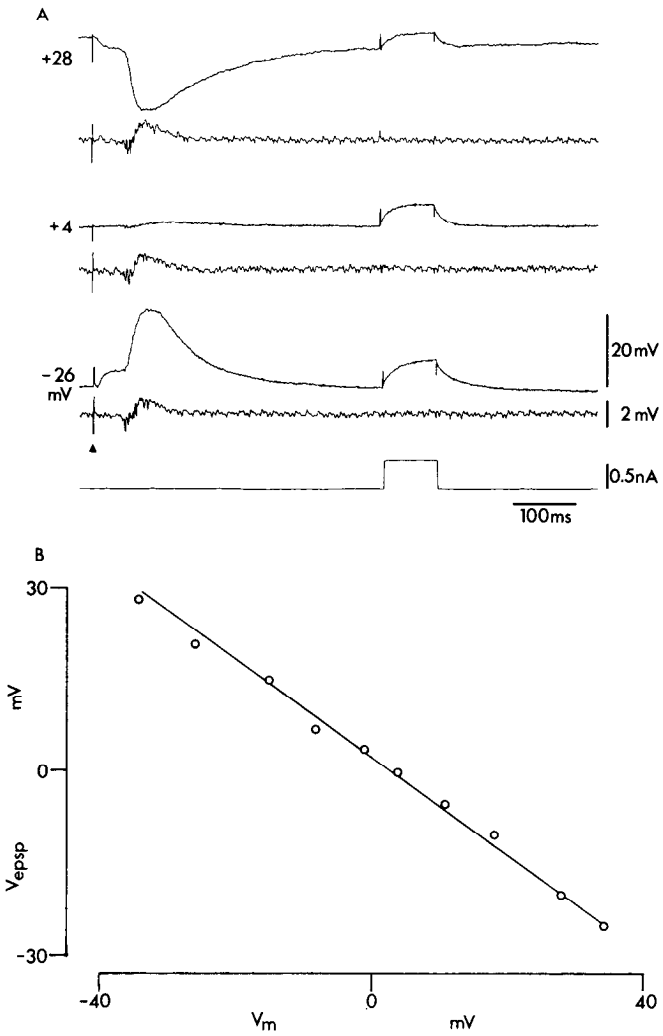

Figure 5. Synaptic potentials associated with synchronized population discharges induced on exposure to 10^{-5} M picrotoxin. *A,* Intracellular recordings (*upper traces*) made 25 min after penetration of a CA3 neuron with an electrode containing 0.5 M Cs fluoride, 0.05 M QX 314, and 0.03 M EGTA. Extracellular field shown by *lower traces.* A constant intracellular depolarizing current pulse (*bottom trace*) was applied. Stratum oriens in the CA1 region was stimulated to selectively activate excitatory synapses between CA3 cells. The resulting EPSP was followed with variable latency (40–100 msec) by a larger EPSP associated with the synchronous population discharge. *B,* Amplitude of the synchronized EPSP plotted against membrane potential. The synaptic event reversed at +5 mV.

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