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Short communication

Calcium Currents in the Muscle Membrane of the Crayfish in K^+-free Internal Environment

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The analysis of inward calcium currents registered under voltage clamp conditions in intact crustacean muscle fibres is complicated by outward potassium currents which are difficult to block completely from the external side of the membrane. In barnacle muscle fibres this problem was approached by internal dialysis of fibres with potassium free solutions (Keynes et al. 1973). However, this approach had left unsolved the problem of interference of membrane invaginations with the space clamp requirements, as the voltage clamped membrane area was very large. We tried to solve this problem by applying the membrane patch voltage-clamp method (Henček et al. 1969; Henček and Zachar 1977) to cut muscle fibre segments which enable in their turn to exchange the internal medium of the fibre by diffusion (Hille and Campbell 1976). The experimental set-up and the equivalent electrical scheme of the muscle fibre in contact with the circular sucrose gap arrangement is shown in Fig. 1. The procedure was as follows. Single muscle fibre isolated from the m. extensor carpopoditi of the crayfish *Astacus fluviatilis* was exposed to the relaxing 'internal' solution and shortly aftewards cut to about 3 mm long segments. The 'internal' solution had the following composition (in mmol/l): 4 $ATPNa_2$, 1 EGTA, 1 $MgCl_2$ and 0.01 $CaCl_2$, to keep the contractile proteins relaxed (Reuben et al. 1971; Brandt et al. 1972) and 240 Tris propionate as an inert electrolyte. The concentrations of both free Ca^{2+} ions and other internal components were calculated for $pH = 7.5$ and $t = 17\,°C$ (experimental conditions) by means of the program COMPLEX (Ginzburg 1976), translated into BASIC. In the calculations all complex-forming equilibria with known stability constants (Martell and Smith 1977) were taken into account.

The muscle fragment was mounted on the top of the sucrose isolating device and pressed to it by means of a perspex spoon (Fig. 1). Only isolated circular membrane area was in contact with the 'external' solution. In most experiments this solution had the following composition (in mmol/l): 20 $CaCl_2$, 220 Tris Cl, $pH = 7.5$. After closing the voltage-clamp circuit we measured the residual resting potential in order to bring the resting potential back to -80 mV (inside negative)

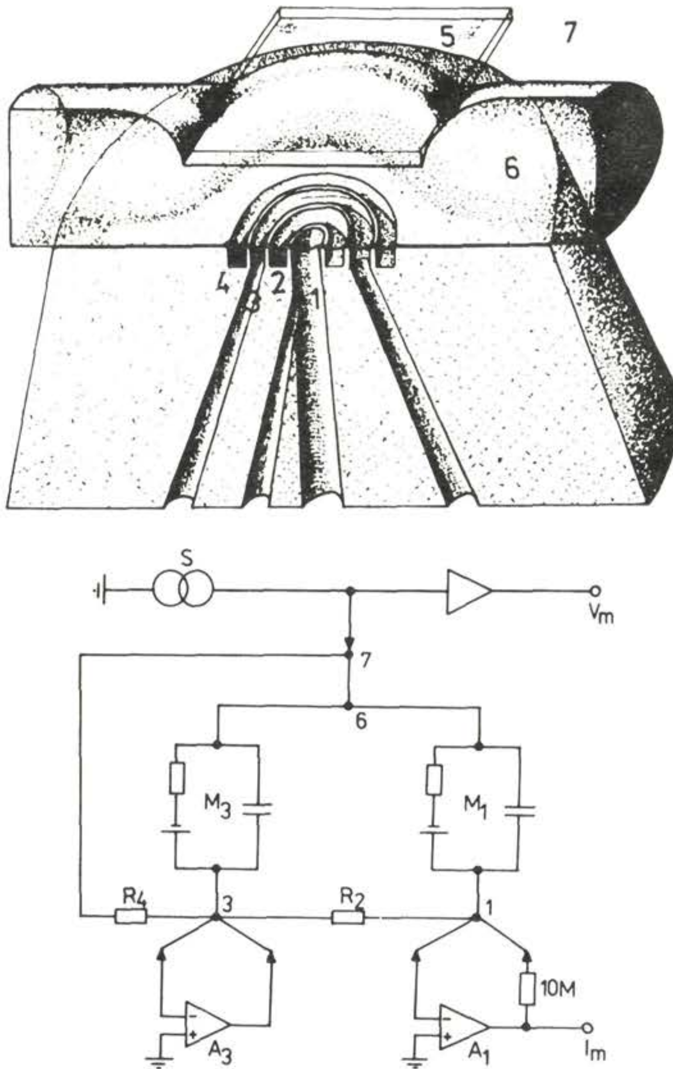

Fig. 1. *Top:* Cross-section through the cylindrical fragment of the muscle fibre fixed on the top of the measuring chamber. 1, 3 — the central channel and the guard ring filled with the external solution: 2, 4 — insulating rings filled with an isotonic sucrose solution; 5 — pressing plate; 6, 7 — intracellular space and the chamber filled with the 'internal' solution. *Bottom:* Equivalent electrical scheme. Numbers 1—7 correspond to those shown above. M_1 — voltage clamped membrane; M_3 — membrane covering the guard ring; R_2, R_4 — resistances of the inner and outer insulating sucrose rings; A_1, A_3 — measuring and grounding operational amplifiers; S — stimulator (the source of stimulating and holding potentials). Black triangels represent Ag/AgCl eletrodes with agar salt bridges. I_m — membrane current recording. V_m — recording of the clamping voltage and of the membrane potential.

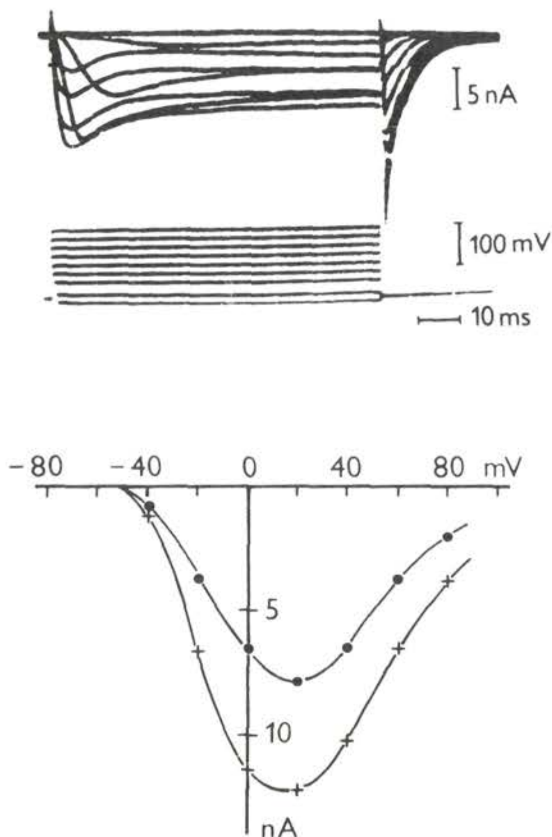

Fig. 2. *Top:* Membrane currents in an about 2 mm long muscle fibre segment equilibrated in K-free internal saline for 47 minutes following the completion of the single fibre dissection. Holding potential −80 mV. *Bottom:* Membrane current-voltage realtions: +, peak inward current; ●, final steady level of the inward current (at 60 ms).

using external source. The capacitive and leakage components of the membrane currents were compensated.

If the experiment started immediately after cutting the muscle segment it was possible to follow the decay of outward potassium currents due to decline of the internal concentration of K^+ ions. Stabilization of the membrane currents was achieved after more than 30 minutes (depending on the fibre segment size).

Fig. 2 shows the typical membrane currents after abolishion of the outward potassium currents, and the corresponding membrane current-voltage relations. It is evident that in the whole range of depolarizing voltages the inward currents are exclusively recorded. At depolarizations more positive than 100 mV onset of

a nonspecific inward current was observed. The ratio of the inward current peak to the steady level was not constant for different preparations.

If the records of I_{Ca} in Fig. 2 are compared with those obtained in intact fibres by means of the subtraction procedure (Henček and Zachar 1977) the most conspicuous difference concerns the time course of inactivation. The possible prolongation of the inactivation by drastic preparation procedure was ruled out by the reversibility of the long lasting treatment of intact fibres with 'internal' solution and application of long lasting depolarization. The presence of slow inactivation in intact fibres is supported by the time course of I_{Sr} and I_{Ba} (Henček, Zachar and Zahradník, unpublished results) separated from the total membrane current by means of the subtraction procedure (Henček and Zachar 1977). Slow inactivation was also observed in intact fibres in the presence of 150 mmol/l TEA at $t \leq 10\,°C$ (Poledna and Zacharová 1981).

The non-inactivating currents were characterized by the following qualitative tests (Zahradník and Zachar 1980a, b). Application of 100 mmol/l TEA^+ to the external solution did not influence the amplitude or the time course of the inward currents. Because TEA^+ in this concentration blocks considerably the outward currents (Henček and Zachar 1977), it can be assumed that no I_{Ca} is flowing through the potassium channels. If Ca^{2+} ions are withdrawn from the external saline, or are substituted with Mg^{2+} ions, the inward currents dissappear which indicates that they are pure calcium currents. The inward currents are diminished or abolished after addition of calcium channel blockers, Co^{2+} ions (20 mmol/l) or verapamil (1 mmol/l). It can be thus concluded that the whole inward current flows through Ca channels. When the Ca^{2+} ions are substituted with Sr^{2+} or Ba^{2+} ions the inward currents increase, but their time course remains unchanged.

From the above findings it can be concluded that the inward currents recorded from muscle fragments of the crayfish in K free solutions are pure divalent cation currents through two populations of calcium channels with similar chemical properties but with different kinetics of inactivation and probably of activation as well.

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