

Calcium Channels in Crayfish Muscle Fibre Fragments Studied by Means of the Vaseline Gap Technique

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Abstract. Calcium currents in crayfish muscle fibres were studied by means of the vaseline gap voltage clamp technique. Overlapping potassium currents were fully suppressed using fibre fragments equilibrated in K^+ -free intracellular solution. The design of the recording chamber tailored to crayfish muscle fibres is described in detail. Ca currents recorded had a two-component time course. The transient ($I_{Ca,T}$) component (peaking in about 10 ms) attained, on average, maximal overall density of $26.4 \mu A/cm^2$ at depolarization to -4.6 mV from a holding potential of -80 mV. The steady ($I_{Ca,S}$) component attained $16.7 \mu A/cm^2$ (evaluated at the end of a 70 ms pulse) at $+13.8$ mV. The average overall surface area of the clamped membrane surface (including invaginated parts) was about $0.07 cm^2$. The $I_{Ca,S}$ component could be separated from $I_{Ca,T}$ using short inactivating prepulses. Voltage and time dependence of the transient component inactivation, as well as its recovery from inactivation, were in agreement with a Ca-dependent mechanism. Independent behaviour of the two Ca current components and differences in their properties support the hypothesis concerning the existence of two populations of Ca channels in the surface membrane of the crayfish muscle.

Key words: Calcium currents — Skeletal muscle — Crayfish — Vaseline gap technique — Inactivation

Introduction

Crustacean muscle fibres are widely used in contractility and excitability studies, since the surface membrane of these fibres operates on the pure calcium electrogenesis principle. Several attempts were made to voltage clamp the surface membrane of these cells (for review see Zachar 1981). Recently, three methods have been applied to muscle fibres of the crayfish. The annular double sucrose gap technique has been used to study the kinetics of both the calcium (Henček

and Zachar 1977) and the potassium conductances (Zachar et al. 1978). The three microelectrode technique developed by Adrian et al. (1970) has been used by Jdaïâa and Guilbault (1986), and the two microelectrode clamp by Finger and Stettmeier (1980).

All the above methods have some drawbacks in common. First, the ionic conductances cannot be satisfactorily separated; second, the geometry of the fibre surface under voltage control is uncertain; and third, the access resistance to invaginated parts of the surface membrane distorts the space control of membrane potential.

We solved the first problem by voltage clamping fragments of crayfish muscle fibres equilibrated in potassium-free internal solutions (Zahradník and Zachar 1980, 1982a). Under these conditions pure Ca currents were measured directly. From their time and voltage dependence the existence of two populations of Ca channels was suggested. The third problem is inherent to this cell type and cannot be solved definitely (Zachar 1981; Hagiwara and Byerly 1981).

In the present paper calcium currents measured by the vaseline gap voltage clamp method of Hille and Campbell (1976) tailored to crayfish muscle fibres are described. This technique was used to solve the second problem mentioned above. Due to substantial methodological improvements, the present work could confirm previous findings under qualitatively better conditions and brings new results concerning inactivation of Ca currents.

Parts of this work have been already published in a short form (Zahradník et al. 1984; Zahradník and Zachar 1985).

Materials and Methods

Solutions: The exposed muscle was kept in crayfish saline consisting of (mmol/l): 5.4 KCl; 208.4 NaCl; 13.5 CaCl₂; 5.6 MgCl₂; pH = 7.4 (5 TrisCl). The internal solution usually contained 1.0 EGTA; 1.0 MgCl₂; 4.0 Na₂ ATP to keep the contractile apparatus in relaxed state, and 240.0 CsGlut as the inert electrolyte. In the experiments described here an equimolar amount of CsGlut was replaced by 10.0 TEA₂SO₄ to block potassium channels, and by 10.0 Cs₂Ox to prevent release of Ca²⁺ from the internal stores. The extracellular solution contained 20.0 GaGlut₂; 220.0 CsGlut, and 10.0 HEPES. pH of both solutions was set to 7.5 by 10.0 HEPES and about 10 Cs₂CO₃. The vaseline for insulating strips was prepared by dissolving 1 g of Parafilm in 5 ml of paraffine oil at temperature just below the decomposition point.

Preparation: Muscle fibre fragments were dissected from the musculus extensor carpopoditi of the crayfish *Astacus fluviatilis* in two stages. First, the muscle was exposed according to Zachar et al. (1964); then, the fast procedure of Hille and Campbell (1976) for dissection of fibre fragments was

Abbreviations: ATP — adenosine triphosphate; EGTA — ethylenglycol-bis-(2-aminoethylether)-N,N,N',N'-tetraacetic acid; Glut — glutamate; HEPES — N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid; Ox — oxalate; TEA — tetraethylammonium; Tris — tris(hydroxymethyl)aminomethane

applied instead of isolating the single fibre. The dissected bundle of 2–6 fibre fragments was placed into a perspex chamber containing 2.5 ml of the internal relaxing solution. Subsequently, the fibres were separated from each other by peeling them apart with a fine forceps. During this procedure they attained original length and diameter. Then, the damaged ends were cut off and fragments of final length of 4–5 mm and about $300\text{ }\mu\text{m}$ in diameter were obtained. After about 10 min equilibration the healthy pieces could be recognized by glassy and soft appearance which was maintained for several hours. The selected fibre fragment without connective filaments was quickly transferred into the recording chamber by means of a forceps.

The fragment was installed in the recording chamber in the same way as described by Hille and Campbell (1976) with the exception that no second cut of the fibre ends was needed. Vaseline strips were applied over the fibre by means of a syringe through a $350\text{ }\mu\text{m}$ (i.d.) blunted needle. All seals were thoroughly inspected at high magnification and if necessary, improved using a stick with an eyebrow hair.

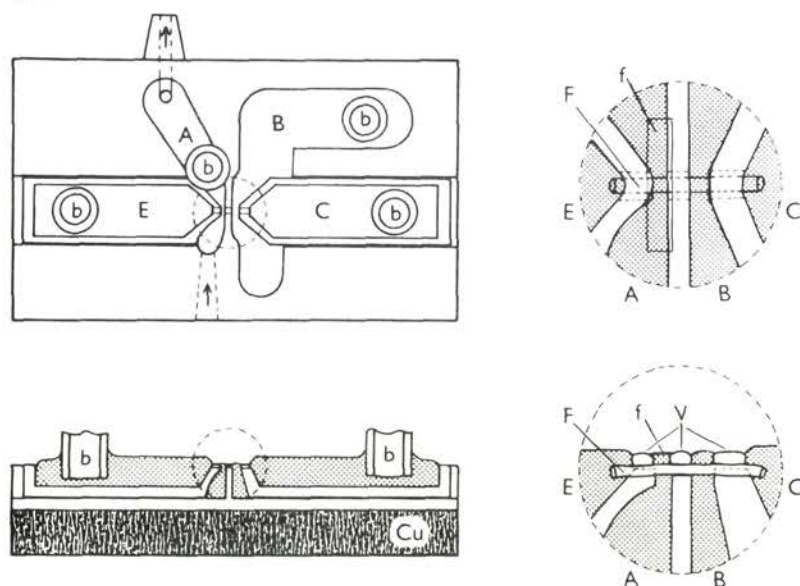


Fig. 1. The recording chamber. The drawing is approximately in scale. *Left* — a view from the top and a longitudinal section along the E — C axis. *Right* — details of the sealing region with the muscle fibre fragment in position. A — testing compartment (the flow of external solutions is shown by arrows). B — grounded, C — input, and E — output compartments. b — agar bridges. F — muscle fibre fragment. V — vaseline strips. f — cellophane cover. The recording chamber was mounted through its copper base (Cu) on the top of a water-cooled stage. Temperature in pool A was about 19°C .

Recording chamber: The design of the recording chamber is shown in Fig. 1. The voltage electrode bridge (b) is placed from above at the outlet side of the test compartment (A), just touching the upper level of the solution and as close as possible to the fibre. This, together with the boat-like shaping of the movable dish (E) and the small piece of cellophane (f) serving as a cover, have substantially improved the flow and exchange of the external solutions and minimized the access resistance.

The dimensions of partitions between the pools are: $EA = 700\text{ }\mu\text{m}$, $AB = 550\text{ }\mu\text{m}$, $BC = 900\text{ }\mu\text{m}$. Such wide gaps are essential for good insulation between compartments since muscle

fibres of the crayfish have relatively thick basement membrane and numerous longitudinal clefts (Uhrík et al. 1980). Perfect sealing of thick fibres by the vaseline strip is a serious problem. We solved it satisfactorily by drilling a groove (500 μm in diameter and 250 μm in depth) in all the three partitions along the longitudinal axis. The surface of these grooves is covered with a somewhat thicker layer of vaseline than the adjacent surface of the partitions. This forms a "bed" for the fibre fragment and makes perfect sealing easier.

The pools E, B and C are filled with the internal solution. In order to increase membrane resistance, CoSO_4 was added to the internal solution in pool B to a final concentration of about 10–20 mmol/l.

The widths of compartments A and B were usually set to 180 μm and 360 μm , respectively. These values were found as optimum by experience and they satisfy the criteria of Hille and Campbell (1976).

The internal solution in pool A was replaced by the external solution immediately before installing the voltage clamp regime. When the external solution was applied very quickly a contraction of the fibre in A often developed, apparently because of the influx of Ca^{2+} through a leaky membrane. However, the contraction could be prevented by slow perfusion and installing the voltage clamp and the holding potential between 10 and 30 seconds after the start of perfusion. If the holding potential was turned on later a slow contraction could be again observed.

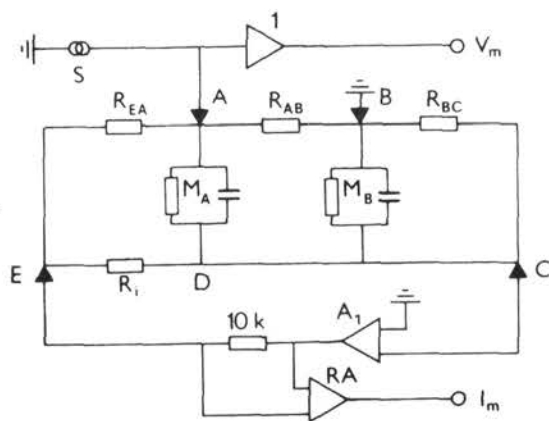


Fig. 2. The equivalent electrical scheme. For symbols A, B, C, and E refer to Fig. 1. S is the source of the holding and stimulating voltages. R_{EA} , R_{AB} and R_{BC} are resistances between compartments EA, AB, and BC. R_i — resistance of the fibre interior. A_1 — clamping amplifier. RA — differential amplifier (reads membrane current I_m). 1 — unity gain amplifier (reads membrane potential V_m). M_A and M_B are membranes of the fibre in compartments A and B. Point D is kept by A_1 at the ground potential (see Hille and Campbell (1976) and Zachar et al. (1982). Black triangles are Ag/AgCl electrodes.

Electronics: The electronic layout of the method is shown in Fig. 2. The design of the feedback circuitry was the same as described elsewhere (Henček and Zachar 1977; Zachar et al. 1982). Membrane currents were measured by means of a differential amplifier as voltage drop on a 10 k Ω resistor placed in the feedback. No attempt was done to compensate for the series resistance because its major part is distributed in the lumen of invaginated membranes, and other possible sources are minimized by the design of the experimental chamber.

Results

An example of membrane currents observed under our experimental conditions is shown in Fig. 3. The figure was obtained from individual sweeps applied every

5 seconds by superposition onto a memory oscilloscope screen. The zero current line recorded at ± 10 mV voltage pulses from $V_h = -80$ mV shows the quality of the leakage and capacitive currents compensation. At the threshold depolarization to -50 mV a small steady current is activated which does not decrease at pulses lasting for several seconds. At higher depolarizations a two-phasic current waveform is activated. The activation of the transient component slightly accelerates with depolarization, which is manifested in the shift of the peak from 11 ms at -40 mV to 5 ms at 0 mV. Distortions of current onsets at depolarizations above $+40$ mV are caused by some nonlinear properties of the preparation. Interestingly, the rate of inactivation markedly decreases with depolarization. At very positive voltages practically no inactivation is seen at the time scale of the records used.

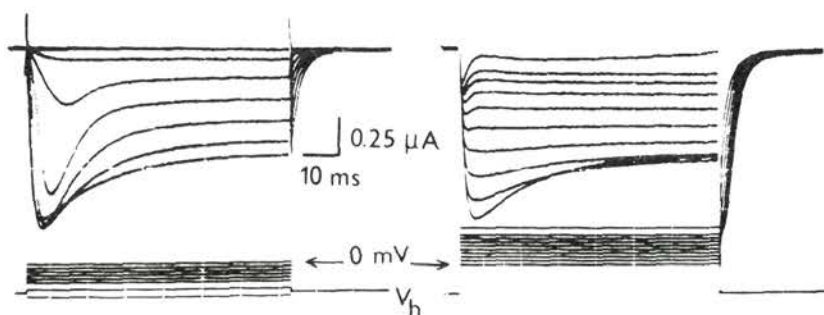


Fig. 3. Calcium currents recorded by means of the vaseline gap technique. Family of Ca currents (upper traces) in response to voltage clamp pulses (lower traces) is divided into two groups for clarity. *Left* — voltage range from -90 mV to 0 mV. *Right* — voltage range from 0 mV to $+100$ mV. Holding potential $V_h = -80$ mV. Experiment (100683) on a fibre fragment equilibrated for 84 min in the internal solution, and 7 min after installing voltage clamp regime.

The transient component relaxes to the steady component of the inward current. The decrease of the steady inward current is very slow and can be followed at very long pulses lasting up to several seconds. In contrast to the transient component, the decrease of the steady one is faster for large current amplitudes.

At the end of the pulse a brief tail current is flowing for about 15 ms. Its amplitude estimated at 3 ms after the pulse end increases with depolarization and attains maximum at $+40$ to $+60$ mV, so that it shows an S-shaped current-voltage curve with half-maximal activation at about 0 mV (not shown).

It should be noted that only inward currents are recorded over the whole range of membrane depolarizations. They are carried by Ca^{2+} ions through calcium channels as may be judged from the composition of the experimental

solutions and from the current-voltage curves (Fig. 4, also see Zahradnik and Zachar (1982a) for further evidence).

Current voltage curves (Fig. 4) corresponding to I_{Ca} shown in Fig. 3 were constructed from the peak values of the transient component and from the last values of the steady component at the end of the 70 ms pulses. As no transient can be identified at high depolarization, in this case the peak amplitude coincides with the steady one.

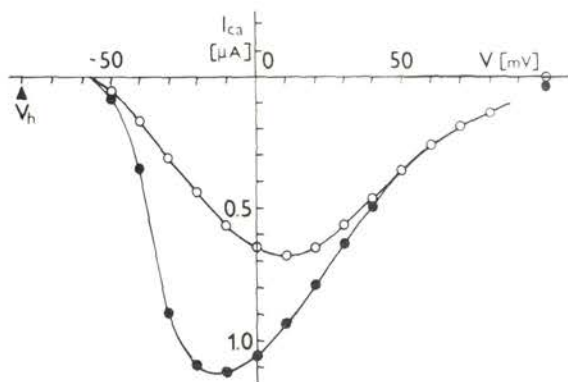


Fig. 4. Current — voltage curves of the transient and steady Ca currents. Constructed from traces shown in Fig. 3; full symbols — peak values; empty symbols — steady values. The lines were drawn by eye.

The potential of maximal transient $I_{Ca, T}$ activation is $V_{m, T} = -14$ mV and that of half-maximal activation is $V_{1/2, T} = -36$ mV. The corresponding values for the steady Ca current $I_{Ca, S}$ are: $V_{m, S} = +10$ mV and $V_{1/2, S} = -28$ mV. The differences between T and S values are highly significant as can be seen from the mean values shown in Table 1.

Table 1. Mean values of Ca currents and characteristics of the tested membrane area.

I_{Ca}	V_m [mV]	$V_{1/2}$ [mV]	maximal I_{Ca} density	
			apparent [mA/cm ²]	overall [μA/cm ²]
Transient	-4.6 ± 6.7	-31.9 ± 5.7	1.03 ± 0.49	26.4 ± 12.6
Steady	13.8 ± 4.5	-22.6 ± 5.6	0.65 ± 0.29	16.7 ± 7.4
T — S	18.4 ± 4.2	9.4 ± 1.5		
S/T			0.66 ± 0.06	
Geometry: $A = 0.180 \pm 0.025$ mm			$d = 0.304 \pm 0.037$ mm	
$P_a = 0.174 \pm 0.039$ mm ²			$P_o = 6.79$ mm ²	

Mean values ($\pm$ standard deviations) were calculated from experiments on 8 muscle fibre fragments excised from different animals. V_m — potential of maximal Ca current ($I_{Ca}^{\max}$) activation; $V_{1/2}$ — potential of half-maximal ($1/2 I_{Ca}^{\max}$) activation; T = $I_{Ca, T}^{\max}$; S = $I_{Ca, S}^{\max}$; A — width of pool A; d — fibre diameter; P_a — apparent surface area of the tested membrane $P_a = \pi \cdot d \cdot A$; P_o — overall area calculated as $P_o = 39 \cdot P_a$ (Uhrík et al. 1980).

The descending part of the I — V curves is linear for a small range of positive voltages. For $I_{Ca,T}$ it is between 10 to 40 mV and for $I_{Ca,S}$ between 30 to 60 mV. At more positive potentials, the I — V relationship is sublinear and tends to cross the potential axis above 100 mV. A true reversal potential V_R cannot be measured reliably as in this voltage range there is a relatively large error caused by nonlinearities of the preparation and by the leakage current subtraction technique. At very high depolarizations it is possible to observe also outward currents, but these are not stable during the life-time of the preparation. They progressively decrease, and at the end of the experiment even an "extra" current of inward polarity appears.

Maximal amplitudes of calcium currents measured by this voltage clamp technique are usually within a range of 0.8 to 3.2 μ A. As the dimensions of the measured portion of the fibre fragment are known, it is possible to evaluate surface current density. It is usually between 0.5 to 2.0 mA/cm² and in the case shown in Fig. 3 it is 1 mA/cm². Mean values of Ca current density given in Table 1 were calculated in two ways. The apparent density is related to the surface of the measured membrane taken as a simple cylinder. The overall density was calculated taking into account branching of the surface membrane into invaginated parts. This branching increases the apparent surface area by a factor of 39 in average (Uhrik et al. 1980).

The steady I_{Ca} is on average 2/3 of the transient I_{Ca} at their I — V maxima. In running current records, the greatest difference is observed at about -20 mV before maximal $I_{Ca,T}$ activation (see Fig. 3).

It is obvious that the transient and the steady components of the Ca current behave independently. Differences in both I — V curves and inactivation properties led us (Zahradnik and Zachar 1982a) and Jdaïãa and Guilbault (1986) to conclude that there are two populations of Ca channels in the surface membrane of the crayfish. The method used in the present work allows to test this conclusion under qualitatively better conditions. A two-pulse experiment (Fig. 5) showed selective depression of the transient Ca current by inactivating prepulse. The pulse protocol is shown in the inset. $I_{Ca,T}$ elicited by the test pulse decreased proportionally to the I_{Ca} amplitude activated by the prepulse. The decrease was the greatest at a prepulse potential of -10 mV. At higher depolarizations I_{Ca} at the prepulse decreased and inactivation of $I_{Ca,T}$ at the test pulse also slightly decreased. At the same time, no changes in $I_{Ca,S}$ amplitude at the end of the test pulse could be observed. This means that after attaining a steady state I_{Ca} flows through a different population of Ca channels than during the transient phase.

The decrease of the transient I_{Ca} may, in principle, be caused by Ca dependent activation of an outward current carried by a different ion. This possibility should be tested since Henček et al. (1978) have identified an early potassium conductance activated by Ca entry in crayfish muscle fibres. The test experiment

is shown in Fig. 6A. Two pulses of equal amplitudes (60 mV) and divided by a constant gap (10 ms) were applied in succession. The length of the first pulse was increased between individual sweeps from 10 to 80 ms. The superposition of records enables direct comparison of the time course of I_{Ca} inactivation during the first pulse, of peak $I_{Ca, T}$ decrease at the second pulse, and of the tail current amplitude decrease at the end of the first pulse. Kinetic analysis of these three processes (Fig. 6B) confirms that all of them have the same rate and consequently are of the same origin. This could be expected since under our experimental conditions, Cs^+ ions are the only candidate for charge carrier through these K channels. The equilibrium potential of Cs^+ is about 4 mV; thus they can give outward currents at positive voltages only. Therefore, a decrease of the tail currents which flow at very negative voltages should reflect a decrease in the number of open channels at the end of the first pulse.

The experiment illustrated in Fig. 6 also extends the conclusion from Fig. 5, namely that the decrease of $I_{Ca, T}$ at the test pulse is proportional not only to the amplitude of I_{Ca} at the prepulse but also to its duration. In other words,

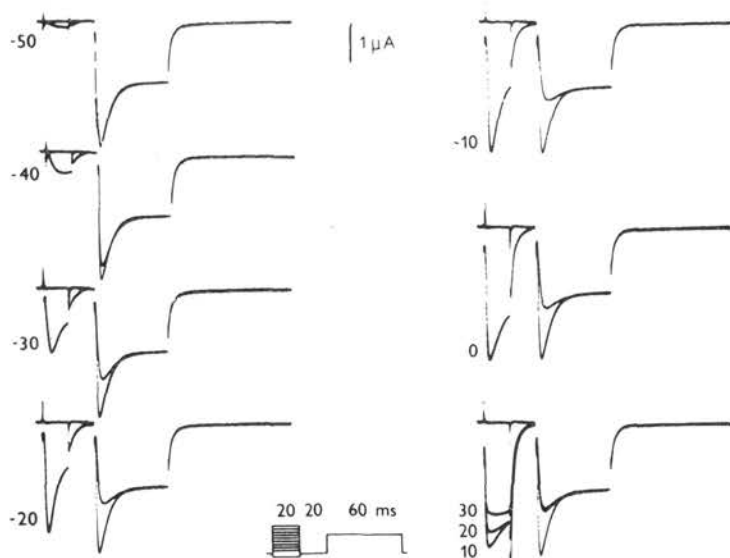


Fig. 5. Inactivation of the transient Ca current by a conditioning prepulse. The pulse protocol is shown in the inset (constant test pulse to -10 mV is preceded by variable prepulses). Each record is a superposition of two sweeps (except of the lower right). The first sweep, with a prepulse to -90 mV, is the same for all records. Prepulse potentials of the second sweep are given at the corresponding current traces. Experiment 220483, equilibration 44 min, 26 min under voltage clamp.

inactivation of the transient Ca current is proportional to the total amount of current carried by Ca^{2+} ions.

A closer inspection of the currents shown in Fig. 6 reveals that there are remarkable changes in their time course. Activation of the prepulse I_{Ca} is evidently faster than I_{Ca} activation at the last test pulse (time to peak is 9 and 14 ms respectively). Also, the tail current after a 10 ms prepulse looks like a single exponential but the last tail is clearly biexponential. It may be concluded that the activation process of the transient Ca current is faster than that of the steady Ca current. The difference is probably even greater as can be observed because fast processes are impeded by the time response of the preparation.

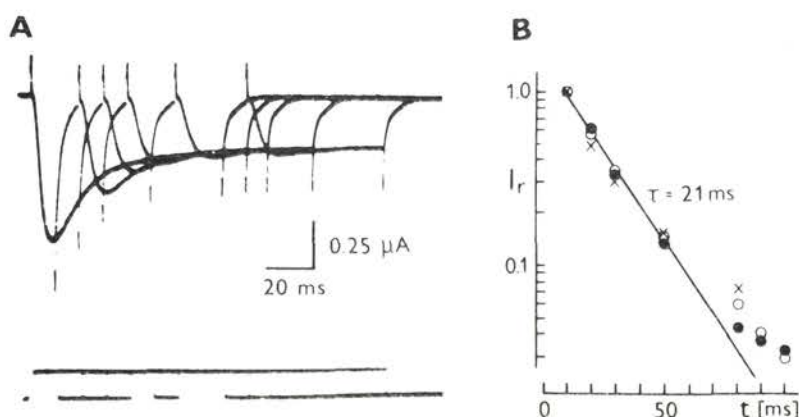


Fig. 6. Time dependence of the transient Ca current inactivation. *A*: Superposition of 5 records differing in the duration of the prepulse (-20 mV; 10–80 ms). The test pulse (-20 mV; 60 ms) follows the prepulse after a constant interval (-80 mV; 10 ms). *B*: Semilogarithmic plot of relative current amplitudes against time, constructed from records shown in *A*. (●) — relative decrease of the prepulse Ca current in time. (○) — relative decrease of the peak amplitude of tail currents against prepulse duration. (x) — relative decrease of the test pulse peak Ca current amplitudes against prepulse duration. The straight line was drawn by eye and it gives a time constant of inactivation $\tau = 21$ ms. The deviation from linearity is due to a slow component of inactivation. Experiment 240383, equilibration 75 min, 65 min under voltage clamp.

Recovery from inactivation can be studied in experiments similar to that shown in Fig. 7. When the pause after a constant prepulse is prolonged in individual sweeps, the test pulse Ca current progressively increases. In the time scale of Fig. 7, recovery was completed to only about 92 % and occurred with two time constants, 80 and 730 ms, respectively. These time constants are several times greater (slower) than the time constant of $I_{\text{Ca},T}$ inactivation at the prepulse; hence they may be hardly supposed to express simple backward reaction steps of physical nature. Rather they may reflect some sequence of

biochemical events. In any case, this experiment can be considered as evidencing that transient I_{Ca} is not a result of a loose clamp in the tubular membrane network but rather a real process associated with a population of inactivating Ca channels.

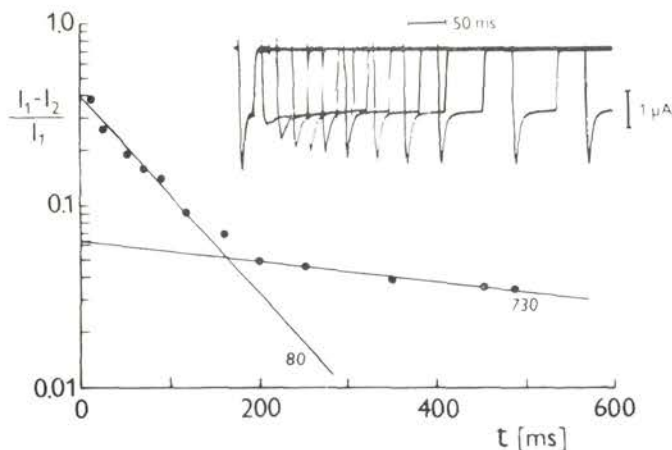


Fig. 7. Recovery of the transient Ca current from inactivation. Semilogarithmic plot of the relative peak amplitudes of the test pulse Ca current (I_2), related to the peak amplitudes of the prepulse Ca current (I_1), against duration of the gap between pulses. Data are taken from records shown in the inset. The time constants of the recovery are given at the straight lines. The inset is a superposition of several sweeps in which the prepulse (0 mV, 20 ms) was followed by the test pulse (0 mV, 60 ms) after a progressively prolonged gap (−80 mV, 10–450 ms). Experiment 060583, equilibration 50 min, 39 min under voltage clamp.

Discussion

Calcium currents in the crayfish muscle fibres have a biphasic time course if overlapping potassium currents are sufficiently depressed. This has been already demonstrated by us (Zahradník and Zachar 1982a), and Jdaiãa and Guilbault (1986) using different techniques. It was hypothesized that two types of Ca channels differing in their inactivation mechanisms are present in the surface membrane.

Ca currents measured by the vaseline gap technique are well developed and free from artefacts over a wide range of membrane voltages (Fig. 3). This is better than could be expected if one considers that the greater part of the membrane (75 %) is localized behind the series resistance (Poledna et al. 1978).

However, coincidence of three factors makes voltage clamp conditions better than expected. First, overall I_{Ca} density is really low (see Table 1). Second, total membrane current (leak + Ca current) is close to zero up to 0 mV membrane potential, as was observed without leakage current compensation. Third, I_{Ca} is

inward so it drives membrane potential to more positive values in regions where the voltage control is not sufficiently fast, and compensates for the voltage drop on the series resistance. As a result, fidelity of the I_{Ca} measurement is satisfactory for many purposes. The good quality of voltage control is confirmed by the activation range of $I-V$ relationships (Fig. 4), which amounts to 40 and 60 mV for the transient and the steady Ca currents, respectively.

Adequate experimental conditions can be easily disturbed by some interventions. For instance, if Ba is substituted for Ca in the extracellular solution, inward current increases about 4-fold, and as a result, increasing depolarization from the threshold by 10 mV leads to the activation of maximal Ba current with clear signs of loose spatial voltage control. Similarly, at the end of the experiment certain swelling of the fibre segment tested occurs. In this case, inappropriate spatial clamp is obtained even with smaller Ca currents. Thick fibres (above 350 μm) are also unsuitable from these reasons.

Limitations of the method are given by passive electrical properties of the preparation, particularly by its relatively large time constants. Capacitive transients measured at small hyper- and depolarizing pulses declined with 2 time constants: the fast one was about 0.9 ms and the slow one up to 3 ms; 90 % of the capacitive current decreased within 3 ms. It is clear that slow charging of membrane capacitance must influence the kinetics of Ca current activation. Therefore no experiments were done in this direction.

More favourable conditions are at later times, when membrane potential is settled, because small Ca current density will not significantly disturb it. Therefore, it is reasonable to suppose that the decline of the transient Ca current is a real process reflecting inactivation of Ca channels as is usual in other cell types (for a review see Eckert and Chad 1984; Tsien 1983; Hagiwara and Byerly 1981).

Three types of experiments give evidence for the above: inactivation elicited by different prepulse amplitudes (Fig. 5), inactivation by different prepulse duration (Fig. 6), and recovery from inactivation (Fig. 7). The following conclusions can be drawn:

1. Decline of the transient Ca current is not an artefact due to instability of voltage in the invaginated membranes, nor a result of outward current activation. Such a case was analysed already in the giant muscle fibres of the *Balanus* which are similar in morphology to crayfish muscle fibres. Keynes et al. (1973) and Hagiwara et al. (1974) have shown that the apparent inactivation of Ca current in the former preparation is a result of interaction between Ca and K conducting systems in deeply invaginated membranes. This is not our case as K currents are fully removed and no other outward currents are activated (Fig. 6). Moreover, the presented Ca currents are free of oscillations, their activation is smooth, without delay, and tail currents are fast and without an overshoot.

Recovery is too slow to be explained by stabilization of the membrane potential after the prepulse.

2. Transient and steady Ca currents behave independently. They can be separated by short inactivating prepulses (Figs. 5, 6) which strongly suppress the transient current and leave the steady one intact. They have different ranges of activation voltages and significantly differing potentials of the maximal current activation. The threshold potential of activation seems to be slightly more negative for the steady current than for the transient one. Ca current decline to a steady level was observed under certain experimental conditions also in other cell types which possess Ca channels inactivating by a Ca-dependent mechanism. This effect was explained by the "calcium domains" hypothesis (Chad and Eckert 1984); however, in those circumstances "steady" Ca current always depends on peak current and it cannot be separated from the former.

3. Inactivation of the transient Ca current depends on preceding integral Ca current rather than on membrane potential. This is evidenced by a decreasing rate of decline with increasing depolarizations (Fig. 3), and by partial release from inactivation at very positive prepulses (Fig. 5). Such observations are typical for the Ca-dependent mechanism (Eckert and Chad 1984). Henček and Zachar (1977) proposed a voltage-dependent mechanism of Ca current inactivation of crayfish muscle fibres by analogy to Na channels. However, their technique of separating Ca currents from K currents did not allow them to study inactivation at positive voltages. Both voltage- and Ca-dependent mechanisms operating simultaneously have been supposed by Jdaïâ and Guilbault (1986) and Zahradník and Zachar (1982b). This problem needs further investigation.

4. All these results taken together give further support to the hypothesis concerning the existence of two populations of Ca channels in the surface membrane of the crayfish muscle fibres. Their inactivation mechanisms and distribution in the membrane will be analysed in our forthcoming papers.

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