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SHORT COMMUNICATION

MOTOR NEURONES OF THE CRAYFISH WALKING SYSTEM POSSESS TEA$^+$-REVEALED REGENERATIVE ELECTRICAL PROPERTIES

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In crustaceans, some motor neurones (MNs) have been shown to be part of the central pattern generator in the stomatogastric system (Harris-Warrick *et al.* 1992; Moulins, 1990), the swimmeret system (Heitler, 1978) or the walking system (Chrachri and Clarac, 1990). These MNs induce changes in the central rhythm when depolarized and are conditional oscillators in the stomatogastric ganglion. Moreover, in the walking system, rhythmic activity can be triggered by muscarinic cholinergic agonists (Chrachri and Clarac, 1987). We have recently analyzed the role of muscarinic receptors in crayfish walking leg MNs (D. Cattaert and A. Araque, in preparation) and demonstrated that oxotremorine, a muscarinic agonist, evoked long-lasting depolarizing responses associated with an increased input resistance. The outward current blocked by oxotremorine is likely to be carried by K^+, as is the case for the M current (I_M) in vertebrates (Brown and Adams, 1980). In most neurones, K^+ conductances play a principal role in maintaining the membrane potential at rest: for example, I_M is active at the resting membrane potential, thus contributing to its maintenance, and the 'delayed-rectifier' (I_K) assists the fast repolarization after an action potential. Some K^+ conductances are Ca^{2+}-dependent ($I_{K,Ca}$) and are activated by an increase in internal Ca^{2+} concentration. In such cases, Ca^{2+} currents may result in hyperpolarization of the neurone through activation of $I_{K,Ca}$. In opposition to these K^+ currents, the direct effect of Na^+ and Ca^{2+} conductances is to depolarize the neurone. For example, the persistant Na^+ current (I_{Nap}) that is responsible for the slow subthreshold depolarization termed slow pre-potentials (Gestrelius *et al.* 1983; Leung and Yim, 1991) participates in the formation of pacemaker depolarization (Barrio *et al.* 1991) and generates plateau-type responses in control conditions (Barrio *et al.* 1991; Llinas and Sugimori, 1980). Similarly Ca^{2+} or non-specific (Na^+/Ca^{2+}) conductances generate such events in *Aplysia californica* burster neurones (Adams and Benson, 1985), crustacean cardiac ganglion (Tazaki and Cooke, 1990), insect neurones (Hancox and Pitman, 1991) and crustacean stomatogastric

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ganglion (Kiehn and Harris-Warrick, 1992). Since crustacean MNs can participate in rhythm production, such depolarizing conductances may exist in most of them and may contribute to the long-lasting MN depolarizations and spike bursts present during locomotion.

Plateau potentials have been demonstrated in several systems, such as the lobster pyloric rhythm generator (Russell and Hartline, 1982; Dickinson and Nagy, 1983), the locust flight and respiratory systems (Ramirez and Pearson, 1991), cockroach MNs (Hancox and Pitman, 1991), the leech heartbeat system (Arbas and Calabrese, 1987) and turtle spinal cord MNs (Hounsgaard and Kiehn, 1985). In the locomotor system of the crayfish, some MNs have been shown to display occasional plateau properties (Sillar and Elson, 1986; Chrachri and Clarac, 1987). However, from these studies, the extent of regenerative behaviour in walking leg MNs is not clear. In the present study, we used tetraethylammonium (TEA^+) to block K^+ currents, in order to unmask the presence of voltage-dependent depolarizing conductances, which are normally concealed by K^+ currents.

Male and female crayfish, *Procambarus clarkii* (Girard), weighing 25–30 g were used. The results described are based on experiments performed on 16 animals. The *in vitro* preparation of the thoracic locomotor nervous system was employed as described previously (Chrachri and Clarac, 1987). It consists of the three thoracic ganglia (Chrachri and Clarac, 1987, 1990) along with the motor nerves from the fifth ganglion of the promotor, remotor, levator and depressor muscles. The thoracic nerve cord and the nerves were pinned dorsal side up onto a silicone elastomer (Sylgard)-lined Petri dish and the fourth and fifth ganglia were desheathed to improve superfusion and to allow intracellular recordings from the MNs (Fig. 1A). The nervous system was continuously superfused with oxygenated saline (in $mmol\,l^{-1}$: 195 NaCl, 5.5 KCl, 13.5 $CaCl_2$, 2.5 $MgCl_2$ and 10 Tris at pH 7.6). Intracellular recordings from the neurites of the MNs (Fig. 1B) were carried out using pipettes filled with either KCl ($3\,mol\,l^{-1}$) or TEA^+ chloride-($1\,mol\,l^{-1}$) and having a resistance of 12 and 30 MΩ, respectively. In some experiments, tetrodotoxin (TTX) was added to block Na^+ channels, in others Ca^{2+} was partially replaced by Co^{2+} in order to block Ca^{2+} conductances. An Axoclamp (Axon Instruments Inc.) was used in either the bridge or the single-electrode discontinuous current-clamp mode (rate 3.5–4 kHz). Recordings were made from the four proximal MN groups (promotor, remotor, levator and depressor) commanding the fifth leg. They were identified from extracellular recordings of spikes in the motor nerve following intracellular injection of depolarizing current into the MN (Fig. 1C) and by antidromic stimulation of the motor nerves (Fig. 1D). Thirty-two MNs from the fifth thoracic ganglion have been analyzed. Data were stored on a tape recorder (Biologic DTR 1800) and either displayed or printed on a four-channel digital oscilloscope (Yokogawa).

In all MNs that did not express plateau properties in control conditions (Fig. 2Ai), injection of TEA^+ was capable of unmasking plateaux above a threshold depolarization, as revealed by depolarizing current pulses at increasing intensities (Fig. 2Aii). These TEA^+-induced plateaux persisted in the presence of Ca^{2+} channel blockers (e.g. $10^{-3}\,mol\,l^{-1}$ Co^{2+}) (Fig. 2Aiii). In contrast, they were suppressed by bath application of TTX ($10^{-6}\,mol\,l^{-1}$) (Fig. 2B). The above findings suggest strongly that plateaux result from the activation of I_{Nap} without the participation of Ca^{2+} currents.

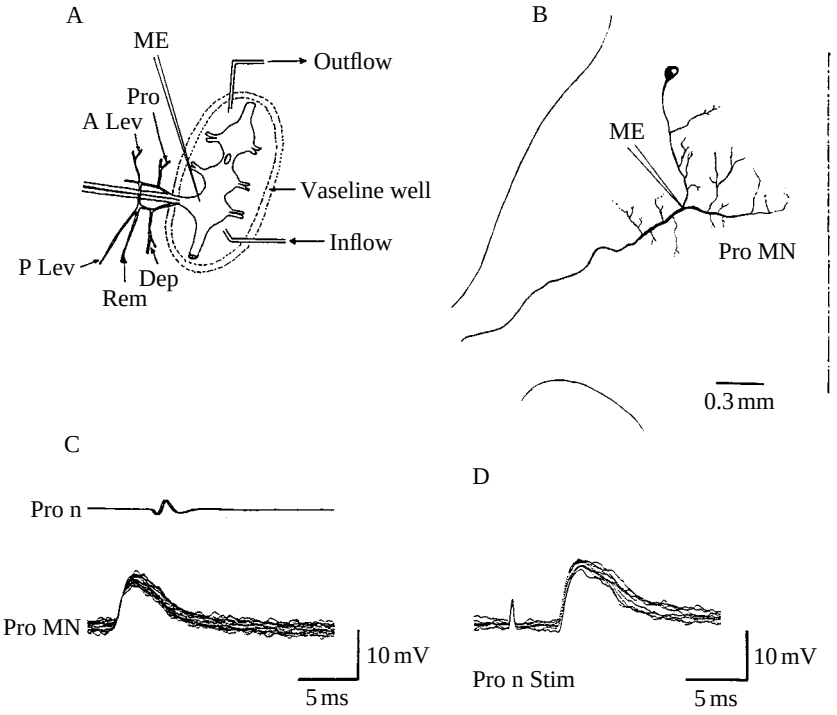

Fig. 1. Diagram of the experimental arrangement. (A) The crayfish thoracic nerve cord with the nerves to the fifth leg attached. The proximal motor nerves of the fifth leg (Pro, promotor; Rem, remotor; A Lev, anterior levator; P Lev, posterior levator; Dep, depressor) were dissected along with ganglia 3–5. A Vaseline wall was placed around the ganglia to allow restricted perfusion of the central nervous system with saline containing Co^{2+} (10^{-3} mol l^{-1}) or tetrodotoxin (TTX) (10^{-7}–10^{-6} mol l^{-1}). One microelectrode (ME) was used to record intracellularly from the neurite of a motor neurone (MN) within the ganglion. (B) Diagram showing the typical arrangement of the electrode and the recorded MN. The Pro MN was drawn from a Lucifer Yellow dye injection. (C,D) Each MN (in this case a Pro MN) was identified in intracellular recordings by injecting depolarizing current in order to elicit spikes that could be recorded extracellularly in the corresponding proximal motor nerve (Pro n) (C) or by antidromic stimulation (Stim) of the motor nerve (D).

Whereas application of TTX (10^{-6} mol l^{-1}) to TEA^+-filled MNs totally blocked both spikes and plateaux after 25 min of superfusion, only spikes were blocked while plateaux remained unaffected for the first 10 min of superfusion (Fig. 2Bii,iii). The time differences in the action of TTX suggest that the transient spike-generating Na^+ channels could be more sensitive to TTX and are, therefore, different from the persistent Na^+ channels involved in plateau development. However, the dissimilarity in response between spikes and plateaux may also be due to different diffusion barriers to the spike- and plateau-generating sites, in which case the two should be located at separate sites. Indeed, plateaux seemed to be generated more centrally (closer to the recording site in the neurite) than axonic spikes, since intracellularly recorded spikes were inactivated at the beginning of the plateau while they were still present in the nerve.

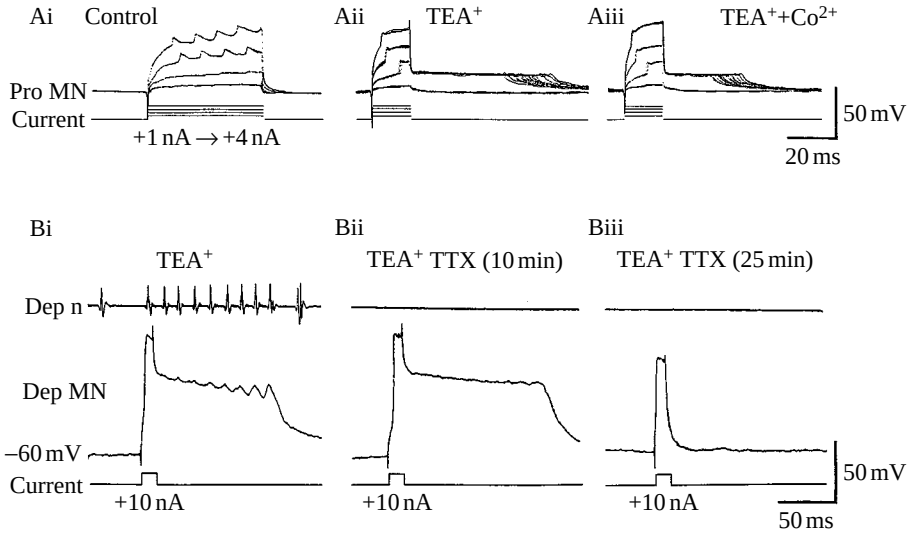

Fig. 2. Ionic channel blockers and plateau potentials in TEA^+-filled MNs. (Ai) Before injecting TEA^+ into a Pro MN, pulses of depolarizing current of increasing intensities (+1 to +4 nA) did not evoke plateaux in the recorded MN. (Aii) After injection of TEA^+ into the neurite by depolarizing current (+5 nA, 10 min), plateaux were evoked above a threshold membrane potential in control saline. (Aiii) The amplitude of plateaux was not modified by Co^{2+} (10^{-3} mol l^{-1}) although, in some instances, their duration was slightly reduced in the presence of Co^{2+}. (B) After TTX (10^{-6} mol l^{-1}) application (Bi), the extracellular MN spikes recorded at the nerve (Dep n) were blocked within 10 min (Bii), whereas the intracellularly recorded plateaux were suppressed only after 25 min (Biii).

In the five TEA^+-filled MNs, brief rhythmic plateaux and spike bursts were either spontaneous (period between 200 ms and 50 s) (N=3) (Fig. 3A) or were elicited by sustained depolarization of more than −50 mV (N=2) with continuous current injection (Fig. 3A,B). Plateaux were followed by a hyperpolarization and then by a progressive membrane potential depolarization until the threshold for the next plateau was reached (i.e. the pacemaker potential). The slope of the pacemaker potential increased as a function of the injected current intensity (Fig. 3B), thus accelerating the rhythm of the plateaux. Plateaux displayed a slower rhythm in the MNs that were silent at resting potential. As shown in Fig. 3C, the MN that evoked plateaux around the resting potential (open squares) had a higher discharge frequency than the other unit (filled triangles). Thus, the results reveal the voltage-dependence of rhythmic plateaux and suggest different voltage sensitivities in different MNs. Both pacemaker and plateau potentials were suppressed by TTX (10^{-6} mol l^{-1}), again suggesting the participation of Na^+-mediated currents.

This study demonstrates that intracellular TEA^+ injections, which block several outward K^+ currents, unmask a slowly inactivating or persistent TTX-sensitive current. This current, which is likely to be carried by Na^+, since it is blocked by TTX and is insensitive to Co^{2+}, has been termed I_{Nap} and has been demonstrated in all of the recorded MNs. Since these neurones did not display plateau properties before TEA^+ injection, we

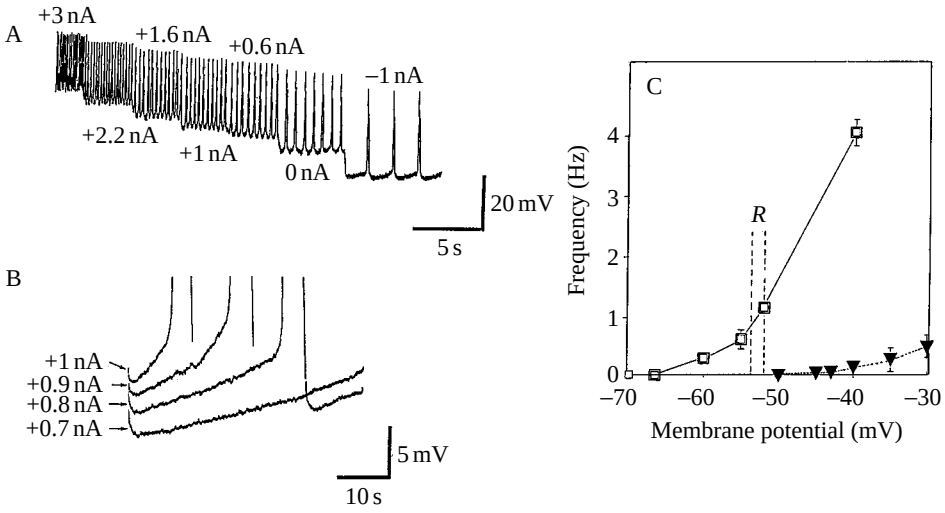

Fig. 3. Intracellular TEA^+-induced rhythmic plateau and pacemaker potentials in two different MNs. (A) The TEA^+-induced rhythmic plateau activity was voltage-dependent. This remotor MN produced bursts in the absence of current injection. (B) Pacemaker potential trajectories leading to plateaux under different current intensities in a depressor MN. In the absence of current injection, this MN was silent. Increasing current injections (+0.7 to +1 nA) elicited rhythmic bursts, the period of which depended upon the amount of depolarizing current injected. (C) Voltage-dependence of the rhythm frequency for the two MNs in A (open squares) and B (filled triangles). The resting potential (*R*) was −52 mV for the Rem MN and −53 mV for the Dep MN.

conclude that plateaux were prevented from forming by the opposing K^+ currents. The presence of I_{Nap} has been demonstrated in other neurones (Barrio *et al.* 1991; Weiss and Horn, 1986; Yawo *et al.* 1986), where it may coexist with other depolarizing currents and may play different functional roles (Gestrelius *et al.* 1983; Llinas and Sugimori, 1980; Brachi, 1987; Gilly and Armstrong, 1984; Rudy, 1978; Stafstrom *et al.* 1985). More recently, a slow Na^+ current (I_{NaS}), probably involved in the development of TTX-insensitive plateaux, has been demonstrated in striatal and hippocampal CA1 pyramidal neurones (Hoehn *et al.* 1993). I_{NaS}, being TTX-resistant, is therefore different from I_{Nap}. Hoehn *et al.* (1993) described two other classes of non-inactivating or slowly inactivating Na^+-dependent currents with characteristics different from those of I_{Nap}: a TTX-resistant Na^+ current blocked by Co^{2+} in mammalian primary sensory neurones and a cofactor-dependent voltage-dependent Na^+ current in gastropod neurones, frog MNs and mammalian neurones.

In the present experiments, spikes were continuously produced during plateaux, demonstrating that the transient spike-generating Na^+ current (I_{Na}) and I_{Nap} are simultaneously active in crayfish MNs. Although the transient I_{Na} shows essentially identical kinetics and TTX sensitivity in all systems studied (Brachi, 1987), I_{Nap} has different properties and TTX sensitivities (Gilly and Armstrong, 1984; Weiss and Horn, 1986), suggesting different activation–inactivation mechanisms in the different systems. Although more research is needed to determine whether the different Na^+ spike and

plateau kinetics of crayfish MNs result from distinct channel populations or represent two different types of behaviour of the same channel, the present results suggest that two different channels may exist.

In some experiments, spike peaks were somewhat more positive than plateau potentials (Fig. 2). The voltage-dependence of the steady-state inactivation process of I_{Na} (Hodgkin and Huxley, 1952) indicates that, at the sustained near-zero potential values attained by the plateau depolarization, the transient Na^+ conductance could be completely inactivated. Consequently, the transient voltage responses superimposed on plateau depolarization (Fig. 2Bi) may not be generated in the same region of the MN. They are probably initiated at axonic sites where, presumably, the plateau depolarization does not inactivate the transient Na^+ current (Barrio *et al.* 1991). Indeed, in all experiments in which intracellular recordings of spikes have been made from the neurite of the MN, the spikes have displayed relatively small amplitudes (15–30 mV), whereas axonal recordings reveal overshooting spikes from the same MNs. The above findings provide strong evidence that two distinct Na^+ channel populations exist, one in the neurite and one in the axon, as has been reported in mammalian neurones (Llinas and Sugimori, 1980).

In this study, we used TEA^+ as a tool to unmask conductances that could sustain regenerative properties that probably contribute to the prolonged MN plateau-like depolarization and spike bursts during walking. Under normal conditions, neuromodulators could play such a role and, thereby, trigger locomotion and modulate burst firing.

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