

Ionic Nature of the Outward-Going Rectification in Crab Skeletal Muscle Fibres

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Abstract. The outward-going rectification was examined in crab muscle fibres under current clamp and voltage clamp conditions. Experiments were performed on large diameter crab muscle fibres; these generate all-or-none responses. Under current clamp conditions, the outward-going rectification can be elicited in the muscle membrane in a choline chloride solution; it however disappears in Cl^- free artificial sea water (ASW). Under voltage clamp conditions, in the absence of the inward Ca current (ASW plus Mn^{++} ions), step depolarizations produce an outward current with a single tail-current component, which is well separated from the initial capacity current. Shifts of the apparent reversal potential of the outward current produced by modifications in Cl or K concentration suggest that this current is not exclusively carried by K^+ ions, but Cl^- ions as well.

Key words: Voltage clamp — Skeletal muscle fibre — Outward-going rectification — Chloride conductance — Crab

Introduction

The experiments described in the present paper were undertaken in order to investigate the ionic nature of the delayed outward current in the crab muscle fibre.

The outward-going rectification can be produced by variations in K conductance and abolished by tetraethylammonium ions (TEA) in frog skeletal muscle (Katz 1949; Stanfield 1970; Adrian et al. 1970) and in single barnacle muscle fibre (Keynes et al. 1973; Hagiwara et al. 1974). In the crab walking leg muscle fibre generating graded responses, under voltage clamp conditions using the double sucrose-gap technique (Mounier and Vassort 1975), the existence of a late outward current which corresponds to the K^+ current inhibited by TEA ions was demonstrated. Its activation during step depolarizations exhibited voltage and time dependence. However, Atwood et al. (1965) demonstrated the existence of an all-or-none response and inward-going rectification in large diameter crab fibres. In addition, Haudecoeur and Guilbault (1974a, b), using the current clamp technique with the crab fibre selected for an all-or-none response could show that

the outward-going rectification is abolished in the absence of Cl^- ions from the extracellular solution. However, if a solution containing choline chloride was only used, the outward-going rectification could still be produced by a depolarizing current despite the presence of choline ions which are known to inhibit partially K currents (Hille 1967).

Furthermore, Hutter and Warner (1967a, b, 1972) and Warner (1972), using frog skeletal fibres, showed that the time and membrane potential dependent effect of pH on membrane conductance, is associated with movements of Cl^- ions. The results of Palade and Barchi (1977) have also indicated the existence of a dynamic Cl conductance in muscle fibres of the rat diaphragm. Under voltage or current clamp conditions with crab muscle fibre, Haudecoeur (1975), Brûlé et al. (1976) found that outward-going rectification only occurs when Cl^- ions are present in the external solution. The outward-going rectification which can be partially inhibited by TEA ions seems to be due either to modifications in Cl or K conductance which would be dependent upon extracellular Cl concentration. In order to distinguish between these possibilities, voltage clamp and current clamp experiments were performed on crab muscles generating all-or-none responses. Some of these results have been published in a preliminary form (Brûlé et al. 1976; Haudecoeur et al. 1978).

Material and Methods

Materials

Experiments were performed at 20°C on bundles of muscle fibres isolated from the meropodite extensor muscle of the crab (*Carcinus maenas*) walking leg. These fibres correspond to those already described by Atwood et al. (1965). They generally respond to electrical stimulation by producing an all-or-nothing response and a phasic contraction. Their diameter varies from 250 to 500 μm . Fibres with diameters ranging from 280 to 300 μm were used. Following isolation bundles of fibres were put into physiological solution or artificial sea water (ASW) for 20 min.

Recording

Membrane potential. Microelectrodes were used to measure membrane potential. In order to eliminate muscle movements, all solutions were supplemented by MnCl_2 (10 mmol/l), since Mn^{++} ions are known to abolish the inward Ca current (Ozeki et al. 1966; Takeda 1967; Chiarandini et al. 1970; Matsumura 1972; Haudecoeur and Guilbault 1972).

Voltage-clamp conditions. Membrane currents were recorded under voltage-clamp conditions using three microelectrodes inserted near the end of the crab skeletal muscle fibre. The theoretical basis and limitations of this method have been discussed elsewhere (e. g. Adrian et al. 1970 in frog fibres, Bertrand et al. 1979 in crab muscle fibre). The method is suitable for analysis of the outward current in the absence of the inward current, which, in crab muscle fibres, is carried by Ca^{++} ions (Haudecoeur and Guilbault 1972; Mounier and Vassort 1975). Three microelectrodes were implanted in a muscle fibre near its end. Two microelectrodes, spaced at a distance of l and $2l$ from the end of the fibres, were used to record the membrane potential. The microelectrodes were filled with 3 mol/l of KCl; their resistance

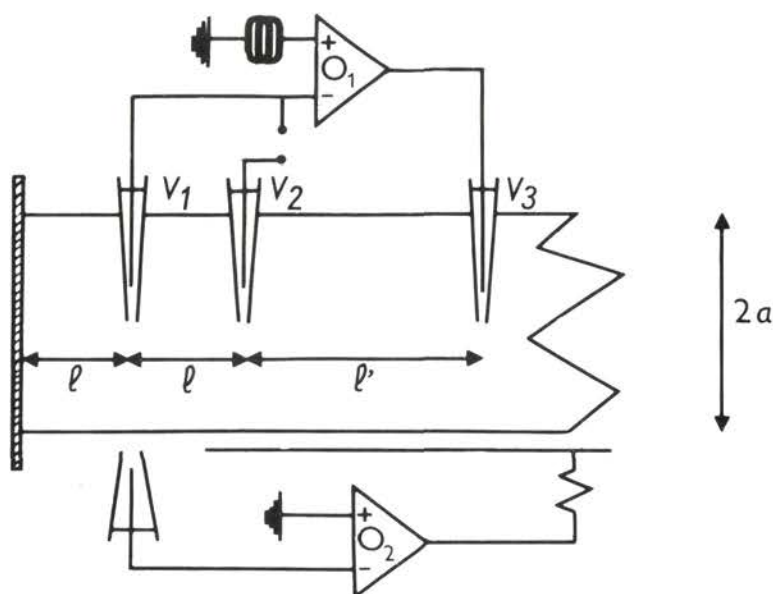


Fig. 1. Experimental set up to record the membrane current under voltage clamp conditions — microelectrodes 1, 2, 3 are inserted at l , $2l$ and $2l + l'$ from one end of the fibre, respectively; V_1 , imposed transmembrane potential; $V_2 - V_1$, potential difference proportional to the membrane current (i_m); O_1 , feedback amplifier to control the membrane potential, V_1 ; O_2 , feedback amplifier to maintain the potential of the external surface of the membrane at the referred potential.

was between 5 and 10 M Ω and tip potential -5 mV. The membrane potential was controlled by a feed-back amplifier connected to the electrode impaled at the distance l . The characteristics of the operational amplifier have been described elsewhere (Bertrand et al. 1979).

Fig. 1 illustrates the experimental set up. The potential difference recorded at l is called V_1 . The potential difference across the membrane recorded at $2l$ is V_2 . The third microelectrode, filled with 3 mol/l of KCl, had a resistance of between 1 and 2 m Ω and was used to pass current; it was impaled at a distance of $2l + l'$ from the fibre end. If there is no regenerative inward current flow, the membrane current flowing in the vicinity of the microelectrode V_1 is proportional to $V_2 - V_1$, provided that $(V_2 - V_1)/V_1$ is less than 6, or l/λ is less than 2, and $l' \geq 4/3 a$ (a , the radius of the fibre; λ , the length constant). Under these conditions met in our experiments, $i_m \cong 2(V_2 - V_1)/3r_i l^2$, where r_i is the internal resistance per unit of length (for further details see Adrian et al. 1970 and Bertrand et al. 1979).

Current clamp conditions. Usually two microelectrodes were introduced into the same fibre, one to pass current through the membrane, the other at a distance of 100 μ m to record membrane potential.

Solutions

Table 1 shows the composition of solutions (pH = 7.8).

Table 1. Solutions used to study crab muscle fibres. Concentrations are given in mmol/l. pH was always 7.8

Solution	Cl ⁻	HCO ₃ ⁻	Na ⁺	K ⁺	Ca ⁺⁺	Mg ⁺⁺	Other ions or substances
I (ASW)*	596.7	2.6	515.6	12.9	11.8	23.6	—
II	596.7	2.6	2.6	—	—	—	choline ⁺ : 596.7
III	596.7	2.6	2.6	—	—	—	choline ⁺ : 496.7 TEA ⁺ : 100
IV	—	2.6	515.6	12.9	11.8	23.6	methylsulphate ⁻ : 525.9 SO ₄ ⁼⁼ : 23.6 propionate ⁻ : 23.6
V	596.7	2.6	515.6	12.9	11.8	23.6	Mn ⁺⁺ : 10 methyl sulphate ⁻ : 20
VI	179	2.6	515.6	12.9	11.8	23.6	Mn ⁺⁺ : 10 methylsulphate ⁻ : 437.7
VII	59.7	2.6	515.6	12.9	11.8	23.6	Mn ⁺⁺ : 10 methylsulphate ⁻ : 525.9 propionate ⁻ : 23.6 SO ₄ ⁼⁼ : 3.75
VIII	596.7	2.6	515.6	12.9	11.8	23.6	Mn ⁺⁺ : 10 SO ₄ ⁼⁼ : 58.05

* ASW — artificial sea water

Results

Current-voltage relations under current clamp conditions

Fig. 2 illustrates current-voltage relations in crab muscle fibres under current clamp conditions. Fig. 2A demonstrates that depolarizing step current can only induce an outward-going rectification (curve *b*) which can partially be inhibited by perfusion with TEA (100 mmol/l) (sol. III) in the presence of choline chloride (596 mmol/l; sol. II, Table 1). Curve *a* was obtained in artificial sea water (ASW) using large hyperpolarizing steps and small depolarizing steps with amplitudes below those required to elicit depolarizing responses. Curve *b* shows that after the exposure of the fibre to choline chloride solution for 25 min (the time needed to obtain steady current-voltage relations), the membrane potential was increased and that, with large depolarizing current steps, the outward-going rectification was still present as in the normal ionic environment (Mounier and Vassort 1975).

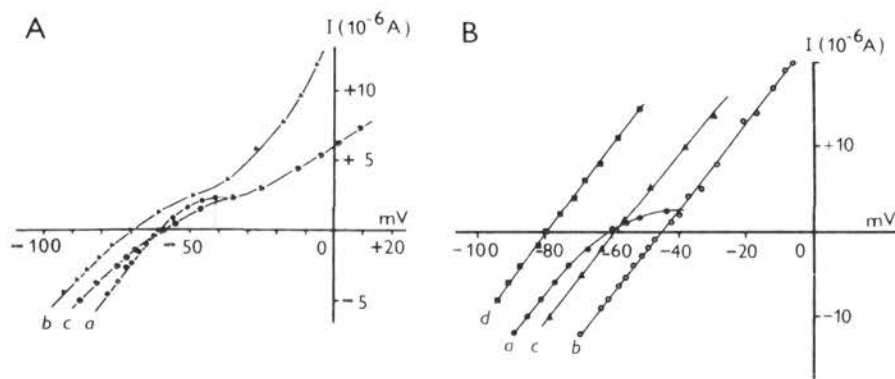


Fig. 2. Current-voltage relations in crab muscle fibres under current clamp conditions obtained in the presence (**A**) and absence (**B**) of Cl^- ions. **A**: the muscle fibre was successively bathed in solutions I, II and III; curve *a* was determined under normal ionic conditions (ASW — sol. I); curve *b* after 25 min in choline chloride solution (sol. II); curve *c* after 30 min in choline chloride solution plus TEA (sol. III) **B**: another fibre successively placed in solutions I and IV: *a* — control saline (sol. I); *b* — after 15 min in Cl^- -free ASW (sol. IV); *c* — as in *b*, but after membrane repolarization to the initial membrane potential; *d* — as in *b*, but after hyperpolarization of the membrane. The amplitude of electronic pulse was measured at the end of the current pulse (125 ms).

The outward-going rectification can partially be inhibited by TEA (100 mmol/l), as shown by curve *c* in Fig. 2A. In Cl^- -free ASW (sol. IV, Table 1), the cell became depolarized (20 mV on the average) as demonstrated in Fig. 2B (*b*). The current-voltage relationship was linear; the outward and the inward-going rectification were abolished and were absent even if the membrane potential had been brought back to the initial value of the resting potential (*c*), or hyperpolarized (*d*) by inward polarizing current.

These results suggest that, similarly as in vertebrate muscle fibres, outward-going rectification can also be produced in crab muscle fibres generating all-or-nothing responses by variations in the K conductance; this rectification is however dependent on the external Cl concentration as well.

Analysis of the outward current under voltage clamp conditions.

The ionic nature of the outward current was determined in the absence of Ca inward current by adding Mn^{++} ions to ASW. In the absence of Ca current, it is possible to evaluate changes in the reversal potential of the outward current produced by modifications of the external concentration of Cl^- or K^+ ions and thus to determine the ionic nature of the outward current. For this purpose the reversal potential of the outward current elicited by a large depolarizing pulse was obtained

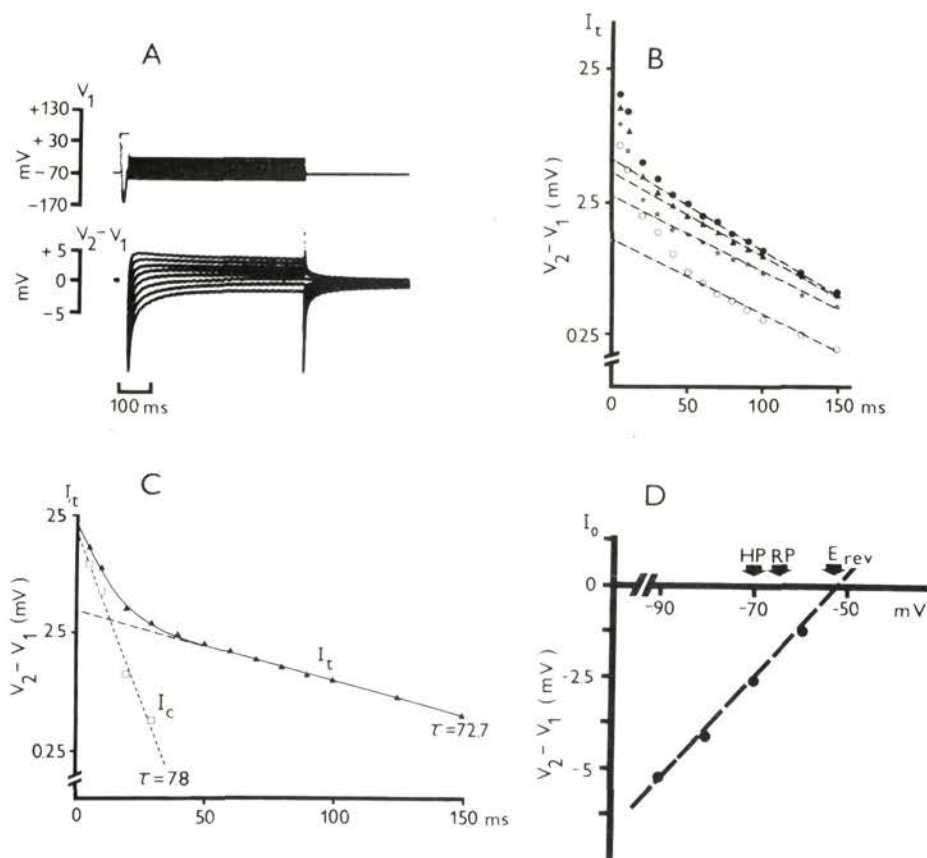


Fig. 3. Determination of the reversal potential of the outward current in crab muscle fibres bathed in ASW with the addition of Mn^{++} ions (sol. V, Table 1) using the double-pulse voltage clamp method. **A:** Records of membrane potential, V_1 , and membrane current expressed as $V_2 - V_1$ (HP = -70 mV; RP = -68 mV). **B:** Tail current-time relations at membrane potentials of the second pulse. \bullet -90 mV; \blacktriangle -80 mV; \ast -70 mV; \circ -50 mV. **C:** Evidence for the existence of a single component in the tail current at a membrane potential (second pulse) of -80 mV. The first component is a capacitive current, I_c ($\tau = 7.8$ ms), similar to values obtained at different membrane potentials $\tau = 7.3$ ms for -90 mV; 7.8 ms for -70 mV; 8.2 ms for -60 mV). **D:** Current-voltage relationship of the instantaneous values of the tail currents (I_0) extrapolated to the beginning of the second pulse from the linear relationship given under **B**. $E_{rev} = -52$ mV, in this fibre; the average $E_{rev} = -50.3 \pm 5.2$ mV (mean \pm SEM; $n = 12$). The muscle fibre was bathed in sol. V for 20 min with subsequent insertion of the microelectrodes.

in the usual way, i.e. by repolarizing the fibre to different levels after turning on the membrane conductance responsible for the dynamic outward current produced by the initial depolarization. The tail current at the end of the voltage step may be either outward or inward, depending upon the level of repolarization, the ionic nature of the outward current may be deduced from the shift in the reversal potential of the outward current due to variations in the external concentration of K^+ or Cl^- ions.

Reversal potential at normal concentrations of Cl^- and K^+ ions.

Fig. 3 illustrates the determination of reversal potential in a fibre placed in ASW with the addition of Mn^{++} ions (sol. V, Table 1).

First, the membrane was depolarized by about 100 mV (command depolarization) in order to obtain a full activation of the outward-going rectification, and it was clamped (A) to different potentials (second pulses) as soon as the outward current had reached maximum values. The semilogarithmic analysis of the tail currents as a function of time for different values of the membrane potential corresponding to the second pulse shows (B) that only one component is present in the outward current activated by the first depolarizing step. The plots show two components in the tail current, the first being due to the capacitive current (I_c) as shown by Fig. 3B and C.

For each value of the membrane potential corresponding to the second pulse, the relation between capacitive current-associated current tails amplitude and time was obtained by subtracting the steady-state current (value at the end of the second pulse) from the total current.

Extrapolation of the instantaneous amplitudes of the tail currents, I_o , at different membrane potentials corresponding to the second pulse enabled the determination of the current-voltage relationship (Fig. 3D). The reversal potential obtained from this linear relationship had a value of -52 mV; the slope of the relation corresponded to the conductance at the end of the first command pulse. The mean value of the reversal potential was -50.3 ± 5.2 mV (mean \pm SEM, $n = 12$).

Variation of the reversal potential with the external concentration of K^+ ions.

Upon exposure of the fibre to a high K solution (129 mmol/l), the reversal potential shifts towards depolarization levels.

Fig. 4 gives an example of the results obtained from a fibre exposed to a high potassium concentration with the calcium currents being inhibited by Mn^{++} ions

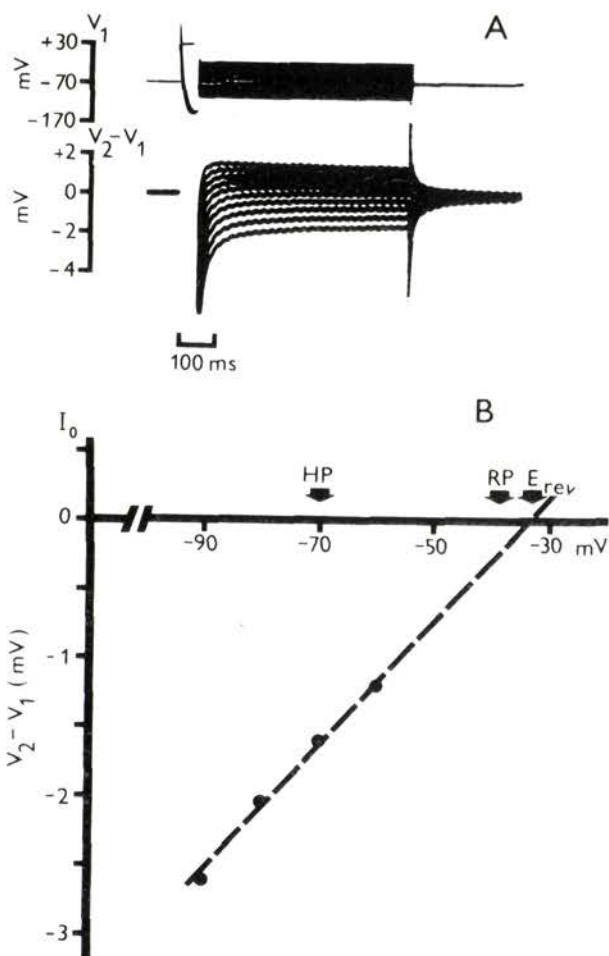


Fig. 4. Determination of the reversal potential (E_{rev}) of the outward current in a fibre bathed in high potassium ASW plus Mn^{++} (sol. VIII, Table 1). **A:** Records of V_1 and $V_2 - V_1$ (HP = -70 mV; RP = -39 mV). **B:** Current (I_0)-voltage relationship. E_{rev} = -33.5 mV, for this fibre; the average E_{rev} = -31.6 ± 2.4 mV (mean \pm SEM; $n = 8$). The fibre was bathed in solution VIII for 20 min with subsequent insertion of the microelectrodes.

(sol. VIII, Table 1). Fig. 4A shows records of the membrane potential V_1 and the voltage difference, $V_2 - V_1$, proportional to the membrane current density. The reversal potential determined from the current-voltage relationship (Fig. 4B) was about -33.5 mV. This relation was constructed as above (Fig. 3) from the instantaneous values of the tail currents, I_0 , extrapolated for different membrane

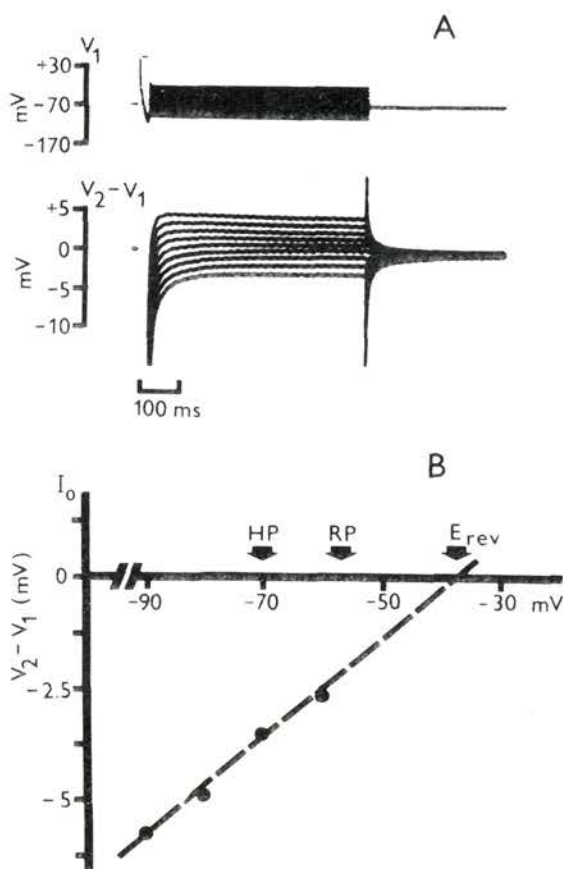


Fig. 5. Determination of the reversal potential (E_{rev}) in a fibre bathed in low Cl ASW containing Mn^{++} ions (sol. VI, Table 1). The external concentration of Cl ions was one third of the control value (ASW). **A:** Records of V_1 and $V_2 - V_1$ (HP = -70 mV; RP = -58 mV) **B:** Current (I_0)-voltage relationship. $E_{rev} = -37.5$ mV, for this fibre; the average $E_{rev} = -37.2 \pm 3.1$ mV (mean \pm SEM; $n = 5$). The fibre was bathed in solution VI for 20 min with subsequent insertion of the microelectrodes.

potentials to the beginning of the second pulse. The average reversal potential was -31.6 ± 2.4 mV (mean \pm SEM, $n = 8$). The results with variation of the external K concentration show that the outward current elicited by a large initial depolarizing step is partially, but not exclusively, carried by K^+ ions, the shift in the reversal potential in depolarizing direction being too small, 18.7 mV compared to 58 mV as expected from the Nernst equation.

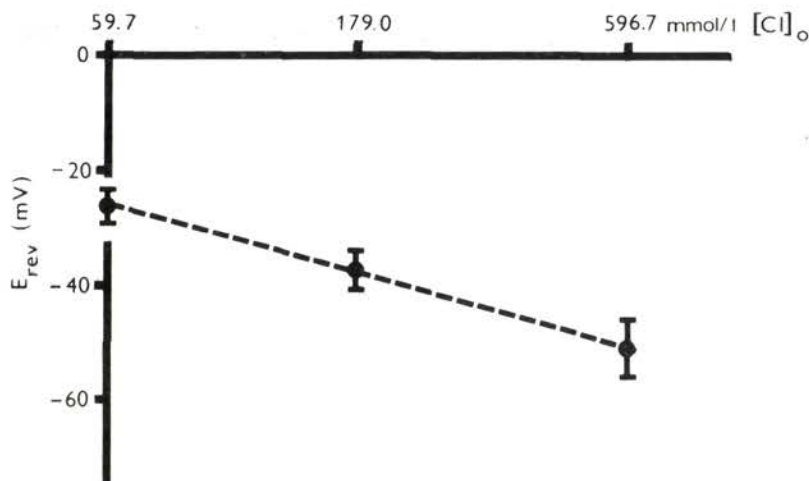


Fig. 6. Relationship between the reversal potential (E_{rev}) and the logarithm of the external Cl concentration in ASW containing Mn^{++} ions.

Variation of the reversal potential with the external Cl^- ion concentration

As mentioned above, the mean value of the reversal potential of the outward current in ASW containing Mn^{++} ions was -50.3 ± 5.2 mV (Fig. 3). Fig. 5 shows an example of results obtained with reduced (1:3) external Cl concentration (sol. VI, Table 1). Fig. 5A shows records of the membrane potential, V_1 and the resulting voltage difference, $V_2 - V_1$, which was proportional to the membrane current density. The reversal potential (Fig. 5B) was -37.5 mV; the mean value determined from 5 different fibres was -37.2 ± 3.1 mV (mean \pm SEM). Thus, the shift occurring at the external Cl concentration being reduced to one third of the normal value was about 13 mV in average. When the Cl concentration was reduced 1:10, the shift in the reversal potential in the depolarizing direction was greater, 23.6 mV in average (Fig. 6). The mean value of E_{rev} was -26.7 ± 2.9 mV (mean \pm SEM; $n=8$). The relationship between the reversal potential and logarithm of the external Cl concentration was linear (Fig. 6). The change in the reversal potential was 23.6 mV for a ten-fold change in the external chloride concentration. The linear relationship between E_{rev} and the logarithm of Cl concentration strongly suggests that a significant proportion of the outward current is carried by Cl^- ions.

Discussion

The results presented in this paper concern the ionic nature of the conductance activated by depolarizing steps and producing, in crab muscle fibres exhibiting all-or-nothing responses to electrical stimulation, an outward current.

There has been evidence for a voltage-dependent outward current which is partially inhibited by TEA and which is not exclusively carried by K^+ ions. Mounier and Guilbault (1970), Haudecoeur and Guilbault (1974*a* and *b*) could show in crab muscle fibres that the outward-going rectification is abolished in Cl^- -free extracellular solution. Our results show that the outward-going rectification can largely be inhibited by TEA and may still be elicited if Cl^- ions are present in the extracellular solution, suggesting that the delayed rectification is not solely due to an increase in the K conductance. The ionic nature of the outward current has been studied by means of microelectrodes under both current clamp and voltage clamp conditions. Both methods yielded results that are in perfect agreement. Under current clamp conditions, an increase in the resting membrane resistance was observed when the fibre was washed by an external solution containing choline chloride (596.7 mmol/l) alone (Haudecoeur and Guilbault 1974*b*), even in the absence of divalent cations. Our results show that, in the presence of choline ions which are known to block the K currents (Hille 1967) and in the absence of external Ca^{++} ions, depolarizing pulses always elicit an outward current. The existence of the outward current under these ionic conditions can be taken as evidence for the existence of an outward current component carried by Cl^- ions.

TEA⁺ ions are known to block the outward ionic current. This would explain the inhibition of the outward-going rectification in crab muscle fibres as tested with depolarizing currents. Under voltage clamp conditions, the crab muscle fibre generates a voltage-dependent outward current which may be inhibited by TEA. The sensitivity of the outward current to TEA may mean that it is a K current (Mounier and Vassort 1975). In frog muscle fibre, however, the outward current does not disappear in the absence of extracellular chloride (Katz 1949; Adrian and Freygang 1962*a, b*; Adrian 1964; Stanfield 1970); whereas it disappears in crab fibre after the fibre has been washed by Cl^- -free ASW for 20 min.

The results presented in this paper provide some evidence for the existence of a voltage dependent chloride conductance. They also show that only a portion of the outward current is carried by the K^+ ions. The results obtained in crab muscle fibres are in agreement with results reported by other authors. In crayfish muscle fibres, Girardier et al. (1963) could demonstrate Cl permeability in the tubular system and suggested the existence of the dynamic Cl conductance. In Purkinje fibres, Fozzard and Hiraoka (1973) showed that the amplitude of the outward current elicited by the depolarizing step is greatly decreased after the external Cl concentration has been lowered from 143 mmol/l to 5.6 mmol/l. A role of Cl^- ions

in carrying the ionic current in the cardiac muscle has been suggested by Hutter and Noble (1961), Carmeliet (1961), Dudel et al. (1967), Peper and Trautwein (1968). An external Cl^- concentration dependent delayed component of the outward current was demonstrated in cardiac muscle fibres by Lenfant and Goupil (1977). However, Kenyon and Gibbons (1979) have shown that in cardiac Purkinje fibres the membrane chloride conductance was not the major determinant of the total current at voltages more positive than -20 mV.

Our results prompted us to postulate the existence of the voltage dependent Cl^- conductance in crab muscle fibres generating all-or-nothing responses. These results differ from those obtained by Mounier and Vassort (1975) who used a double sucrose-gap technique in crab muscle fibres, producing graded responses in normal ionic environment. The latter results were confirmed in crayfish muscle fibre by Henček and Zachar (1977), who used a patch clamp technique. According to these authors, two outward currents carrying K^+ ions (I_{K1} , I_{K2}) exist in crustacean muscle fibres. The fast current component, I_{K1} , is dependent upon the inward Ca current (I_{Ca}). In the absence of I_{Ca} the I_{K1} current component cannot be elicited. The evidence for a single outward current component which follows from our results (Fig. 3B and C) is in agreement with the results of these authors, since our solutions contained Mn^{++} ions to block I_{Ca} .

Our results, however, show that the outward current is not exclusively carried by K^+ ions, but by Cl^- ions as well. This discrepancy may be explained in supposing that the surface membrane is preferentially permeable to K^+ ions while the tubular membranes are selective for the chloride conductance (Girardier et al. 1963). This hypothesis is supported by the following considerations:

i) Henček and Zachar (1977) employed a patch clamp technique which permitted to study a small membrane surface area. No outward current possibly present in the tubular membranes of the crayfish can be recorded under these conditions.

ii) Recently, we have shown (Delorme et al. 1978; Bertrand et al. 1979) that sucrose produces detubulation of fibres; this agreed with the results of Fatt and Katz (1953), who considered sucrose as toxic for crab muscle fibres. The similarity between records obtained by Mounier and Vassort (1975) and by Henček and Zachar (1977) can be explained by assuming that the double sucrose gap technique also causes detubulation, since outward current in the tubular membranes would not be recorded. The existence of detubulation by sucrose is also confirmed by the following facts: Mounier and Vassort (1975) report a value of the membrane resistance in the test gap of $1 \text{ M}\Omega$; this value is very large compared to that calculated from the specific membrane resistance ($110 \Omega \cdot \text{cm}^{-2}$) reported by Fatt and Katz (1953). As the surface membrane in their extreme conditions could vary from $4.71 \times 10^{-4} \text{ cm}^2$ to $9.42 \times 10^{-4} \text{ cm}^2$, the membrane resistance in the test gap

would range from 200,000 Ω to 100,000 Ω , which is considerably less as compared to 1 M Ω . Also, the time constant of the capacitive current calculated by us ($\tau \doteq 7.8$ ms; Fig. 3) exceeds significantly that which can be estimated from tracings published by Mounier and Vassort (1975);

iii) Finally, as suggested by Henček et al. (1978), in muscle fibres with invaginations, the double sucrose-gap technique is expected to lead to oscillations in the total current under normal ionic conditions. They stated: "So far three methods have been used for voltage clamping the muscle membrane of crustacea. A muscle analogue of the nerve axon voltage clamp method (Hodgkin et al. 1949) was introduced by Hagiwara and Naka (1964) and Keynes et al. (1973). The problems connected with membrane invaginations remain, however, unsolved. Basically the same applies for the method of the artificial "Ranvier node" which was applied to crab muscle fibres by Mounier and Vassort (1975)".

We conclude that crab muscle fibres exhibiting all-or-nothing behaviour possess an outward-going rectification which is not exclusively due to changes in potassium conductance, but to chloride conductance as well. Our results can be brought to a common basis with those of Mounier and Vassort (1975) and Henček and Zachar (1977), in postulating that, in crustacean muscle fibres, the surface membrane is preferentially permeable to K^+ ions while the tubular membranes exhibit a selectivity for Cl ions. The fact that a single component was only found in the total outward current might be explained on the assumption that both components of the outward current have the same activation kinetics.

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