Short communication

Low Calcium Treatment Provokes Persistent Spiking in Tonic Crayfish Muscle

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The fibres of the tonic abdominal flexor muscle (TAF) of the crayfish do not usually exhibit autoregenerative action potentials (AP) of an "all or none" type, but graded responses (GR) which are accompanied by delayed rectification (Lehouelleur 1978). This type of electrical response is known to be dependent on a single calcium inward current, and several, outward, potassium currents, which have different kinetic properties (Henček et al. 1978; Mounier and Vassort 1975; Mounier 1979.). However, it is possible to modify the electrical response of TAF fibres by surgical denervation of the muscle (Lehouelleur et al. 1983) or by the action of the alkaloid — veratridine (Lehouelleur et. al. — in preparation). The dependence of these phenomena on the newly developed sodium conductance has been discussed. Reuben et al. (1967) reported another case of AP induction in crayfish muscles following the removal of calcium ions from the extracellular medium. In these conditions he observed spontaneous AP's, which were sodium-dependent, blocked by tetrodotoxin (TTX) and were not modified by the alteration of extracellular potassium and chloride ion concentrations.

In our experiments we have elicited transformation of the TAF response to electrical stimulation by means of transient perfusion of the preparation in an extracellular medium with the calcium concentration reduced to 0.1 to 0.25 of the normal. The novel finding was that once the ability to develop AP's had been evoked, it remained for several hours, in contrast with the short time-span expected if they were due to any well-known influence of the lowered calcium level on membrane excitability (Hagiwara and Takahashi 1967; McCleskey and Almers 1985; Sheu and Blaustein 1983).

The experiments were performed on muscle fibres of the tonic abdominal flexor (TAF) taken from the third abdominal segment of *Procambarus clarki.* The crayfish were obtained from Dahl Co. (Berkeley) and maintained in a controlled thermal and light environment. The TAF preparation was continuously superfused with fresh van Harreveld solution (VH) containing (in

mmol/l) : NaCl 207; KCl 5.4; $CaCl_2$ 13.5; $MgCl_2$ 5.3; HEPES 5.7; pH adjusted to 7.2; temperature 13—16°C.

The solution was modified during the experiments by the removal of some components and introduction of appropriate replacements, or by addition of specific agents, as indicated in the text. The chemicals were purchased from Sigma.

The fibres were studied using the conventional current clamp technique with two glass microelectrodes impaled into a single fibre at a distance not exceeding 50 μm. The voltage electrode was filled with 3 mol/l KCl and had a resistance of 10—16 MOhm, while the current electrode was filled with 2 mol/l citrate-K and had a resistance of 6—10 MOhm. In some experiments the current electrode was filled with 3 mol/l KCl or 1 mol/l EGTA-K. The fibre membrane was held at a steady potential of -80 mV by passing a direct current which did not exceed 30 nA. Most other details of the experimental procedure were similar to those described by Lehouelleur (1978) and Lehouelleur et al. (1983).

The input resistance of TAF fibres superfused with normal, unmodified VH ranged from 0.5 to 1.5 MOhm. In 48 independent trials in these conditions, the fibres displayed only GR's of the delayed rectification type, even if the membrane was depolarised from $-90 \div -80$ mV to a potential level close to 0 mV. In one case only did a TAF fibre give an electrical response of delayed rectification type, but which was intermediate between a typical GR and AP. These observations were consistent with the results of Lehouelleur (1978).

The same fibres were superfused with VH solution in which the calcium concentration was lowered from 13.5 mmol/l to $1 \div 3$ mmol/l (26 independent trials). The calcium ions removed were replaced by an equivalent quantity of sodium ions (in 22 trials) or magnesium ions (4 trials). No changes occurred by the end of the superfusion with reduced calcium and magnesium substituted VH or after the subsequent return to normal VH. On the contrary, superfusion with reduced calcium and sodium substituted VH, followed by a return to normal VH, caused a $2 \div 3$ fold increase in the input resistance of the fibre membrane, which reached values of $2 \div 5$ MOhm. Subsequently overshooting AP's developed, these could be evoked by depolarising pulses from a membrane potential held at $-65 \div -100$ mV, and were triggered at a potential level of about -40 mV. (Fig. 1*A*). In several cases (5 out of 22) these AP's were fully developed only after $1 \div 2$ hours following return of the fibre to normal VH. The AP's obtained continued even after 24 hours superfusion in normal VH; moreover, once they had been induced, they could not be abolished by subsequent superfusion with VH solution enriched with calcium ions (4-fold) or with magnesium (8-fold) (2 trials). These results establish that the AP's depended neither on disruption of the surface bivalent cation layer of the membrane, which would be the expectation according to the surface charge density hypothesis of Hagi-

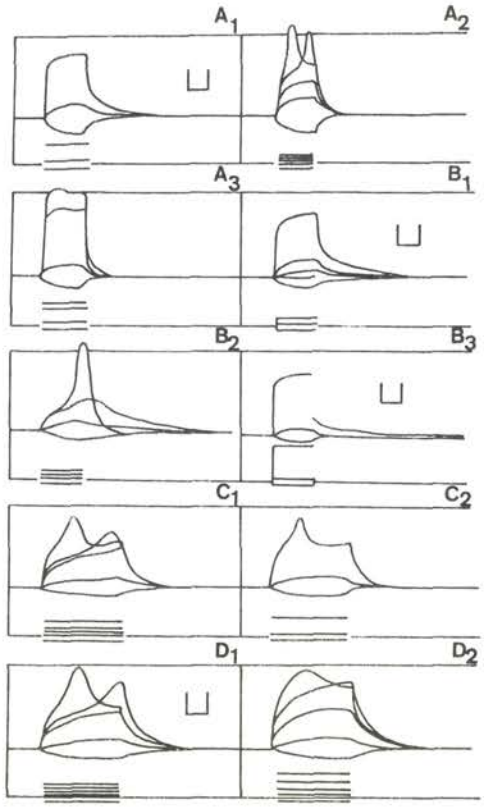

Fig. 1. Records of spikes obtained in crayfish tonic muscle fibres: A_1, B_1 — control, before treatment of the fibre; A_2, B_2, C_1, D_1 — spikes after treatment with lowered external calcium; A_3 — blockade of the spike by 0.3 mmol/l La^{3+}; B_3 — effect of Na^+ removal from extracellular solution; C_2 — lack of effect of TTX (12 μmol/l); D_2 — effect of Mn^{2+} (10 mmol/l); Calibration: A_{1-3}, C_{1-2}, D_{1-2} — 20 mV, 100 ms, 100 nA; B_{1-2} — 20 mV 50 ms, 100 nA; B_3 — 20 mV, 50 ms, 500 nA. Resting potential: −80 mV.
Upper line: 0 mV. *Lower trace*: current pulses.

wara and Takahashi (1967), nor on the loss of calcium channel selectivity, which was proposed by McCleskey and Almers (1985) to be the effect of low calcium treatment on calcium channels. If the AP's were dependent on these effects they should be both short lasting and reversed by superfusion with normal VH. Nevertheless, transient disturbance of the normal balance of extracellular monovalent and bivalent ions seemed to be important for the induction of AP's in fibres that are usually nonspiking.

The induced AP's could not be blocked by tetrodotoxin (TTX) at a con-

centration of 12 μmol/l (Fig. 1*C*), but they were completely blocked while the fibre was superfused with a physiological solution in which all the sodium ions were replaced by Tris (Fig. 1*B*). In the sodium-free environment the input resistance of the fibre was reduced about 10-fold to less than 0.5 MOhm (3 trials). The return to normal VH did not reestablish the AP's. The insensivity of the AP's to TTX did not allow us to explain their mechanism as dependent on the newly developed sodium conductance, as it was for similar observations made by Lehouelleur et al. (1983), Lowe et al. (1978), Reuben et al. (1967) and Schwartz and Stühmer (1984). The fact that AP's disappear in the absence of extracellular sodium could not be used to support the sodium hypothesis because, in such conditions, the observed diminution of the input resistance indicated increased leak currents which could both prevent the regenerative response and diminish the amplitude of the graded response.

The calcium blockers: manganese ions (5 ÷ 10 mmol/l) and lanthanum ions (0.3 ÷ 0.5 mmol/l) prevented the induction of AP's (6 trials), (Fig. 1*A* and 1*D*); this blockade was fully reversible. This action of calcium blockers precluded possibility that there is a TTX insensitive sodium conductance, which has been shown to exist in some preparations (Koyima and Sperelakis 1983; McLean et al. 1974). This also allowed the categorisation of the AP's as being dependent on a calcium current (Hagiwara and Byerly 1981). At the same time, the idea that there exist nonspecific cation channels which are sensitive to La^{3+} and activated by removal of the extracellular calcium (Sheu and Blaustein 1983) seemed to be incorrect for the reason given above.

The transformation of GR's into AP's was observed only in those fibres which were impaled with microelectrodes throughout the experimental manipulations. The neighbouring TAF fibres showed no transformation of the electrical response until they were impaled and treated with reduced calcium. In four fibres tested with current electrodes filled with EGTA-K (the potent and specific calcium chelator) there was no AP induction in any of the experimental conditions that usualy induced AP's. Similarly, there was no AP induction when the current electrode was filled with KCl. This result allowed us to reject the hypothesis that leakage of citrate to the interior of the fibre and chelation of intracellular calcium might cause the diminution of the calcium dependent potassium currents and the enhancement of the calcium current. These, in turn might provoke AP induction, as proposed by Hagiwara and Byerly (1981). Moreover, it is possible that the injected citrate becomes a substrate for the aerobic energy metabolism of the fibres; however, if this effect were to be established as dependent on chelation, it would have to be observable without any additional manipulation, such as diminution of the extracellular calcium concentration.

Although a transient reduction in the extracellular divalent cation con-

centration is essential for the observed effect the mechanism of its action must differ substantially from those discussed above. The large increase in input resistance, observed in our experiment, can be considered as the primary effect of low calcium treatment as well as the basis of the fibre's capacity to develop AP's. This increase in resistance may be due to reduced passive chloride or potassium conductances, constituing background leak currents, or it may be due to a reduction in an early transient potassium current which usually prevents fast depolarization and large changes of membrane potential. This explanation needs the assumption that the transient lowering of extracellular calcium elicits a persistent modification of these conductances, and consequently, the modification of these types of channels. It is possible that the postulated modification is similar to the inactivation of channels accompanied by disruption of their gating mechanism.

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