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ANALYSIS OF MECHANISMS OF SPIKING IN NORMALLY 'NON-SPIKING' MOTONEURONE SOMATA IN CRAYFISH

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Summary

The soma membrane of the abdominal giant motor neurone (MoG) of the crayfish was studied by use of current injection and by measurements of total and local membrane current under voltage-clamp. Depolarization of the soma from the recorded resting potential (about −70 mV) did not produce a regenerative potential. Sodium and calcium currents were observed under voltage-clamp. It was shown that the sodium spike cannot develop at the usual resting potential since sodium channels are already partially inactivated; the spike can appear after the removal of inactivation by a hyperpolarizing prepulse. It was also shown that an early outward current prevents the Ca^{2+} regenerative potential; the potential can appear after inactivation of this outward current by a depolarizing prepulse.

Introduction

Many arthropod neuronal somata do not produce action potentials. In these non-spiking neuronal somata, it has been shown that extracellular tetraethylammonium (TEA^+) or 3-aminopyridine (3-AP), as well as intracellular EGTA or citrate which reduces the intracellular free calcium concentration, transform a graded response to depolarization into an all-or-none action potential. These regenerative responses are either Ca^{2+}-dependent (Pitman, 1979, in cockroach; Kuwada, 1981, in crayfish) or depend on both Na^+ and Ca^{2+} (Goodman & Heitler, 1979, in locust).

A voltage-dependent transformation from a non-spiking to a spiking state has also been observed in arthropods. The 'non-spiking' visual interneurones of the fly will generate spikes in response to a pattern-movement stimulation if the cells are hyperpolarized (Hengstenberg, 1977). In an interneurone from the lobster commissural ganglion, which shows spontaneous oscillations, spikes appear only between membrane potential values of −30 and −60 mV (Robertson & Moulin,

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1981). The ionic mechanisms of the regenerative potentials were not studied in either of these cases.

The results cited above show that at least some seemingly inexcitable neurones do have channels capable of producing regenerative depolarizing potentials. The experiments with TEA^+ and 4-AP show that the K^+ channels are principally responsible for preventing the regenerative potential changes that are mediated by the Na^+ and Ca^{2+} channels.

The soma of the motor giant (MoG) of the crayfish *Procambarus clarkii* is also non-spiking (Takeda & Kennedy, 1964; Selverston & Remler, 1972; Wine & Mistick, 1977; Kuwada & Wine, 1981). In previous papers (Czternasty & Bruner, 1981; Czternasty *et al.* 1984; Bruner *et al.* 1986) we have shown that a sodium action potential can be induced in this soma after a conditioning hyperpolarization, and a calcium spike can be produced after a depolarization. The present paper analyses the mechanisms responsible for the demasking of the inward conductances and characterizes certain properties of the sodium and calcium currents carried by these conductances in this cell.

Abstracts containing preliminary work have already been published (Czternasty *et al.* 1982*a*,*b*; Czternasty, 1985).

Materials and methods

Experiments were performed upon giant motoneurones (MoG) of the second, third or fourth abdominal ganglion of crayfish *Procambarus clarkii*. Animals, measuring 5–10 cm in length from rostrum to telson and of either sex, were shipped by air from Carolina Biological Supply (USA) and maintained in aerated water tanks for up to 3 months.

Crayfish were anaesthetized by cooling and the abdomen was removed and submerged in cold (5–10 °C) saline. The abdominal chain of six ganglia was then isolated by cutting all roots and removing the main artery and excess connective tissue. The preparation was pinned to Rhodorsil (Rhone-Poulenc) bottomed chamber of 1 ml. The chamber was continuously superfused with saline at about 5 ml min^{-1}. The normal saline (NS) had the composition (in mmol l^{-1}): NaCl, 195; KCl, 5·4; $CaCl_2$, 13; $MgCl_2$, 2·6; Tris–maleate NaOH, 10 (pH 7·2). In more recent experiments a solution containing Hepes buffer was used (in mmol l^{-1}): NaCl, 207·5; KCl, 5·4; $CaCl_2$, 13·5; $MgCl_2$, 5·3; Hepes, 5·7; (Kawagoe *et al.* 1981). No difference was found between results obtained in these solutions. '$0Na^+$' solution was made by replacing NaCl with equimolar choline chloride (Merck); in reduced-Ca^{2+} saline calcium chloride was replaced by magnesium chloride. Solutions containing tetrodotoxin (TTX, Calbiochem) were made by adding stock solution (1 mg TTX in 1 ml H_2O) to NS. Tetraethylammonium (TEA^+) experiments were performed either by ionophoretically injecting TEA^+ into the cell from a micropipette filled with 0·5 mol l^{-1} TEA–Br (Koch & Light; usually 20–25 m injection with 0·5 s, 100 nA pulses at 1 Hz were necessary before the cell started to

show the action potential) or by substitution of TEA–Cl for some of the NaCl in the bathing solution.

The MoG soma was exposed by removing the overlying sheath, and was impaled with voltage-recording and current-passing electrodes filled with 3 mol l^{-1} potassium chloride. The recording electrode was connected to a high input-impedance, unity gain d.c. amplifier. The output of the amplifier was displayed on a storage oscilloscope (Tektronix 5301N) and filmed (Nihon-Kohden camera). Intrasomatic current pulses to polarize the membrane were delivered from a constant-current generator and were monitored *via* the Ag/AgCl bath reference electrode with a standard current-to-voltage converter using an operational amplifier with high input impedance.

In voltage-clamp experiments the resistance of the potential recording electrode was 5–8 MΩ and it was 1–4 MΩ for the current electrode. Both electrodes were shielded to reduce capacitative coupling. Local membrane currents were recorded with a pair of extracellular pipette electrodes which measured the extracellular voltage drop. Their tips were displaced lengthwise so that one was about 400 μm behind the other and they were placed at different regions of the soma, perpendicular to the cell surface (Kado, 1973). Local currents recorded at the soma showed that the soma still had a capacitative current when the recorded intracellular potential was flat (see Fig. 3). This result indicates that at least some of the soma membrane was not fully at the command level for as much as 600 μs after the voltage step.

All current–voltage plots (I/V) were corrected for leakage current. Small hyperpolarizing pulses of different amplitudes were injected into each cell studied in voltage-clamp and the I/V plots for these pulses were extrapolated on a straight line to depolarizing values. The leakage current was seen to be linear for depolarizing potentials when the voltage-dependent inward channels and much of the outward channels were blocked.

Experiments were performed all year round. The temperature of the saline was 15–18°C, with a variation of less than 1°C in the course of a single experiment.

Results are expressed as means ± S.E. unless marked otherwise.

Results

The resting membrane

Motor giant (MoG) somata can be reliably identified from their size (120–150 μm in diameter) and position in the ganglion (Takeda & Kennedy, 1964; Wine & Mistick, 1977). The mean input resistance of the soma determined near the resting potential (about −65 to −75 mV) was 553 ± 56 kΩ (21 cells), close to the previously reported value of 600 kΩ (Takeda & Kennedy, 1964) and similar to that for the synaptic area of the motor giant axon (Furshpan & Potter, 1959).

Mean specific membrane capacitance was 8·9 ± 0·5 μF cm^{-2} (11 cells) calculated from the input resistance and the time constant (4·9 ± 0·8 ms, $N = 11$) and taking the soma diameter to be 150 μm. Analysis of the capacitative current obtained

using low-amplitude voltage-clamp pulses showed that this current was described by the sum of two exponential terms, the values of which were 0·25–0·47 ms and 4·1–12 ms (five cells). Both these results suggest that the cell membrane is considerably infolded. This was confirmed ultrastructurally (Cuadras *et al.* 1985).

The membrane potential varied with external K^+ concentration with a slope of about 50 mV per 10-fold change in $[K^+]_o$ (measured between 5·4 and 100 mmol l^{-1} K^+ in eight cells). The relationship did not change in the presence of 10^{-4} mol l^{-1} ouabain. This indicates that the resting cell membrane had a relatively low permeability to ions other than potassium and that an electrogenic pump did not contribute to the resting potential.

Sodium conductance in MoG somata

When depolarized from rest with short constant-current pulses, the membrane potential was changed at the time constant of the membrane. The rise time of this response was very rapid, in spite of the capacitance of the membrane, because of the low input resistance of the cell. A rapid fall in the potential then took place, giving the initial phase of the depolarization a spike-like appearance (Fig. 1Ai). Since the rise time of the depolarization follows the membrane time constant, regardless of the amplitude of the depolarization, this initial phase is not likely to be due to a regenerative process. The spike-like appearance is more likely to be the result of an increase in a delayed repolarizing conductance which began with the depolarization and required several milliseconds to reach its plateau value.

When the membrane potential was shifted to −84 mV (Fig. 1Aii,Aiii), the input resistance of the cell increased by about twofold. This increased the membrane time constant, slowing the depolarization produced by the current pulse, and led, after some delay, to a regenerative spike. The mean threshold for evoking the spike (starting from around −90 mV) was $-32 \pm 3{\cdot}5$ mV (S.D.) and the mean overshoot was $10 \pm 5{\cdot}9$ mV (S.D.) (in 25 cells). The mean spike duration at half amplitude was 0·31 ms.

When the normal saline was changed to $0Na^+$ saline the spike was abolished (in eight cells). This effect was not due to a shift of the spike threshold, since larger depolarizing pulses were also ineffective. No noticeable change in cell input resistance was observed. Normal saline containing 10^{-8} mol l^{-1} TTX blocked the spike and the effect was only partially reversed. Thus this spike was due to voltage-gated, TTX-sensitive Na^+ channels (Hartung & Rathmayer, 1985).

In voltage-clamp experiments with a holding potential (V_H) near the resting potential (e.g. −70 mV) or more negative (Fig. 1Bi) a fast inward current ($I_{In,f}$) was obtained in all cells on step depolarization. A large outward current was also rapidly established by the depolarizations, which tended to mask the inward current (Fig. 1Bi,ii). When V_H was moved to −50 mV an inactivation of the inward and the outward currents were observed (Fig. 1Biii).

$I_{In,f}$ was activated at around −40 mV (see also Hartung & Rathmayer, 1985). It was maximal at around −20 mV and the shortest time to peak was less than 1 m[illegible] The current became outward at around +25 mV (Fig. 1C).

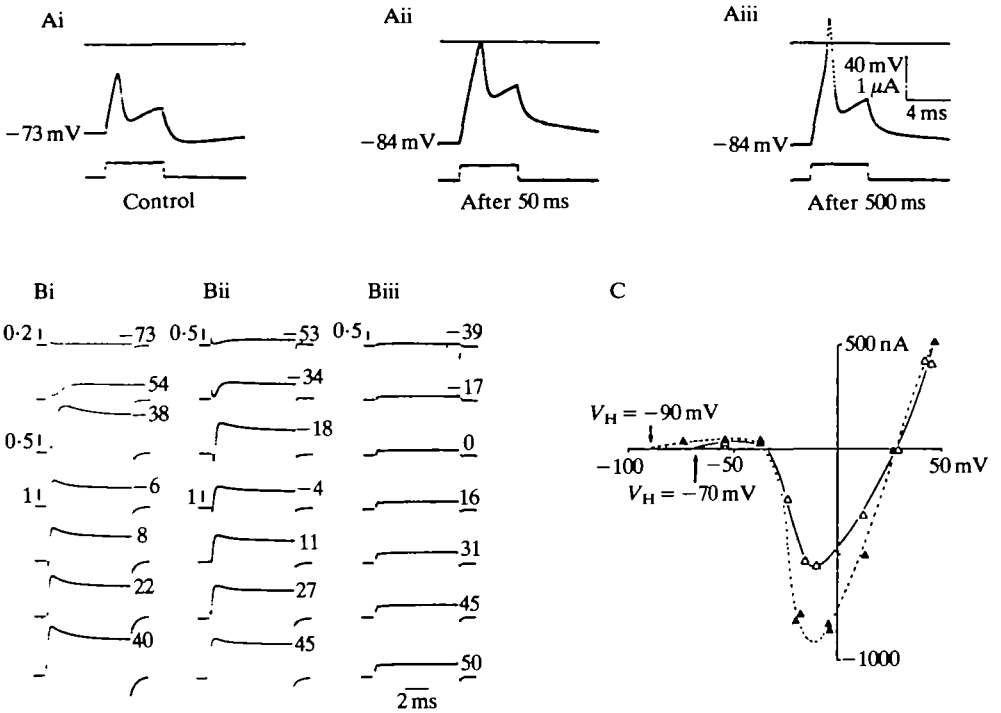

Fig. 1. Electrical properties of the motor giant soma in normal solution. (A) Depolarizing pulses applied from resting potential (Ai) and after 50 ms (Aii) and 500 ms (Aiii) holding at −84 mV. A fast action potential was provoked in Aiii but not in Ai or Aii. (B) Current traces from another cell for voltage-clamp steps to the level shown on the right of each trace from holding potentials of −90 mV (Bi), −70 mV (Bii) and −50 mV (Biii). Calibration (in μA) is on the left of the corresponding records. Note that the fast inward current and the outward current were diminished when V_H was shifted to less negative values. (C) Current–voltage relationship for another cell at two different holding potentials (as indicated in the figure). Current was measured 0·5 ms after the onset of the test pulse.

These results suggest that in current-clamp experiments the sodium spike was evoked because the hyperpolarization removed Na^+ channel inactivation. This was confirmed in experiments on the voltage dependence of the $I_{In,f}$ inactivation.

Inactivation of the sodium current

From a V_H of −95 mV a 10 ms depolarizing prepulse of variable amplitude was applied before a 4 ms test pulse to −38 mV (Fig. 2A). The level of the test pulse was chosen to activate the delayed outward current as little as possible (see Fig. 4C). In these experiments the early outward current was blocked by 2 mmol l^{-1} 4-aminopyridine (4-AP) (see below) and the Ca^{2+} inward current by 2 mmol l^{-1} $CdCl_2$.

The steady-state inactivation curve showed that the peak $I_{In,f}$ did not change for prepulse potentials more negative than about −90 mV. In the range of cell resting

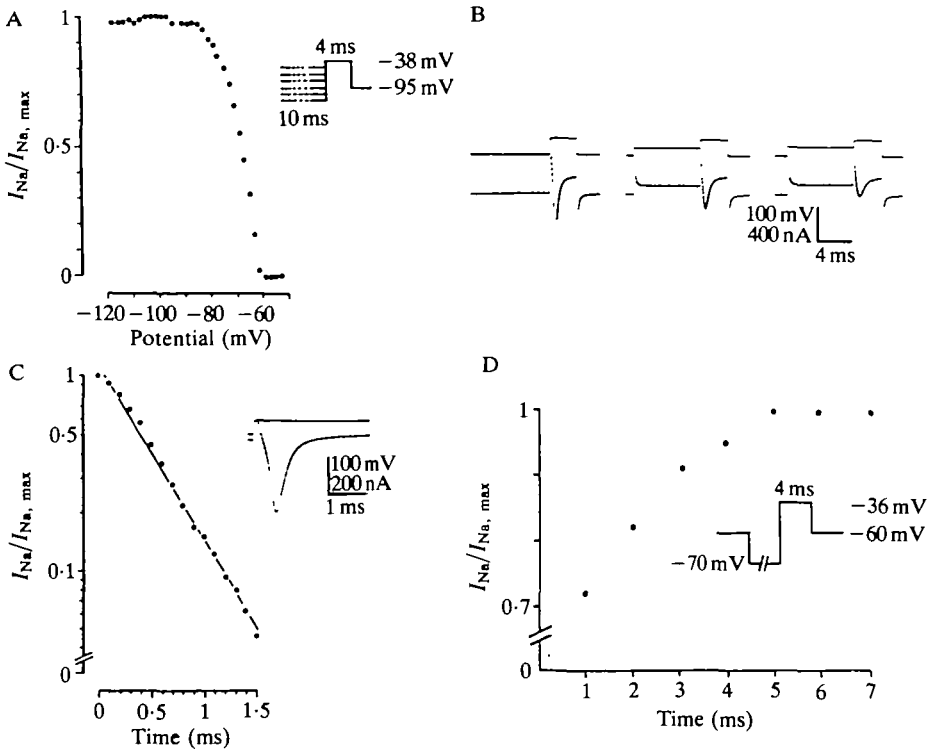

Fig. 2. Sodium current inactivation and recovery from inactivation. The bath solution contained 2 mmol l^{-1} $CdCl_2$ (substituting for $CaCl_2$) and 2 mmol l^{-1} 4-AP. (A) Steady-state inactivation of Na^+ current. The curve was constructed from current records such as those shown in B. Test pulses (to −38 mV for 4 ms) were preceded by 10 ms conditioning pulses to the different membrane potentials (see inset in A) from a V_H of −95 mV. (C) Semilogarithmic plot of the current decay (inset shows an example of a current record) during a 4 ms clamp pulse to −44 mV (V_H = −90 mV). (D) Time course of removal of resting inactivation with conditioning hyperpolarization. The cell was held at −60 mV and stepped to −70 mV for variable periods (shown on abscissa) before a test pulse (4 ms to −36 mV, see inset) was applied.

potential (−65 to −75 mV) the peak $I_{In,f}$ was substantially reduced: the current was half-minimum at around −70 mV for the curve shown in Fig. 2A. Similar results were obtained in three other cells.

Prolongation of the prepulse duration to 600 ms did not modify the inactivation curve. However, the slow variety of the inactivation process, if present in the MoG somata (cf. Simoncini & Stühmer, 1987), was not studied systematically in the present work.

The time course of Na^+ current inactivation was tested with a clamp step to −44 mV from a V_H of −90 mV. The inward current decayed very nearly as a single exponential with a time constant of 1·3 ms (Fig. 2C). Voltage dependence of the time constant of inactivation was not further studied in the present work.

The time course of recovery from inactivation was examined by setting V_H at

−60 mV, applying the de-inactivating prepulses to −70 mV for variable times and then stepping to −36 mV to test the inward current. The deinactivation was about 70 % completed by 1 ms at −70 mV and was 100 % complete between 4 and 5 ms. This rate of recovery of activation of the Na^+ current is very fast and comparable to that obtained for the squid giant axon (Hodgkin *et al.* 1952). Deactivation by hyperpolarization was not examined for holding potentials less negative than −60 mV. However, this result showed that at the normally recorded resting potentials, around −60 mV, some of the sodium channels were inactivated. A conditioning hyperpolarization would then be necessary to obtain a Na^+-dependent action potential (see Fig. 1A).

Somatic localization of the sodium current

Since our experiments were not performed on isolated somata, it was necessary to localize $I_{In,f}$. Simultaneous transmembrane and extracellular current recordings were performed which showed that $I_{In,f}$ could be detected in several places close to the soma membrane (Fig. 3B), even in areas opposite the exit of the axon. Perfusion of the preparation with saline containing 10^{-8} mol l^{-1} TTX blocked the fast current in both records in less than 2 min. These effects were also observed in two other cells. Finally, in eight other cells, $I_{In,f}$ could not be obtained when the NaCl in the saline was replaced with choline chloride. In both cases, the slow inward Ca^{2+} current (see below) was not reduced.

In two cells, delayed inward clamp currents on 'notches' were observed which shifted towards the beginning of the voltage step as the depolarization was increased. They were not recorded by the extracellular electrodes at the soma (Fig. 3A) and were not blocked by TTX at concentrations which eliminated the somatic $I_{In,f}$ (Fig. 3C). These observations suggested that the inward current notches were due to spikes triggered in the neurite deep in the neuropile. It should be noted that there is a diffusion barrier between the perfusion fluid and the neuropile of the crayfish abdominal ganglion (Zucker, 1972; Brown & Sherwood, 1981).

The early outward current

It has been shown in a previous paper (Shimahara *et al.* 1986) that transformation of the non-spiking soma into a Ca^{2+}-dependent spiking one necessitates the reduction of the outward current, for example by a pre-depolarization to −50 mV, and cannot be explained by facilitation of a Ca^{2+} conductance *per se*. Moreover, in the present work we have observed (see Fig. 1B) that the depolarizing pulses activated a very fast outward current which was strongly reduced when V_H was held at −50 mV. These observations suggested that the early, voltage-dependent outward current (also known as the A-current and first demonstrated by Hagiwara & Saito, 1959; see also Rogawski, 1985) is present in the MoG soma and that inactivation of this conductance could switch the MoG from a non-spiking to a Ca^{2+}-dependent spiking soma. This was confirmed in the following experiments.

The early outward current showed a very rapid activation (time to peak less than

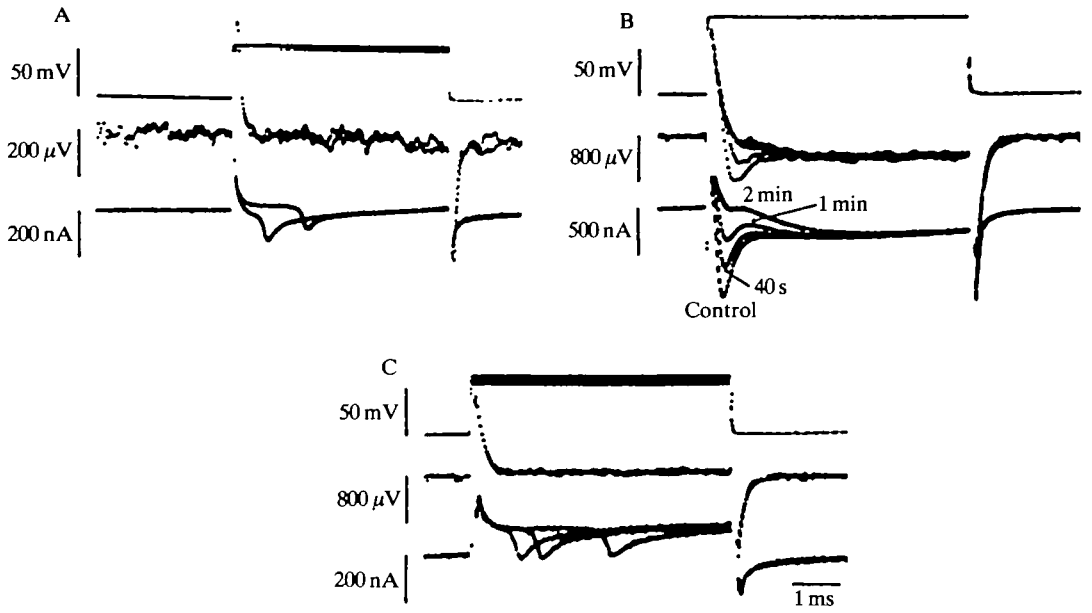

Fig. 3. Simultaneous recordings of membrane potential (upper trace), local (middle trace) and total (lower trace) current of a voltage-clamped cell, demonstrating the somatic fast and slow inward current (TEA^+-injected cell, $V_H = -90$ mV). (A) At low-amplitude depolarizing pulses a small inward current, observed only in the total current record, is probably due to a regenerative potential in a poorly clamped part of the neurite. Note that this current appeared in an all-or-none fashion and shifted to the left when the test pulse amplitude was increased (see also C). (B) Stronger depolarization activated a two-phase inward current, fast and slow (control), present in both current records. 10^{-8} mol l^{-1} TTX was then added which, in 2 min, blocked the fast component. The slow component persisted in both records. (C) Weaker depolarizations applied in the presence of TTX show that the current of neurite origin was resistant to this drug.

5 ms) and an increase in current amplitude when activated from a V_H between -70 and -90 mV (Fig. 4Ai,Bi). When 1 mmol l^{-1} 4-AP was added to the perfusing solution the early current was eliminated (Fig. 4Aii,Bii).

The activation potential for the early outward current was determined from I/V plots for currents measured at different times following the step depolarizations from a V_H of -90 mV. Since the early outward current showed a peak at about 2 ms, I/V curves were made for the currents at 2 ms and at 30 ms. The currents were diminished by about 10-fold by 4-AP at 0 mV and even more when TEA^+ and 4-AP were applied together (Fig. 4C).

Whereas in the absence of blockers, the outward current was activated at about -60 mV, in the presence of 4-AP the outward current was activated at about -30 mV. Addition of TEA^+ further decreased the outward current but did not influence the activation potential, which was more clearly seen in the I/V curve at 30 ms (Fig. 4Cii). The outward current was not completely suppressed by the

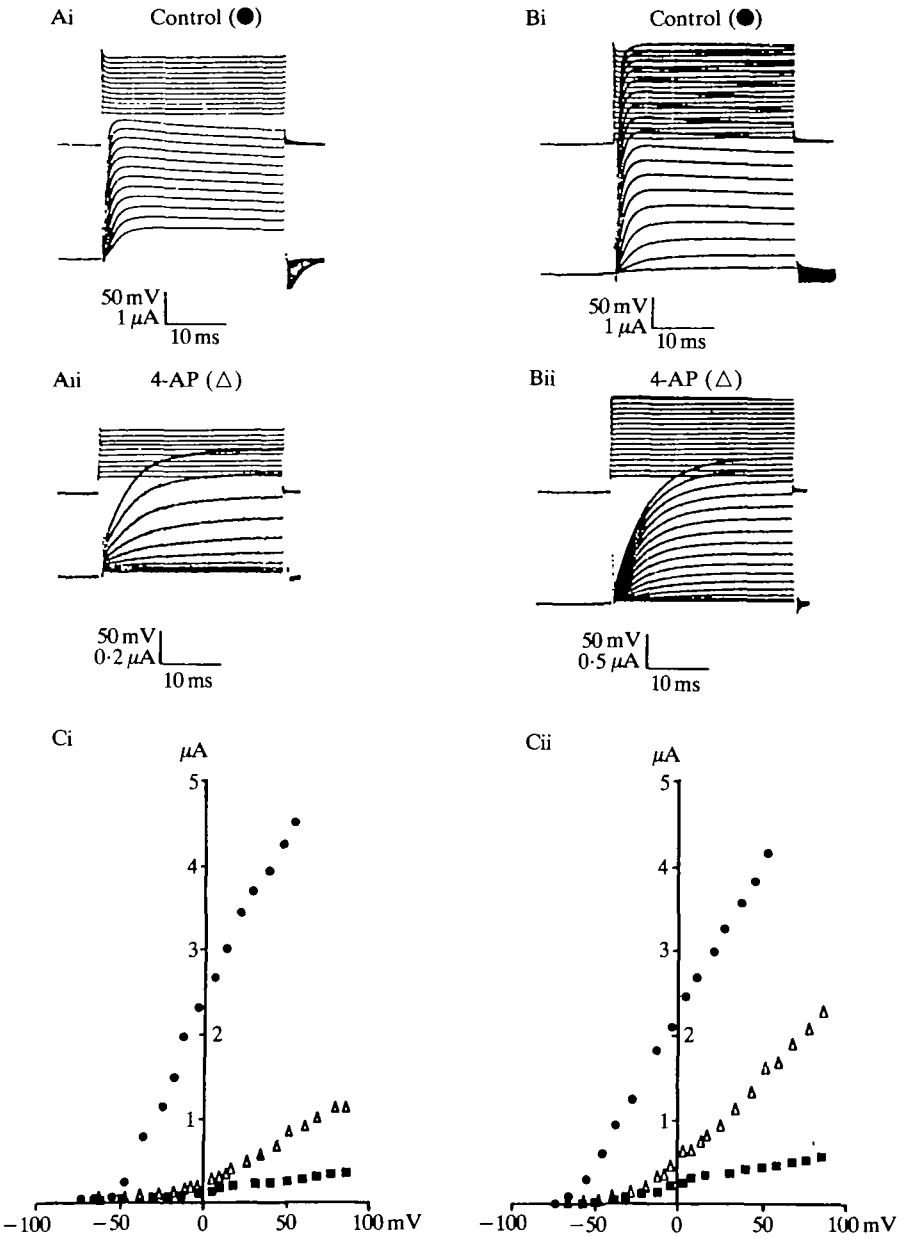

Fig. 4. Early outward current in the motor giant soma. Bath solution contained TTX (10^{-7} mol l^{-1}) and Cd^{2+} (2 mmol l^{-1}). Examples of current records for clamp pulses at $V_H = -90$ mV (Ai) and -70 mV (Bi) and after addition of 2 mmol l^{-1} 4-AP (Aii and Bii). (C) Current–voltage plots established in the same experiment in bathing solution as above (●), after adding 2 mmol l^{-1} 4-AP (△), after adding, in addition, 50 mmol l^{-1} TEA^+ (■). (Ci) Currents measured 2 ms after the onset of the test pulse, (Cii) currents measured at 30 ms.

presence of 1 mmol l^{-1} 4-AP and 50 mmol l^{-1} TEA^+, or by the injection of TEA^+ into the MoG soma (see Fig. 9B). The origin of the remaining outward current has not been studied further.

Since 4-AP at low concentrations is a fairly selective blocker of the early potassium current (Thompson, 1977), these experiments suggest than an I_A-like current exists in the motor giant somata and would be the dominant voltage-dependent current activated at the onset of the conditioning depolarization to −50 mV.

Inactivation of the early outward current

The early outward current was also suppressed by modifying the holding potential. Fig. 5A shows outward currents for test pulses to −25 mV, which is just above the value for slow outward current activation. Progressive decreases in the early outward current were observed when V_H was set from −90 mV to less negative values. For $V_H = -40$ mV only the small, very slowly activating outward current was observed.

Fig. 5B shows an inactivation curve which was constructed by applying conditioning pulses of 7 s at various potentials followed by a test pulse of 30 ms to −40 mV (see inset). The curve decreases from the maximum value at very hyperpolarized voltages (−100 mV) to very small values in the region of −40 mV. Since the second component of the early outward current decayed very slowly (see below) a 'steady-state' inactivation curve cannot be constructed. However, after a 7 s conditioning pulse the outward current had inactivated to very low values before the test pulse was applied.

We have further analysed the time course of the early outward current decay during 5 s depolarizing pulses when the membrane potential was shifted from −90 mV (V_H) to potentials between −74 and −52 mV (e.g. the inset of Fig. 6A). These experiments were performed in normal solution and the depolarization was set below the value of Ca^{2+} and Na^+ conductance activation in the motor giant soma.

To evaluate the time constant of the current decay, the outward current was plotted as $I_K/I_{K,max}$, where $I_{K,max}$ is the maximum value of I_K at the onset of the pulse (Fig. 6A,B). Two phases of decay were revealed, a fast one (τ_1 of 1·5 s) and a slow one (τ_2 of 18·1 s). Only τ_2 was voltage-dependent, as shown in Fig. 6C,D.

Finally, the effects of inactivating the early outward current on MoG soma excitability were investigated under current-clamp conditions. Depolarizations from a potential of about −70 mV to between −50 and −60 mV showed a slow, roughly S-shaped increase in the depolarization to about 50% greater than the initial value (Fig. 7Ai). Input resistances measured by short, hyperpolarizing current pulses during the depolarization showed that a nearly proportional increase in resistance accompanied the slow, depolarizing shift. Addition of 1 mmol l^{-1} 4-AP blocked the slow depolarization and increased input resistance (Fig. 7Aii).

Reduction of the early outward current by a prepulse or by pharmacological

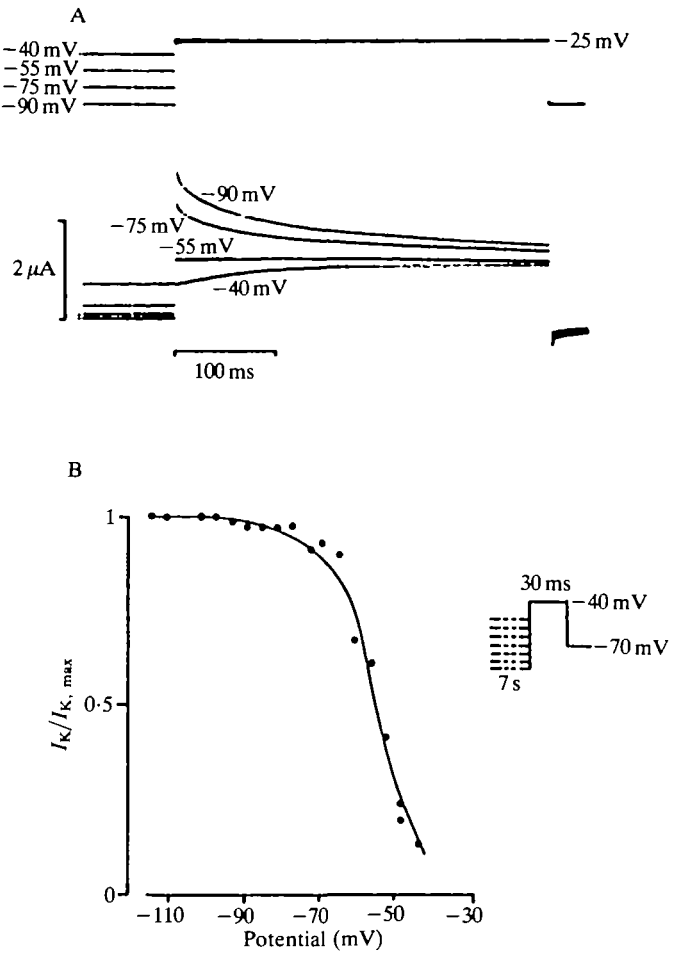

Fig. 5. Voltage dependence of the inactivation of the early outward current. (A) The holding potentials were set to the values indicated before test pulses were applied (to −25 mV for 250 ms) followed by repolarization to −90 mV. (B) Inactivation curve established in another cell by applying the conditioning pulse of 7 s at different potentials (abscissa) followed by test pulse of 30 ms to −40 mV. $V_H = -70$ mV (see inset).

means switches the cell from a non-spiking to a spiking one (Fig. 7B). We should add that the action potential is due to Ca^{2+} influx into the cell, as shown in Arsenazo III experiments (Czternasty *et al.* 1984). The appearance of the action potential was dependent on the duration of the conditioning depolarization, since a full-size action potential could be triggered only after several seconds of depolarization (Fig. 7Bi). Moreover, action potentials were also obtained if, instead of a conditioning depolarization, 4-AP at low doses (0·1–1 mmol l^{-1}) was added to the perfusing solution (Fig. 7Bii). Finally, TEA^+ applied extracellularly

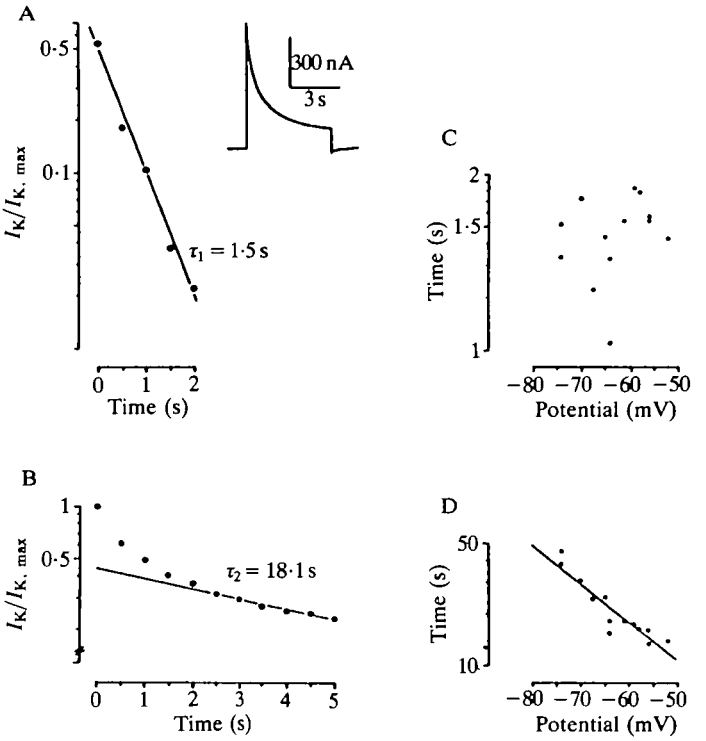

Fig. 6. Time constants of outward current decay and its voltage dependence. (A,B) Semilogarithmic plots on different time scales of the early outward current decay during a 5 s clamp pulse from −90 to −52 mV (see inset). Time constants are calculated from a best-fit straight line through the data points. (C) No voltage dependence of the fast time constant (τ_1). (D) The second time constant (τ_2) shortens with depolarization. Ordinates, time constant in seconds; abscissae, membrane potential during 5 s clamp pulses.

(50 mmol l^{-1}) or ionophoretically injected into the cell had a similar effect (not shown).

Thus, these experiments showed that in normal conditions the early outward current prevents the development of the Ca^{2+}-dependent action potential.

Calcium conductance in the MoG somata

The current responsible for the Ca^{2+}-dependent action potential was not detected in the I/V curves for normal cells when the holding potential was close to the normal resting potential, but was unmasked if the V_H was set at −50 mV (Fig. 8A).

The inward current was also revealed at V_H close to normal resting potential (about −70 mV) if the cells were injected ionophoretically with TEA^+ prior to voltage-clamping. In these conditions a step depolarization produced a rapid inward sodium current followed by a slower inward calcium current (see Fig. 3B).

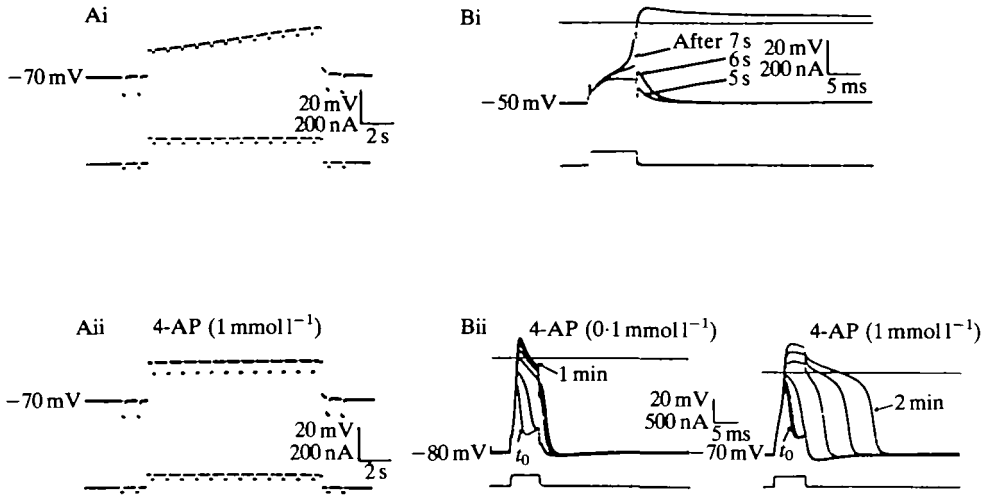

Fig. 7. Conditions permitting a Ca^{2+} regenerative potential. Two cells are shown in different bathing media, cell A in TTX (10^{-7} mol l^{-1}) and $CdCl_2$ (2 mmol l^{-1}) and cell B in normal media. (Ai) The effects of a depolarizing pulse of current on the membrane potential and resistance. (Aii) The effect of adding 4-AP (1 mmol l^{-1}) to the medium. Cell B had a resting potential of −75 mV. The potential was shifted with current to −50 mV before applying fixed depolarizing current pulses at different times after the shift (Bi). The same kind of regenerative potential was obtained from the more hyperpolarized potentials of −80 or −70 mV in the presence of 0·1 or 1 mmol l^{-1} 4-AP, respectively (Bii). The current pulses were given at different times after adding 4-AP at time zero.

The maximum amplitude of the slow inward current occurred 2–4 ms after the onset of the depolarizing pulse.

The inward Na^+ current was blocked by TTX (10^{-7} mmol l^{-1}) leaving only the slow inward current. It was activated at around −40 mV, was maximal at −10 to 0 mV (Fig. 8B) and reversed at about +40 mV. This reversal potential was due to a residual outward current which was not blocked by TEA^+ (see below). After a shift of the holding potential to −90 mV this I/V plot did not reveal the existence of a second, low-threshold Ca^{2+} current, as is observed in vertebrate neurones (i.e. Carbone & Lux, 1984). In this respect the giant motor neurone soma resembles *Helix* neurones (Lux & Brown, 1984).

The slow inward current recorded extracellularly (see Fig. 3B) showed the somatic localization of the channels mediating the current. These channels were not blocked by TTX (10^{-8} mmol l^{-1}) and substitution of choline for Na^+ did not modify the slow inward current (not shown). However, this current was blocked by Co^{2+} (10 mmol l^{-1}, Fig. 9B), Mn^{2+} (2·5 mmol l^{-1}) or Cd^{2+} (1 mmol l^{-1}), well-known calcium channel blockers.

Further experiments confirmed that Ca^{2+} was the main charge carrier for the slow inward current. I/V plots established in bath solutions of different Ca^{2+}

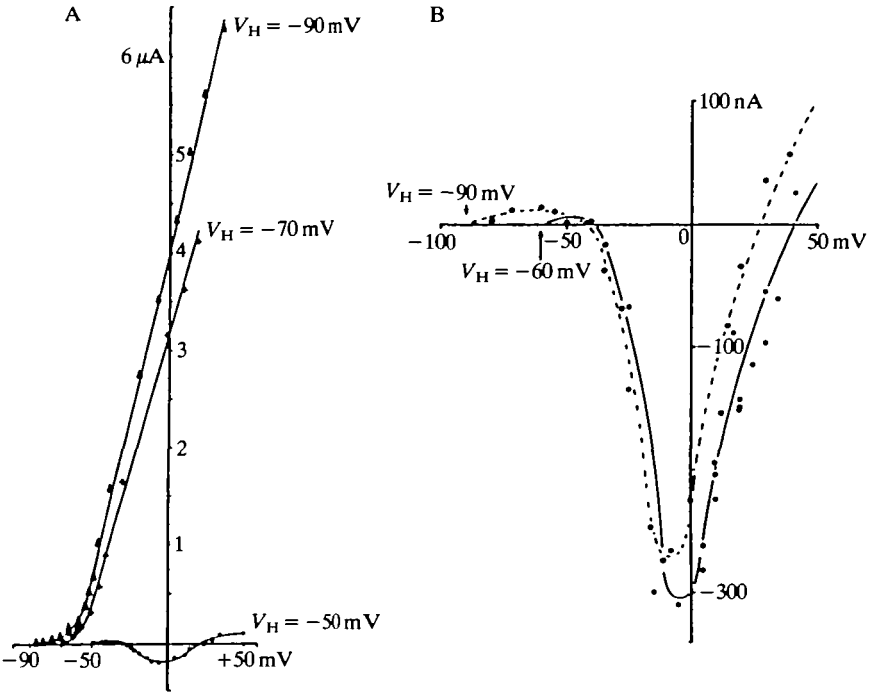

Fig. 8. Slow inward current in a motor giant soma. (A) Current–voltage relationship in a normal cell at three holding potentials, as indicated in the figure. The inward current was unmasked when V_H was −50 mV. Currents measured 2 ms after the start of the test pulse. (B) TEA^+-injected cell. Current–voltage relationship at two holding potentials, as indicated. Currents were measured 4 ms after the start of the test pulse. Both experiments were performed in normal extracellular solution.

concentration (6·5, 13 and 26 mmol l^{-1}) showed an increase of the maximal current with increasing Ca^{2+} concentration in the bath (Fig. 9A).

Comparison of the inward current time courses for identical test depolarizations in these three solutions (Fig. 9Ai) showed that the decline of current become faster as external Ca^{2+} concentration was increased. These results and our previous results with Arsenazo III (Bruner *et al.* 1986) suggest that inactivation of Ca^{2+} entry is predominantly mediated by intracellular free Ca^{2+} concentration.

Subtraction of the current–voltage relationships established before and after adding Co^{2+} to the bath allowed the inward Ca^{2+} current to be estimated (Fig. 9Biii). The resulting I/V plot showed that the calcium current was not reversed for depolarizations up to +80 mV. The inward current asymptotically approached the voltage axis. This I/V curve is in excellent agreement with the plot of peak Arsenazo III absorbance changes *versus* membrane potentials (Bruner *et al.* 1986).

Discussion

A transition from a non-spiking state to two different spiking states, depending

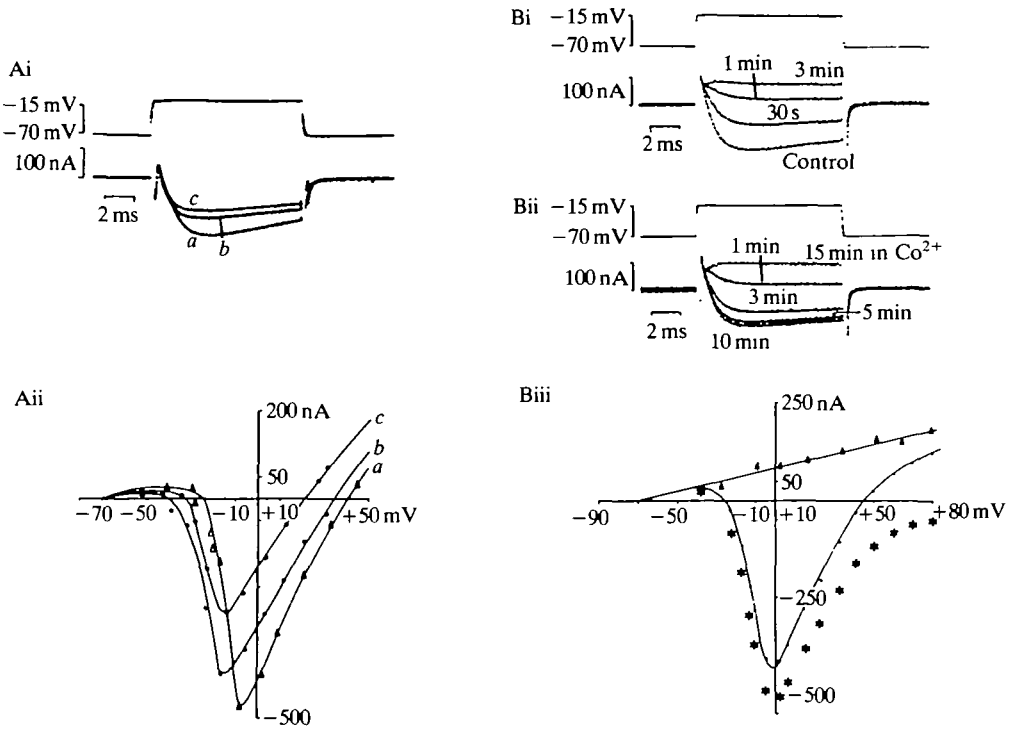

Fig. 9. Dependence of the slow inward current on extracellular $[Ca^{2+}]$. Two different cells injected with TEA^+. (Ai) Examples of current records during clamp test pulses to −10 mV at three extracellular Ca^{2+} concentrations: *a* 26, *b* 13 and *c* 6·5 mmol l^{-1} ($V_H = -70$ mV, 10^{-6} mol l^{-1} TTX present throughout the experiment). Note the increase in the inactivation rate when $[Ca^{2+}]_o$ was increased. (Aii) Current–voltage plots were established for the Ca^{2+} concentrations as indicated above. In A and B, current was measured 2 ms after the start of the test pulse. (B) Current records from another cell before (control) and at different times after the application of 10 mmol l^{-1} Co^{2+} (Bi) and during a wash (Bii). Current–voltage plots (Biii) were established in normal solution (●) and after adding 10 mmol l^{-1} $CoCl_2$ (△) to the bathing solution. Asterisks indicate the calculated *net* inward current (see text).

upon the level of membrane potential, as observed in this study of the crayfish MoG soma, has not been reported in any other neurone. However, neurones are known in which two types of regenerative potential can be evoked *independently*.

The giant motor axon of the jellyfish shows both a Na^+-dependent and a Ca^{2+}-dependent action potential (Mackie & Meech, 1985). They can be evoked separately since the Ca^{2+} spike is activated by depolarization and its amplitude does not exceed the threshold for the Na^+ spike. Fast, escape-type swimming depends on sodium-type motor impulses, whereas slow swimming uses low-amplitude, Ca^{2+} regenerative potentials.

In mammals, studies on thalamic brain slices (Llinas & Jahnsen, 1982) have shown that if the resting potential is more positive than −60 mV, depolarization

results in a Na^+-dependent regenerative potential, whereas if it is more negative than this value, the response is a short burst of inactivating Ca^{2+} spikes.

Sodium current in the non-spiking crayfish soma

The observation that the MoG soma does not produce regenerative potentials in response to depolarization from resting potential confirms earlier data (Furshpan & Potter, 1959; Takeda & Kennedy, 1964; Selverston & Remler, 1972; Wine & Mistick, 1977; Kuwada & Wine, 1981). We have demonstrated that the sodium-dependent, TTX-sensitive inward current (Hartung & Rathmayer, 1985) has a somatic localization. The opening of these channels occurs under conditions where a regenerative potential cannot be induced in current-clamp conditions.

This inward current is similar to the one studied in another non-spiking crustacean cell membrane, the muscle receptor dendrite of the crab (Mirolli, 1981). In both cases the inward currents have fast time courses (time to peak less than 1 ms), similar thresholds for activation (−40 to −50 mV), show complete inactivation at voltages more positive than about −55 mV, are sensitive to 10^{-8} mmol l^{-1} TTX and to the substitution of Na^+ by choline, and are insensitive to calcium channel blockers. This comparison suggests that the fast sodium channels in both preparations could be fairly similar.

Why are sodium spikes not induced without prior hyperpolarization?

In the dendrites of the muscle receptor organ of the shore crab, spike-like transients (which are TTX-sensitive) can be induced after hyperpolarization, and it has been suggested that the sodium channels are partially inactivated at resting potential (approx. −70 mV) (Bush, 1981). In our preparation we have shown that the inward sodium current was increased by holding the membrane potential between depolarizing pulses more negative (i.e. −90 mV) than the resting potential and that the current was inactivated at depolarized V_H (see Fig. 2A). These results show that at potentials from −60 to −70 mV the voltage-dependent sodium channels are partially inactivated and this can account for the absence of the sodium spikes in the current-clamp experiments.

The possible shunt of the sodium current by the fast outward current – as was proposed for the non-spiking dendritic membrane of the crab (Mirolli, 1981) – is not responsible for the absence of the sodium spikes in our preparation. The time to peak of the outward current was about 1 ms, whereas the inward Na^+ current peaked at about 0·5 ms. Therefore, the Na^+ spike could occur before the membrane was shunted by the early outward current.

Finally, Kuwada & Wine (1981) have demonstrated that the MoG somata become excitable a few days after axotomy. Since the normal MoG soma membrane *does* contain voltage-dependent Na^+ channels, further experimentation is required to find out whether axotomy increases their density, the characteristics of their activation or both.

Inactivation of the outward current

A decline in outward current during long depolarizing pulses has been observed in several preparations, e.g. in molluscan central neurones (Aldrich *et al.* 1979). The current decline may be due to a decrease in K^+ conductance, to a time-dependent reduction in the driving force for K^+ produced by accumulation of these ions just outside the membrane, and/or to an increase in the inward current.

The third possibility could not apply under our experimental conditions, since the amplitudes of the test pulses were below the threshold for activation of inward calcium or sodium currents (see Fig. 6). Of the other two possibilities, an accumulation of K^+ just outside the membrane occurs during depolarizing pulses (Lux & Eckert, 1974), and since the crayfish neuronal soma has an extensively infolded membrane (Cuadras *et al.* 1985), the outward current may result in an accumulation of potassium in the infoldings. Our preliminary experiments are in favour of this hypothesis since a K^+ tail current reversal potential, which after a 5 ms depolarizing pulse was around -92 mV, shifted to less negative values after a depolarizing pulse of several seconds.

The increase in cell input resistance during conditioning depolarization suggests a decrease in K^+ membrane conductance. Moreover, the observed depolarizing shift in membrane potential, as the cell's input resistance increased, may be due to this decrease in K^+ conductance. It is less probable that this membrane potential shift is also due to the accumulation of K^+ in the extracellular space, since in this case a decrease rather than an increase in cell input resistance would be expected. A time-dependent decrease in potassium conductance has been clearly demonstrated in voltage-clamp experiments both in neuronal somata of molluscs (Aldrich *et al.* 1979) and in the squid giant axon (Chabala, 1984).

Finally, the MoG soma of crayfish has more than one type of voltage-dependent outward current, as observed in the soma of other invertebrates (Hagiwara *et al.* 1961; Neher, 1971; Meech & Standen, 1975; Thompson, 1977); for example, the voltage-dependent transient outward current (I_A) and the delayed outward rectifier (I_K, see Adams *et al.* 1980, for a review). The kinetics of the early current decline showed two phases (see Fig. 6). The fast phase is probably due to a time-dependent K^+ conductance inactivation, the slow phase may be due to the decrease of the driving force for K^+ due to K^+ accumulation outside the cell membrane, or to both phenomena (see Chabala, 1984, for references). The shortening of the slow-phase time constant upon application of stronger depolarizations (see Fig. 6D), which should be accompanied by an increased K^+ efflux, suggests that the second mechanism could be of importance in the slow, but not in the fast, phase.

Why are calcium regenerative potentials not induced without prior depolarization?

We have shown in a previous paper (Shimahara *et al.* 1986) that the regenerative

character of the Ca^{2+} action potential cannot be attributed to 'facilitation' of a Ca^{2+} conductance during the conditioning depolarization.

Voltage-clamp experiments in the present work showed that the early outward current attained its maximal amplitude in about 2 ms when the membrane was depolarized to the Ca^{2+} action potential threshold established in current-clamp experiments. A small part of the early outward current consists of the TTX-sensitive, Na^+-dependent K^+ current, recently demonstrated in the crayfish MoG soma (Hartung, 1985). Another part of this current has the characteristics of a voltage-dependent early outward current. Thus, these outward currents should be responsible for a very rapid repolarization of the membrane in response to a depolarizing test pulse (see Fig. 1A).

Since the maximum inward Ca^{2+} current was observed within 2–3 ms of the onset of the test pulse, we can postulate that the calcium regenerative potential cannot be induced under normal conditions because the membrane is shunted by a simultaneous development of the above-mentioned outward currents. The conditioning depolarization (i.e. to −50 mV) inactivated these conductances and allowed the relatively small Ca^{2+} current to produce a spike.

The 4-AP block of the voltage-dependent early outward current produced a similar effect on the Ca^{2+} spike. This result implies that the Na^+-dependent K^+ conductance is less important than the A-current in preventing the development of the Ca^{2+} spike.

A mechanism depending on the A-current has been proposed for the delayed inking response in *Aplysia*, where inking may be obtained after a long delay with a single protracted stimulus or immediately if a stimulation has been applied previously (Byrne, 1980). This behaviour is due to the activation of a transient outward current by excitatory synaptic inputs to the motor neurones of the ink gland. These neurones can fire only under conditions where the transient outward current is in the inactivated state.

The physiological role of the 'switching' property of the MoG soma is not known. In the whole animal, the motor giant axon fires only once at the beginning of the repetitive tail flips triggered by a visual or tactile stimulus (Wine & Krasne, 1972; Wine, 1977). Moreover, the synaptic inputs are remote from the soma and the axon spike produces only a small (below 10 mV) depolarization in the soma (Selverston & Remler, 1972) which cannot trigger a regenerative potential.

The motor giant soma membrane appears, however, to be an excellent model for studying the interactions of ionic mechanisms involved in the switching from a non-spiking to a spiking membrane. This is of importance since the membrane properties of dendrites are known to be similar to those of the soma in several other crayfish neurones (Wine, 1975). If the dendrites can change spiking state, this would have an important influence on neural integration.

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