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EVIDENCE FOR A TRANSIENT POTASSIUM MEMBRANE CURRENT DEPENDENT ON CALCIUM INFLUX IN CRAB MUSCLE FIBRE

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SUMMARY

1. Voltage-clamp experiments were achieved on crab muscle fibre with the double sucrose-gap technique.

2. The accuracy of the imposed voltage has been controlled with an impaled micro-electrode connected to an external circuit.

3. Step depolarizations elicit two kinds of records. In type I fibres, the initial current exhibits only an inward calcium component. In type II fibres, the initial current exhibits a hump, transient outward current, mixed with the calcium current; these fibres exhibit always action potentials with fast repolarization.

4. A potassium origin is suggested for this outward current, due to its dependence on $[K]_o$ and its inhibition by TEA.

5. In fibres with a composite initial current, the voltage dependence of the availability of the measured inward current appears complex. It can be shown to be the sum of a simple calcium inactivation (which is observed alone in TEA solution) and a fast potassium inactivation. This potassium conductance is nearly half-available at the resting membrane potential.

6. The origin of the transient outward current is tentatively described. Consecutive to a transient internal increase of calcium ions (due to the calcium current) its activation curve is shifted in an hyperpolarizing direction resulting in an increased activation for an apparent identical depolarization.

7. This fast outward current which overlaps the calcium inward current can account for the low amplitude and the variability of the electrical activity of crab muscle fibres.

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INTRODUCTION

Though ionic movements involved in the electrical activity of crustacean muscle fibres have been extensively reported (Fatt & Katz, 1953; Hagiwara, Hayashi & Takahashi, 1969; Keynes, Rojas, Taylor & Vergara, 1973; Hagiwara, Fukuda & Eaton, 1974), the occurrence of graded responses as well as autoregenerative action potentials on the same or similar fibres have not yet been fully explained.

It is well established that the inward current, responsible for the depolarization, is carried by calcium ions (Fatt & Ginsborg, 1958; Hagiwara & Naka, 1964; Hagiwara & Nakajima, 1966; Hagiwara & Takahashi, 1967). Though Mounier & Vassort (1975) confirm that the electrical response of crab muscle depends upon calcium ions, they also suggest the existence of a fast outward current that partly masks the calcium current.

The aim of the present report is the identification of the hump observed on the current traces during voltage-clamp conditions as a fast outward current, labelled I_{K1}. Some characteristics of this current are described. The results can account for the low amplitude and the variability of the electrical activity of crab muscle fibres.

METHODS

The preparation, method and solutions were essentially similar to those described in the previous paper (Mounier & Vassort, 1975). Crab muscle fibres were isolated from the meropodite and set up in a double sucrose-gap apparatus. The voltage-clamp device (Rougier, Vassort & Stämpfli, 1968) is completed by a micro-electrode (> 7 MΩ) which allows the measurement of the voltage in the test-gap inside, or just outside, the fibre (Fig. 1). When outside, the micro-electrode measures the external voltage which is slightly different from V, the imposed voltage, due to a voltage drop across the serial resistance (R_s). This difference, which never exceeds a few mV, allows the determination of an approximate value of the serial resistance ($\leqslant 5$ kΩ). When impaled, the micro-electrode measures the internal voltage which has to be constant since in this device the feed-back amplifier maintains the interior of the fibre at the ground potential.

RESULTS

Control of the imposed membrane voltage

The double sucrose-gap technique allows the use of large electrodes and thus large instantaneous currents can be delivered. However, the electrodes are far from the surfaces of the membrane to be controlled. In particular the outside resistance which is in series with the membrane resistance can modify the applied voltage; amplified by the negative feed-back amplifier this difference between the applied voltage and the effective membrane voltage could lead to large oscillations and transient variations of the internal voltage.

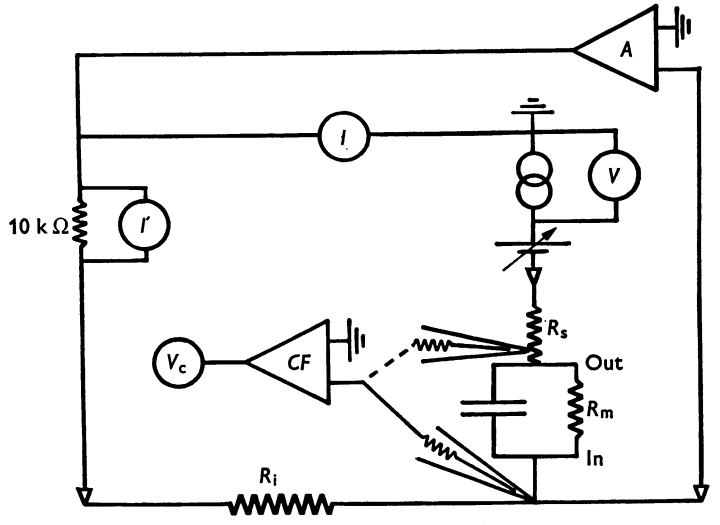

Fig. 1. Diagrammatic arrangement for voltage-clamp recording with extracellular electrodes on the simplified equivalent circuit of the cell. R_m, membrane resistance in the test compartment; R_i, effective longitudinal resistance; R_s, serial resistance; A, feed-back amplifier; V and V_c, oscilloscope (Tektronix 565) for measuring imposed and controlled voltage; I and I', oscilloscope for measuring current as the potential drop across R_i or across a 10 kΩ serial resistance; CF, cathode-follower.

The records on Fig. 2*A* illustrate the current elicited by a depolarization of 38 mV and the controlled voltage, V_c, at the head of a micro-electrode just outside the membrane. V_c shows transient variations due to the voltage drop across the serial resistance. After impaling the micro-electrode (holding potential, $E_r = -66$ mV in this experiment) the current and the controlled voltage are recorded for the same depolarization (Fig. 2*B*). A larger inward current is elicited by lower depolarization (25 mV, Fig. 2*C*), however, in both cases the internal voltage remains constant except for the fast transients corresponding to the capacitive currents. The same accuracy of the internal voltage control is obtained for a fibre which exhibits a large hump during high depolarizations (Fig. 2*D*). This is also true in strontium and TEA solutions where the inward currents are larger (Fig. 2*E*). No noticeable variation is observed according to the localization of the micro-electrode in the test-compartment, including near the current-pool. These results suggest good voltage control with no evidence for oscillations although this does not exclude non-uniformity in the tubular system. It is worth noting that a decrease of the accuracy of the voltage clamp is shown by variation in the intracellular voltage, as well as by a clearly observed inward current which is uncontrolled. In this

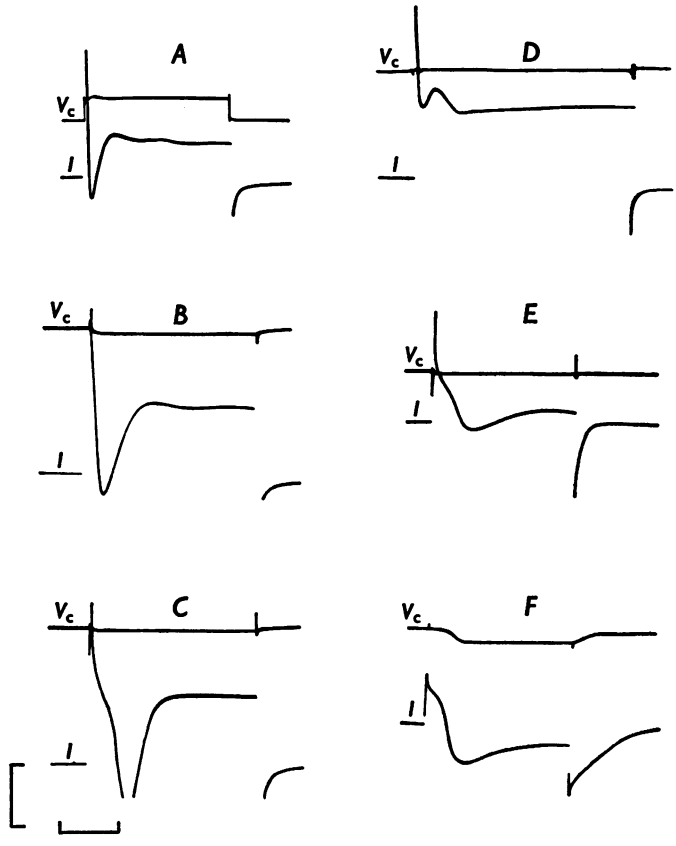

Fig. 2. Simultaneous records of V_c, controlled voltage, and I, membrane current. In A, the micro-electrode is supposed to be just outside the membrane, so that the potential across the membrane equals V_c. $V_c = V - R_s I$, with I positive or negative; $R_s . I$ can be as large as 5 mV at the peak of the inward current. In B, C, D or E, the micro-electrode is inside the cell. These records show that the internal compartment is closely maintained to the ground, there is no transient variation even during the large inward currents or the hump. F is an example of uncontrolled internal voltage. A–B, ASW solution, imposed membrane voltage of + 38 mV; C, ASW solution, imposed membrane voltage of + 25 mV (same fibre); D, ASW solution for another fibre with hump, imposed membrane voltage of + 80 mV; E and F, strontium solution. Vertical scale: V_c = 100 mV in A; 40 mV in B–F. I = 0·4 μA in A, D, E, F; 0·1 μA in B and C. Horizontal scale: 20 msec in A, D, E, F; 10 msec in B and C.

last case, in strontium solution (Fig. 2F), the inward current appears suddenly with its maximal amplitude for a slightly subthreshold depolarization. This current is initiated after a long delay but activates very rapidly. Further increase in the depolarization amplitude decreases the

delay but does not modify the activation rate and the amplitude of the response.

It was impossible to keep the micro-electrode inside the fibre throughout the experiments because the cell membrane appears very fragile and because the fibre develops strong contractions during depolarizing pulses; a large leak current then rapidly develops. Thus, the studied voltage-clamped fibres were selected on the basis of some criteria resulting from the above observations: the amplitude of the inward current and its rate of activation must smoothly increase or decrease respectively with small increase of the depolarizing pulse.

In some experiments, less than 10 %, the inward current was followed by train of large oscillations similar to those described for other crustacean fibres (Strickholm, 1963; Hagiwara & Naka, 1964; Keynes *et al.* 1973). Those fibres were discarded because of a non-uniform voltage control.

Evidence for a transient outward current

ASW solution

The occurrence of different electrical activities (graded or autoregenerative) elicited by stimulation of similar crab muscle fibres is well reported (Atwood, 1965). Further differences are emphasized on electrical responses during current clamps (Fig. 3). The repolarization of given fibres is markedly faster than for other fibres and is followed by a negative post-potential. Similarly differences shown are in the current traces recorded during voltage clamp. With slow repolarizing fibre (type I) the inward current inactivates rather exponentially and is followed by a delayed current. When clamping back to E_r an outward tail-current is observed though often smaller than in the illustrated experiment. On the other hand voltage clamping a fibre the electrical activity of which repolarizes faster (type II) always results in an inward current abbreviated by a fast outward current. These initial currents are followed by a small delayed outward current.

The two current–voltage relationships summarize these typical results (Fig. 3*C*). No global inward current is recorded and this was the situation in most of the experiments. The reversal potential of the inward current, V_{rev}, after extrapolation of the leak current determined during hyperpolarizations is +27 mV (V_{rev} = +92 mV) for a type I fibre. The reversal potential ranges from +25 to +30 mV in different experiments of this kind. For a fibre with a noticeable hump (type II) the reversal potential of the inward current is +15 mV. It ranges between 0–15 mV for different fibres the electrical response of which is generally smaller and with a fast repolarization. In a few cases, the initial current did not exhibit an inward component but only the hump, outward current, could be recorded. The leak current, defined as the current elicited by small depolarizations or

hyperpolarizations, is always larger for a fibre which exhibits a hump. Summarizing five typical experiments of both types, the resting conductance of the membrane was double for fibres of type II.

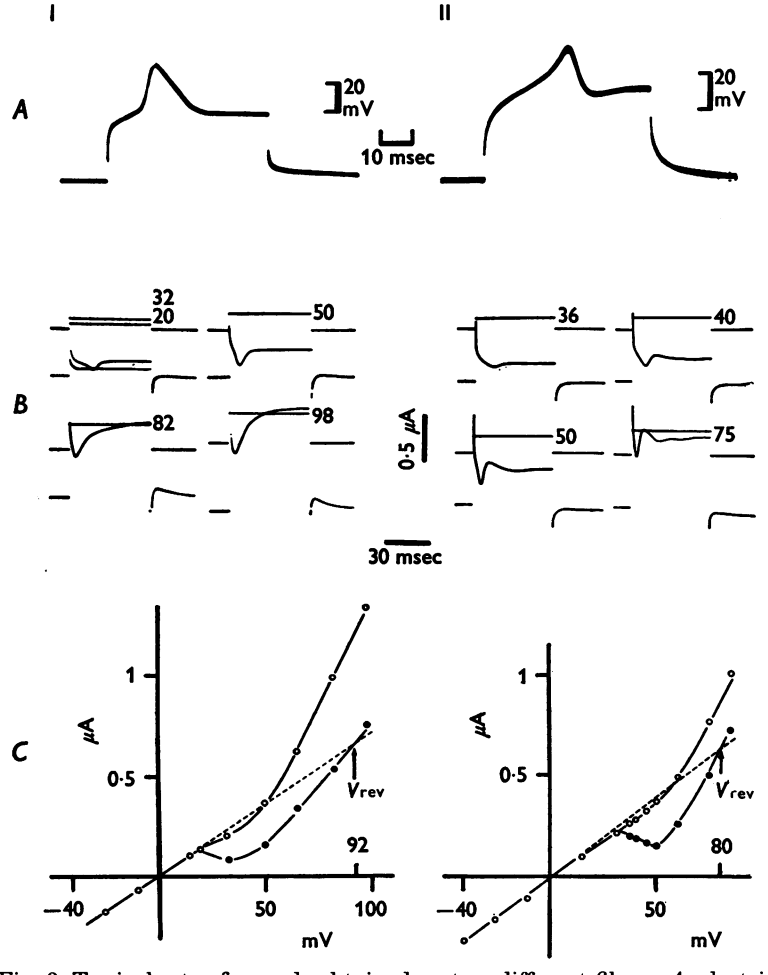

Fig. 3. Typical sets of records obtained on two different fibres. *A*, electrical responses elicited in current clamp. In I, the repolarization is slower than the depolarization. Both phases have the same rate in II, moreover the repolarization is followed by a transient hyperpolarization. *B*, corresponding voltage-clamp traces. A hump is markedly developed in II. An outward tail occurs at the end of a 55 msec-step on type I fibre, though for both fibres I and II holding potential equals the resting potential, E_R. *C*, current–voltage relationships. (Peak inward current, filled circles; maximal outward current measured at the end, open circles.) The reversal potential of the inward current, V_{rev} is larger for fibre of type I ($V_{rev} = +92$ mV) than for fibre of type II ($V_{rev} = +80$ mV).

The fact that different characteristics such as fast repolarization of the electrical activity, existence of a hump, low reversal potential and large resting conductance are always found together on some fibres but none of them on the others, and the described control of the voltage-clamp technique lead us to suppose an ionic origin of the hump and to discard the idea of a damped oscillation. Further investigations on the origin of this fast outward current are necessary. Two ionic species can be thought to carry this current: either outward-going potassium ions or inward-going chloride ions. The use of TEA ions which are known to block at least partially the potassium conductance (Koppenhöffer, 1967; see also Stanfield, 1970) and modifications of the ionic composition of the bathing solution should provide us with the nature of the carrying ion and provide further experimental evidence for the ionic origin of the hump. Chloride-poor solutions do not modify this outward current and their effects are reported elsewhere (Mounier & Vassort, 1974).

Influence of TEA ions

In TEA solution (20 mM) the electrical response, even if graded in ASW solution, becomes always regenerative, increases in amplitude to reach 90–95 mV and is prolonged (Fig. 4*A*). The threshold is generally decreased. When applied to a fibre during voltage clamp, TEA increases the peak amplitude of the inward current as well as its time to peak. Those effects are more marked with fibres which exhibit a hump in ASW solution (type II). Simultaneously with the increase of the inward current the hump decreases and disappears, leading to a rather exponential inactivation of the inward current (Fig. 4*B*). Whatever the kind of fibre, with or without hump, after TEA treatment the inward current reverses for depolarizing steps in the range of 95–100 mV, that is to say an apparent E_{rev} of +30, +35 mV. The peak current–voltage relationships established in ASW solution and then in TEA solution clearly show a shift of the apparent reversal potential by about 25 mV (Fig. 4*C*).

The effects of TEA solution (increase of the electrical activity, increase of the inward current, suppression of the hump and shift of the reversal potential) can be explained by assuming that TEA ions block a fast outward current which flows partly at the same time as the inward current.

Thus, the apparent reversal potential of the inward current in ASW solution is lower than its true reversal potential.

Influence of a potassium rich solution

The disappearance of the fast outward current in TEA solution suggests that it is carried by potassium ions. This is confirmed by its sensitivity to a potassium-rich medium.

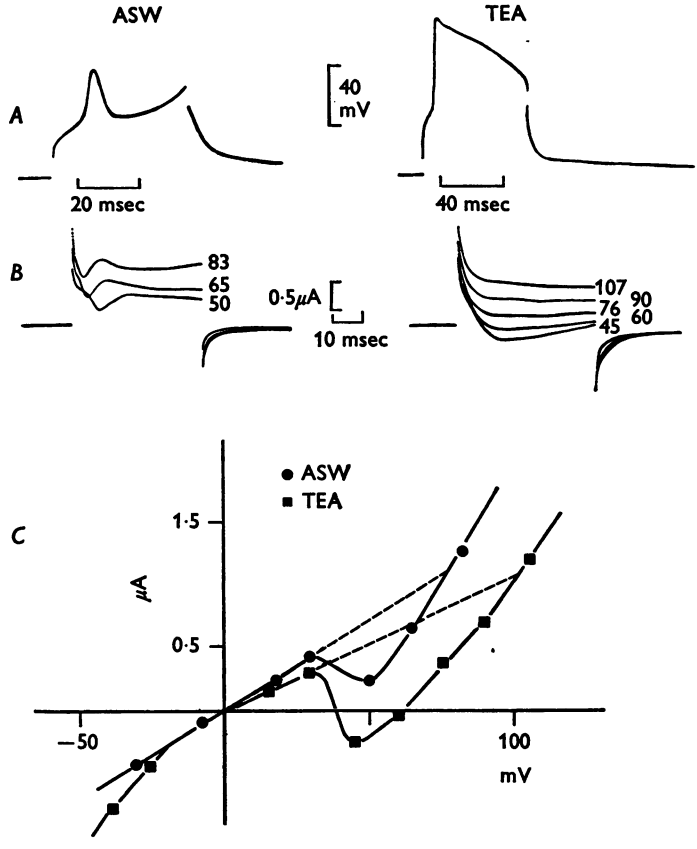

Fig. 4. Action of TEA solution (20 mM, 15 min) on electrical activities of a crab muscle fibre. *A*, the amplitude and the duration of the electrical responses obtained in current-clamp conditions are markedly increased in TEA solution. *B*, correspondingly the hump on the current traces has disappeared and the inward current appears larger. *C*, peak current–voltage relationships established in ASW (circles) and in TEA solutions (squares). Notice the shift (25 mV) of the apparent reversal potential of the inward current in TEA solution as evidenced by the intersection of the two relationships with the respective extrapolated value of the leak current (fibre 1609).

Currents elicited by a 57 mV-depolarization in ASW solution ($[K]_o$ = 12·9 mM) or in potassium-rich solution ($[K]_o$ = 64·5 mM) are illustrated on Fig. 5*A*. The leak current increases considerably in potassium-rich solution; this prevents very detailed experiments. The hump clearly disappears, as would be expected for a potassium current since the driving force for potassium ions is then reduced by 41 mV due to the fivefold increase in $[K]_o$. Moreover, assuming a small delayed current in both cases (ASW

and potassium-rich solutions) so that the late current represents mainly the leak current, the inward current increases noticeably in potassium rich solution and its inactivation becomes nearly exponential with a time constant of 15 msec (Fig. 5*B*).

Voltage dependent inactivation of the fast outward current

According to the preceding observations, during voltage clamp in ASW solution, some fibres (type II) exhibit two initial ionic currents which flow in opposite directions. Though these currents show rather similar time constants in the voltage range studied it may be possible to modify their

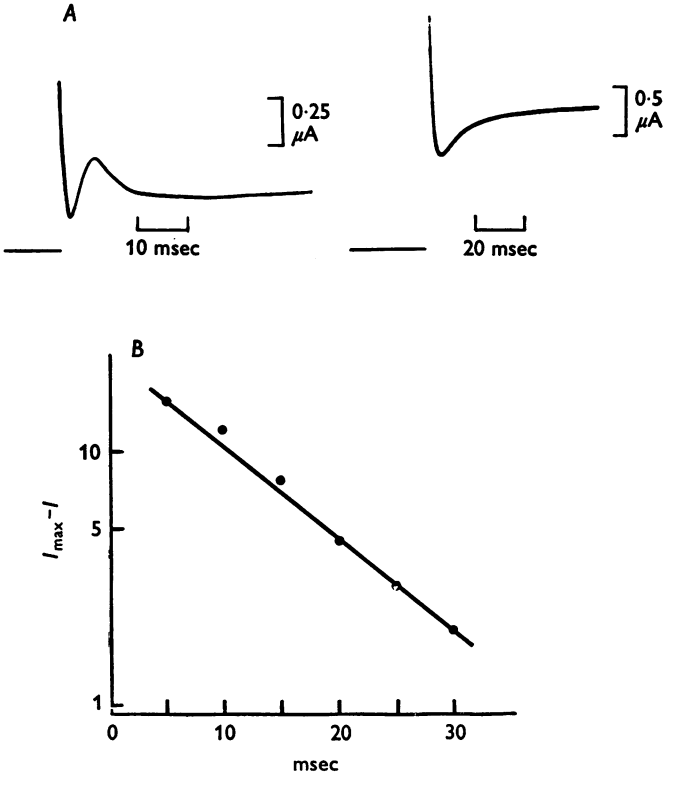

Fig. 5. *A*, current traces recorded for a 57 mV-depolarization in ASW (left) and in potassium-rich solution ($[K_o] \times 5$; right); in *B*, semilogarithmic plot of the inactivation of the inward-current in potassium-rich solution. Note that it is a simple exponential (time constant 15 msec).

relative amplitudes, and thus to dissociate more clearly the two currents, by conditioning polarizations of the membrane if their respective conductances do not have the same voltage dependence of their inactivation

variables. In other words, it is expected that conditioning polarizations of the membrane will alter more specifically the availability of one of these two currents.

The availability of the calcium conductance is a simple function of the membrane potential for crab muscle fibre in TEA solution or for fibre (type I) which does not exhibit a hump on current traces (Mounier & Vassort, 1975; their Fig. 7*B*).

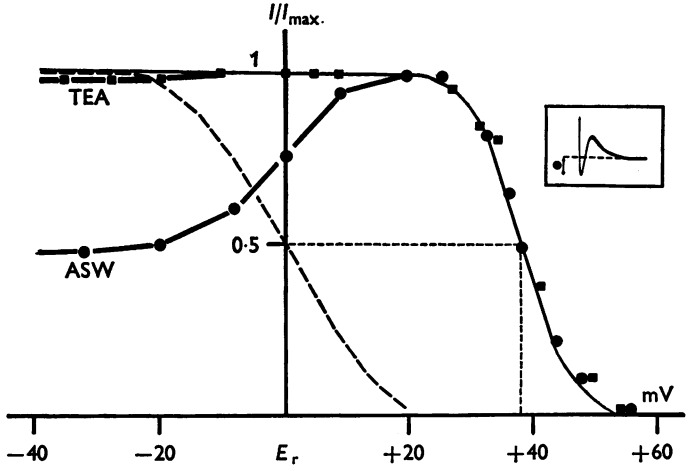

Fig. 6. Voltage-dependence of I_{Ca} and I_{K1}. Voltage-dependence of fractional availability of the inward current measured as the relative value of the peak current during depolarizing step of 56 mV for a fibre showing a hump on the current trace. The curve (thinner continuous line) is drawn according to the equation $I/I_{max} = 1/1 + \exp[(V_H - V)/K]$ where $V_H = 38$ mV and $K = 4$. The apparent decrease of availability of I_{Ca} observed in ASW solution (circles) for conditioning low depolarizations or hyperpolarizations is suppressed by TEA solutions (squares). The dashed line is the relative difference between the two preceding voltage dependences. It gives an approximative shape of the voltage characteristic of I_{K1}; half-inactivation is for the resting potential.

Similar experiments performed with fibres with a noticeable hump on current traces give different results. A 56 mV test pulse is preceded by 95 msec conditioning polarizations of different amplitude. The amplitude of the current is measured after extrapolation to short time of the late and leak current (Fig. 6 inset). The voltage-dependence of the inactivation variable seems a rather complex function (Fig. 6). The inward current is maximal for 20–30 mV conditioning depolarization and decreases for larger conditioning potential; half-inactivation occurs for a 38 mV-depolarization, V_H. Unexpected for lower pre-depolarizations and for pre-hyperpolarizations the amplitude of the inward current is diminished: the initial current appears to be not fully available at the resting membrane

potential and seems further inactivated, though never fully inactivated by conditioning hyperpolarizations. Similar voltage-dependence relationships have been established in four fibres which exhibited a hump on the current traces. After TEA treatment, when the hump has disappeared on the current trace, the voltage dependence of the initial current becomes a simple function (Fig. 6) as it remains for other fibres without a hump.

This complex voltage-dependence of the inactivation variable of the initial current can be accounted for by assuming that two ionic currents flow in opposite directions nearly simultaneously, but each of them has a particular voltage-dependence. The lower the conditioning membrane potential the larger the outward current is and, though the calcium inward current is constant, the smaller the initial inward current appears. Assuming a constant full availability of the calcium channel for membrane potential lower than -45 mV (this is supported by the voltage-dependence relationships established in TEA solution or on fibres without a hump), the inactivation voltage-dependence of the outward current has been extrapolated. Its availability is maximal for membrane potential hyperpolarized by 20 mV or more. This fast outward channel is inactivated by a 15 mV-depolarization of the membrane.

Failure to elicit a noticeable fast outward current in the absence of a calcium inward current

The outward current does not appear activated by depolarizations that are too low to elicit a calcium current since the current trace is flat after the capacity current. It has also been reported that manganese ions inhibit both the calcium inward current and the fast outward current (Mounier & Vassort, 1975). Thus, no idea of the time course of the fast outward current is yet available.

Having determined an availability curve of this current noticeably different from that of the inward current we further investigate the characteristics of the outward current. Step depolarizations of small increasing amplitude are applied to fibres that are hyperpolarized to -20, -30 mV, in order to fully reprime the fast outward current. This procedure always fails to elicit a noticeable outward current before triggering an inward one. Another tentative experiment was performed in the expectation of obtaining a faster re-availability of the fast outward current (Fig. 7). The experimental procedure is as follows: the fibre is depolarized to 75 mV during 75 msec in order to inactivate the inward and the outward currents, then after different durations of repriming at the resting potential, E_r, a depolarization to $+40$ mV is applied. Both currents recover simultaneously as they do when the repriming is carried out at more negative potential.

We have not succeeded in eliciting noticeable outward current in the absence of inward current. In the discussion we give an hypothesis which suggests that the fast outward current is mainly consequent upon the influx of calcium ions.

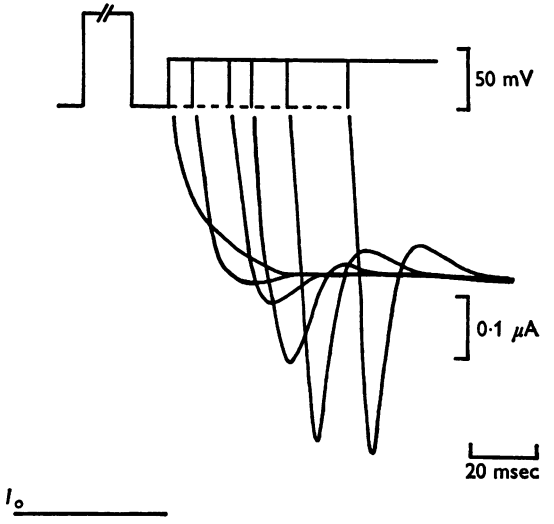

Fig. 7. Re-availabilities of I_{Ca} and I_{K1} in ASW solution. First step depolarization is 75 mV high and 75 msec long in order to inactivate the initial current. After a given delay during which some repriming occurs at the holding potential, E_r, a second 40 mV-step is applied. Both currents seem to recover simultaneously.

DISCUSSION

Voltage-clamp experiments are difficult to perform with crab muscle fibres in the double sucrose-gap particularly because of the complex structure of this fibre. However, control of a constant internal voltage during depolarizations has been achieved in some experiments with a micro-electrode. The whole set of results supports the existence of a fast outward current: a hump on the current trace occurs only with fibres that exhibited fast repolarizing electrical responses. Associated features included a larger resting conductance, a lower apparent calcium reversal potential, an abnormal voltage-dependent availability curve of the inward current. Moreover the sensitivity of this hump to $[K]_o$ and to TEA reinforces its ionic dependence and supports its potassium origin. Rather than supposing abnormal voltage-dependence of the calcium conductance (Geduldig & Gruener, 1970), the inactivation curve (Fig. 6) can be explained by assuming the existence of two currents flowing in opposite direction. An apparent decrease of the net inward current consequent

upon previous hyperpolarization, as well as a similar hypothesis, have been already reported on different tissues (gastropod neurones: Connor & Stevens, 1971 and Neher, 1971; smooth muscle: Vassort, 1974). After TEA the inactivation curve is simple: this pharmacological effect allows us to determine the inactivation curve of the fast outward current. It appears half-available at resting potential. Thus, in most cases, an outward current occurs quasi-simultaneously to the inward one. This result explains the apparent very low E_{Ca} ($+10$ to $+15$ mV) and its small shift by variation of $[Ca]_o$. It also accounts for the slightly higher E_{Ca} ($+25$ to $+30$ mV) and the larger shift for fibres which do not show a hump or which are TEA-treated as previously reported (Mounier & Vassort, 1975).

Very recently Begenisich & Lynch (1974) with internal applied divalent cations on squid giant axons failed to find a marked effect of calcium ions on the kinetics of the potassium current, which is a delayed outward current. However it is worth noting that we are dealing with calcium-spiking cells and that a transient outward current, named I_{K1} here, has been described in nearly all of those cells, except the barnacle. If we fit our experimental availability data using the equation $f_\infty = 1/1+\exp[(V_H - V)/K]$ (Hodgkin & Huxley, 1952), it is noticeable that in fibres which clearly exhibit a hump the data are well fitted when a slope factor, K, of 4 is used (see also Standen, 1974) while with other fibres, without a hump, a slope factor of 7 is best used (Mounier & Vassort, 1975, Fig. 7), as for squid axon (Hodgkin & Huxley, 1952); TEA does not modify the slope factor in these two types of fibres.

Discrimination between I_{K1} and I_{K2}

The fast outward current (I_{K1}) is separable from the late outward one (I_{K2}) though both are mainly carried by potassium ions. The delayed rectification appears only for a membrane potential higher than -15 mV while I_{K1} has a lower threshold. Manganese ions block I_{Ca} and I_{K1}, however they only decrease I_{K2}; TEA inhibits I_{K1} and the inward current inactivation is then exponential but the delayed rectification is only reduced. A simple vizualization of the different origins of I_{K1} and I_{K2} is given by the effects of strontium ions in current-clamp. After switching to a strontium solution, the amplitude of the electrical response increases rapidly to its maximal value while the duration is enhanced more slowly. The existence of two potassium voltage- and time-dependent conductances, one responsible for the undershoot, the other for the delayed rectification, has already been proposed by Werman & Grundfest (1961) on lobster muscle fibres, and is well supported in molluscan neurones (Connor & Stevens, 1971; Neher, 1971; Neher & Lux, 1972).

Influence of double cations, a possible origin of I_{K1}

The absence of noticeable I_{K1} in absence of I_{Ca} suggest a dependence of I_{K1} upon I_{Ca}. In the following a tentative hypothesis is put forward which attributes a major role to divalent cations on both sides of the membrane.

Increase of the external divalent cations concentration induces a shift of the activation curve in a depolarizing direction (Frankenhaeuser & Hodgkin, 1957; D'Arrigo, 1973; Mounier & Vassort, 1975). A similar shift is observed for the activation curve of a potassium current on heart muscle under the influence of adrenaline (Hauswirth, Noble & Tsien, 1968) though it might be attributed to a decrease in internal $[Ca]_i$ and consequently a decrease of positive screening ions on the internal surface of the membrane (McNaughton & Noble, 1973). Moreover, the results of Meech (1972) and Krnjević & Lisiewicz (1972) show that a rise in internal free calcium ions induces an increase of G_K. Those results can be summarized by stating that the electrical field of the membrane depends on the gradient of double-charged cations across it; an increase of the ratio $[X^{2+}]_o/[X^{2+}]_i$ induces a shift towards the right, of the activation and inactivation curves.

We suggest that the fast inward current causes a local transient increase of the calcium concentration just inside the fibre. This results in a transient shielding of the negative surface charges at the inside of the membrane, which shifts the I_{K1} activation curve in an hyperpolarizing direction. This would make possible or, better, markedly enhance the generation of I_{K1}. The rapid decrease of I_{K1} would result from the diffusion of calcium ions inside the cell and thus be a form of deactivation rather than an inactivation. Similar suggestion of a calcium current triggering a potassium current has been recently supported by Meech (1974) and Standen (1974) on snail neurones. Only calcium ions seem to be able to trigger such a variation in I_{K1}. In strontium solution, no I_{K1} is observed (Mounier & Vassort, 1975). With *Aplysia* neurones, Geduldig & Gruener (1970) found an abnormal inward current inactivation curve only in ASW solution but not when calcium ions were substituted by sodium ions.

Control of the electrical activity

The experiments were performed on phasic fibres issued from the same part of the muscle extensor. They all received branches of the 'common inhibitor' (Wiersma & Ripley, 1952; Atwood, 1965). However, different kinds of electrical responses are reported here, as well as in the literature. Generally graded, the electrical activity can be autoregenerative; correspondingly, the net inward current is small and followed by a more or less developed hump, or in some cases larger and without hump. An explana-

tion of the responses might be that the part of the membrane in the test-gap contains more or less extended parts of inhibitory synapses, but also that the potent inhibition varies between fibres (Atwood & Bittner, 1971). The particular behaviour of one fibre results probably from the positions and amplitudes of its membrane-conductance (chiefly G_{K1}) variables.

A simple way to reduce the excitability can result from an increase of membrane conductance G_m by a selective increase of G_{Cl} (Takeuchi & Takeuchi, 1967; Motokizawa, Reuben & Grundfest, 1969) without noticeable variations of the resting membrane potential. The depolarizing calcium current must surpass a larger leak current. The complex inactivation curve described in Fig. 6 suggests another mode of control of excitability. Inhibition can be the consequence of an increase of the availability of I_{K1} after small hyperpolarization of the cell membrane. Such hyperpolarizations have been described (Dudel & Kuffler, 1961; Atwood, 1965; Usherwood, 1973) though these authors also reported some depolarizations. On the other hand, small depolarizations of the cell make autoregenerative spikes more probable. Similar mechanism of modulation of the excitability has been recently described in snail neurones (Daut, 1973). This is formally a situation opposite to the one generally described on excitable cells.

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