*J. Physiol.* (1979), **286**, *pp.* 591–606
*With* 8 *text-figures*
*Printed in Great Britain*



# A DUAL EFFECT OF FORMALDEHYDE ON THE INWARDLY RECTIFYING POTASSIUM CONDUCTANCE IN SKELETAL MUSCLE

BY O. F. HUTTER AND THELMA L. WILLIAMS*
*From the Institute of Physiology, University of Glasgow, G12 8QQ*

(*Received 13 April 1978*)

SUMMARY

1. The inwardly rectifying potassium conductance of the membrane of frog sartorius muscle fibres is greatly reduced by treatment of muscles for 30 min with a solution containing formaldehyde (10 mM).

2. A transient increase in the conductance of the inward rectifier is observed early during formaldehyde action.

3. Analysis of the biphasic time course of the conductance changes, as determined under controlled voltage conditions, suggests that treatment with formaldehyde alters simultaneously, but in opposite ways, two factors that determine the conductance of the inward rectifier.

4. The linear component of the current–voltage relation, which dominates the relation at strongly positive potentials, is not affected while the above changes occur. But on prolonged exposure to formaldehyde the leak conductance increases.

5. The effects of formaldehyde on the inward rectifier are reversible on prolonged superfusion with normal Ringer solution.

6. The slight inward rectification remaining after most of the extracellular K is replaced by Rb, is similarly reduced by treatment with formaldehyde.

7. The results are interpreted in terms of the chemical properties of formaldehyde and present views of the mechanisms of inward rectification.

## INTRODUCTION

The K conductance of the skeletal muscle membrane behaves as though it consisted of several independent components in parallel. The component which can conduct the largest current is the time and voltage dependent *delayed* rectifier. This undergoes activation upon depolarization of the membrane and suffers inactivation on prolonged depolarization. In permanently depolarized (Katz, 1949) or normally polarized resting muscle (Hutter & Noble, 1960) the *inward* rectifier is the dominant component. A *linear* component lies in parallel to the inward rectifier and constitutes the entire conductance at high positive voltages (Adrian & Freyang, 1962*b*).

Several agents, such as Rb (Adrian, 1964), TEA (Stanfield, 1970) and formaldehyde (Hutter, 1969) are known to reduce or abolish the inwardly rectifying

* Present address: Physiology Department, the Medical College of St Bartholomew's Hospital, Charterhouse Square, London EC1M 6BQ.

component without altering the linear component. Formaldehyde also abolishes the contractile response to depolarization, but it spares the delayed rectifier (Hutter, 1969). Pre-treatment of muscle with formaldehyde has therefore proved convenient for the study of the delayed rectifier with only the linear component in parallel (Argibay & Hutter, 1973; Argibay, Hutter & Slack, 1974; and in preparation). In this paper the effect of formaldehyde on the inward rectifier will be described more closely, and implications about the mechanisms of inward rectification will be discussed.

## METHODS

Experiments were performed on isolated sartorius muscles of *Rana temporaria*, at all seasons of the year at a room temperature close to 20 °C. The compositions of the solutions used are given in Table 1. Paraformaldehyde was dissolved in distilled water at a concentration of 0·4 g/l. and stirred continuously overnight at 60 °C. After cooling, this solution was used to prepare solutions D and F.

TABLE 1. Composition of solutions. Concentrations in m-mole/l. pH adjusted to 7·2. A contains $7{\cdot}3 \times 10^{-3}$ mM tubocurarine

| Solution | $Na^+$ | $K^+$ | $Rb^+$ | $Ca^{2+}$ | $Cl^-$ | $CH_3SO_4^-$ |
|---|---|---|---|---|---|---|
| A | 112 | 2·5 | — | 1·8 | 118 | — |
| B | 112 | 2·5 | — | 1·8 | 3·6 | 114·5 |
| C | — | 119·5 | — | 1·8 | 3·6 | 114·5 |
| D | — | 119·5 | — | 1·8 | 3·6 | 114·5 |
| E | — | 5·0 | 114·5 | 1·8 | 3·6 | 114·5 |
| F | — | 5·0 | 114·5 | 1·8 | 3·6 | 114·5 |

| Solution | $H_2PO_4^-$ | $HPO_4^{2-}$ | Tris maleate | Acetyl glycine | Formaldehyde |
|---|---|---|---|---|---|
| A | — | — | 2 | 2 | — |
| B | — | — | 2 | 2 | — |
| C | 0·85 | 2·1 | — | — | — |
| D | 0·85 | 2·1 | — | — | 10 |
| E | 0·85 | 2·1 | — | — | — |
| F | 0·85 | 2·1 | — | — | 10 |

The dissected muscle was pinned out by its attachments onto a sheet of dental wax and covered with solution A. Chloride was removed by repeated washing and soaking in solution B for about 15 min. The muscle was then allowed to undergo contracture and relaxation in solution C and pinned to approximately 1·2 times its *in situ* length, ventral side upwards, in a bath of 0·8 ml. volume, through which solution C flowed at a continuous rate of 3–6 ml./min. The superfusate could be changed with minimal disruption of flow, by turning a tap. Control measurements were made with the muscle in solution C before switching to solution D.

Standard glass capillary micro-electrodes were used throughout. The current electrodes were filled with acidulated 2 M-K citrate and had resistances of 5–8 MΩ. The voltage electrodes were filled with 3 M-KCl and had resistances of 20–30 MΩ. Tip potentials were less than 5 mV.

Membrane conductance was measured under either constant-voltage or constant-current conditions. In the former case we employed the voltage clamp technique of Adrian, Chandler & Hodgkin (1970*a*), in which two micro-electrodes inserted at distances of $1l$ and $2l$ from the pelvic end of a fibre record potentials $V_1$ and $V_2$, and a third micro-electrode at $2l+l'$ supplies the current needed to control the potential $V_1$. The membrane current per unit area of surface was calculated from the measured voltage difference $(V_2 - V_1)$ by

$$I_m = \frac{a}{3l^2 R_i}(V_2 - V_1) \quad \text{(Adrian } et\ al. \text{ 1970}a\text{)},$$

where $a$ is the fibre radius and $R_i$ is the internal resistivity, taken as 170 Ω cm at 20 °C (Nakajima & Hodgkin, 1972). The electrode distance was between 250 and 1000 μm, depending on the range of clamp voltages used and hence conductance values encountered in a given experiment. The current electrode distance $l'$ was about 25 μm. $V_1$ was recorded against a reference Ag–AgCl half-cell placed in the bath, near the pelvic end of the muscle. A d.c. voltage source in series with the reference cell was used for calibration and for balancing the resting potential (between −2 and −4 mV). The current path was completed to earth by another Ag–AgCl half-cell. The total current was measured by the voltage drop across a 4·7 kΩ resistor placed between the half-cell and earth.

Two types of voltage-clamp programme were used for investigating the time course of changes in conductance during formaldehyde treatment. In the first type (Fig. 1), after the resting potential was backed off with the d.c. calibrating voltage, the holding potential was set equal to zero and was applied 500–1000 msec before a square voltage-clamp pulse of 500 msec duration. After a further 10 sec at the holding potential, a second clamp pulse was applied, also of 500 msec duration but to a different voltage, usually of opposite polarity, from the first. This pulse was followed by another 500–1000 msec at the holding potential, and the entire programme was repeated at 20 sec intervals. The second type of voltage programme consisted of four successive steps of voltage, each of 500 msec duration but of different magnitudes, repeated once every 10 sec. In the intervals the muscle remained at the near zero resting potential. In both cases, current and thus conductance was calculated from the values at the end of each 500 msec step. For membrane voltages positive to about −40 mV, no significant change in conductance occurred during this time.

In the constant-current experiments, two micro-electrodes were inserted near the centre of a fibre. The distance between the electrodes was usually about 25 μm, and when larger an appropriate correction was made. Constant-current pulses of 500 msec duration were applied once every 10 sec through the current micro-electrode, while the membrane voltage was recorded with the other micro-electrode. The potential reference electrode and the current path to earth were via half-cells as in the constant-voltage experiments. Membrane current density was calculated by Cole's theorem (Cole & Curtis, 1941), which gives for unit area of membrane

$$I_m = \frac{R_i}{8\pi^2 a^3} I_o dI_c/dV,$$

where $I_o$ is the total current, $R_i$ and $a$ as before.

## RESULTS

### *Abolition of inward rectification*

The inward rectification which is characteristic of skeletal muscle in isotonic $KCH_3SO_4$ solution is evident in the currents recorded in Fig. 1*B* in response to the voltage clamp programme of Fig. 1*A*. In this fibre approximately ten times as much inward current was recorded at −20 mV as was outward current at +20 mV. This inward rectification was nearly abolished by treatment of the muscle for 30 min with a solution which also contained 10 mM-formaldehyde. As seen in the current traces of Fig. 1*C*, the currents after formaldehyde treatment were small and nearly non-rectifying.

The change produced in the relation between current and voltage in another fibre is illustrated in Fig. 2. Before treatment with formaldehyde, the current–voltage plot was concave downwards at negative voltages and also at positive voltages which were less than about 30 mV. Above this voltage, the plot merges into a straight line, so that the relation becomes linear above 50 mV (cf. Adrian & Freygang, 1962*a*, *b*). After formaldehyde treatment, the current–voltage relation was approximately linear over the entire voltage range, and coincided with that portion of the curve which had been linear before treatment.

The current and voltage data of Fig. 2 have been used to calculate the potassium conductance at each voltage. In Fig. 3 is shown the inwardly rectifying portion of the conductance, which has been obtained by subtracting the linear conductance ($G_L$), determined at high positive voltage, from the total conductance measured at each

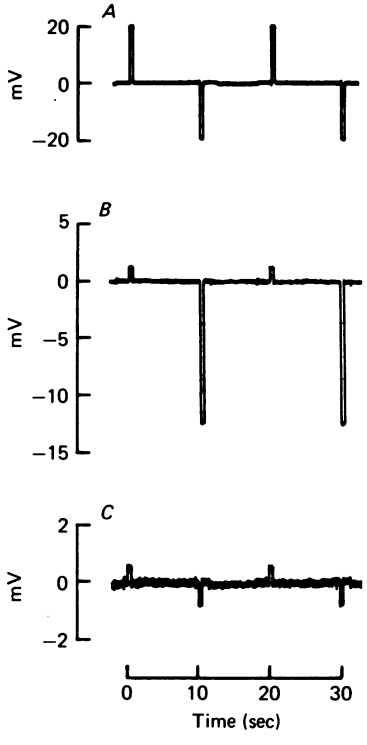


Fig. 1

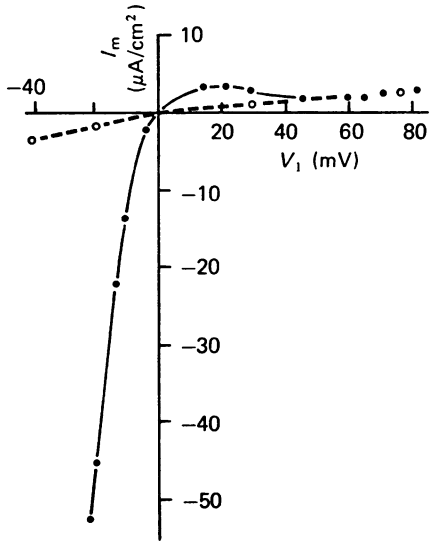


Fig. 2

Fig. 1. Abolition of inward rectification by addition of formaldehyde (10 mM) to isotonic $KCH_3SO_4$ Ringer bathing muscle. Three-electrode technique. *A*, membrane voltage $V_1$. Pulse duration 500 msec. *B*, corresponding membrane current before formaldehyde treatment (solution C), as measured by $(V_2-V_1)$. *C*, membrane current at same membrane voltages after formaldehyde treatment (solution D), at higher gain. Fibre diameter: 85 μm; electrode spacing $l$ = 500 μm. Transient capacity currents not shown in pen-traces illustrated.

Fig. 2. Current–voltage relation in isotonic $KCH_3SO_4$ Ringer before and after formaldehyde treatment. Three-electrode technique. Current measured at end of 500 msec pulse. Fibre diameter = 85 μm. Electrode spacing $l$ = 1 mm. Filled circles before (solution C), open after (solution D), formaldehyde treatment. $G_L$ = 0·04 mmho/cm².

voltage. Before formaldehyde treatment, the conductance decreased steeply with increasing voltage from 2·2 mmho/cm² at −20 mV, through a value of 0·6 mmho/cm² at 0 mV (ordinate intercept), to zero at about 50 mV.

In two fibres, pulse voltages as negative as −40 mV were used. In these fibres, the conductance–voltage relationship exhibited an inflexion point at about −20 mV, such that the curve appeared slightly concave downwards between −40 and −20 mV. Voltages more negative than −40 mV were not used with untreated fibres in order to avoid the complication of time-dependence.

After formaldehyde treatment, the rectifying conductance at $-20$ mV in the fibre of Fig. 3 was reduced to 0·04 mmho/cm², and that at 0 mV to approximately 0·01 mmho/cm². Thus in this fibre, about 98 % of the inwardly rectifying conductance was abolished by formaldehyde treatment. In twenty-two experiments (fifteen with the two-electrode and seven with the three-electrode technique), the average value of the inwardly rectifying conductance at the ordinate intercept was $0{\cdot}78 \pm 0{\cdot}36$ (S.D.) mmho/cm² before treatment with formaldehyde, and $0{\cdot}022 \pm 0{\cdot}015$ (S.D.) mmho/cm² after. The fraction abolished in each of the twenty-two experiments fell within the range of 92–99 %, with an average of 97·0 %. In these same fibres, the linear conductance averaged $0{\cdot}10 \pm 0{\cdot}05$ (S.D.) mmho/cm². Classical cable analysis of three fibres revealed no change in the internal resistivity or low frequency membrane capacitance during formaldehyde treatment.

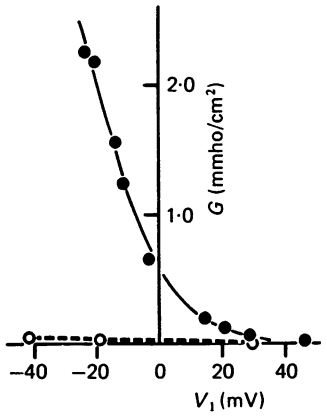


Fig. 3

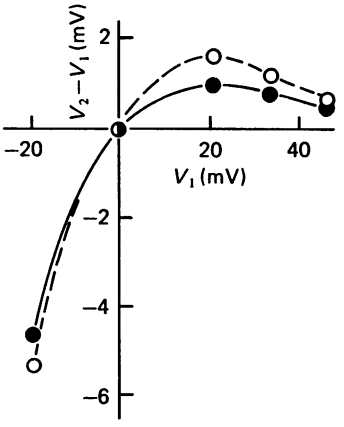


Fig. 4

Fig. 3. Rectifying conductance before and after formaldehyde treatment, calculated from data illustrated in Fig. 2. Ordinate = $I_m/V_1 - G_L$. Symbols as in Fig. 2. Curve before treatment drawn from eqn. (7), with $\bar{G} = 4{\cdot}2$ mmho/cm², $V_h = -21$ mV, and $v = 11{\cdot}5$ mV.

Fig. 4. Current–voltage relation at $t = 0$ (filled circles) and approximately 5 min (open circles) of formaldehyde treatment (solution D). Three-electrode technique. Voltage-clamp programme consisted of four consecutive 500 msec pulses in order of decreasing voltage. Fibre diameter = 125 $\mu$m, electrode spacing $l = 275$ $\mu$m.

The negative-slope portion of the curve of Fig. 2 between +20 and +50 mV is a common but variable finding in such preparations (cf. Adrian & Freygang, 1962*a*, *b*). In some fibres, the slope remained always positive, although decreasing with increasing voltage, until it became constant at about +50 mV. However, the shape of the conductance–voltage curve and its alteration by formaldehyde were similar to that in Fig. 3 whether or not a negative slope region was present in the current–voltage plot.

The effects shown in Figs. 1–3 were obtained after treatment of the muscles with formaldehyde solution for at least 30 min, at which time the conductance changes were complete. In good experiments, the conductance remained essentially unchanged for up to an additional 1·5 hr. Events which occur before this steady state is reached will now be described.

*Early conductance increase*

Exposure to formaldehyde solution causes, after a delay of about 1·5 min, a transient increase in conductance, which reaches a peak in another 3–5 min. Although this increase is substantial at certain membrane voltages, it is small or absent at voltages positive to about 50–80 mV. Thus in the experiment of Fig. 4, the currents recorded at voltages of −20, +20 and +33 mV increased during the first minutes of formaldehyde treatment, whereas the current at +50 mV was little affected. In consequence, the negative slope between +20 and +50 mV became steeper during the early phase. Furthermore, fibres showing no negative slope in this voltage region before treatment often developed one during the first 5 min, as conductance increased at voltages less than +50 mV but remained unchanged over the linear region of the current–voltage curve. These results suggest that the early increase in conductance is confined to the inwardly rectifying pathway.

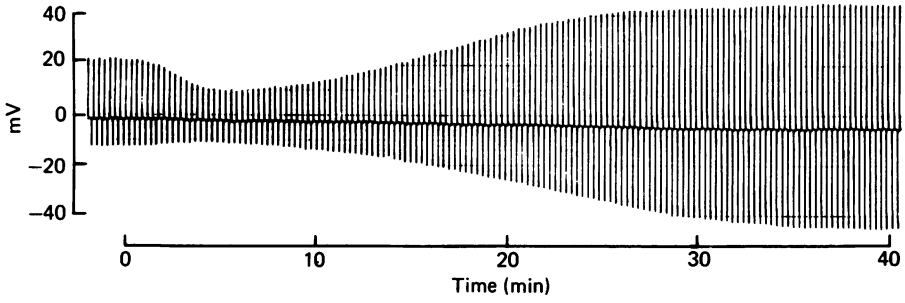


Fig. 5. Effects of formaldehyde on membrane voltage recorded during periodic application of constant-current pulses. Two-electrode technique. Muscle initially in $KCH_3SO_4$ Ringer (solution C). Pulse duration 500 msec, amplitude $1{\cdot}4 \times 10^{-7}$ A, applied once every 10 sec at alternate polarities. Formaldehyde (solution D) introduced at $t = 0$. Pen record retouched slightly.

*Time course of formaldehyde effects*

Exposure to formaldehyde thus produces first an increase and then a decrease in conductance at membrane voltages within the range of inward rectification. The time course of these changes will now be described, beginning with two-electrode constant-current experiments, as these yielded pen records that illustrated the entire sequence.

An experiment in which inward and outward current pulses of 500 msec duration and of equal magnitude were imposed alternately once every 10 sec is shown in Fig. 5. Owing to the slow paper speed used, each voltage response appears in the record as a single line, recording the maximum voltage attained during the pulse. At the beginning of the experiment, the negative voltages recorded in response to inward current pulses were smaller than the positive voltages recorded during outward pulses of the same magnitude. This assymetry reflects inward rectification since a smaller voltage in response to a given current denotes a greater conductance. Within 1 or 2 min after the application of formaldehyde the voltage responses of both polarities began to decrease, indicating an increase in conductance. At each polarity the voltage response passed through a minimum value, denoting maximum

conductance, within the first 5–7 min, and then progressively increased as the inwardly rectifying channel closed.

When constant currents are used, as in the experiment of Fig. 5, the changes in conductance which are observed during the course of formaldehyde treatment are not only due to the direct effect of formaldehyde, but also reflect the dependence of the conductance on the inconstant voltage at which it is tested. In order to separate the effects of formaldehyde and of voltage, the three-electrode voltage-clamp technique was used to arrive at the time course of the conductance change for different clamp

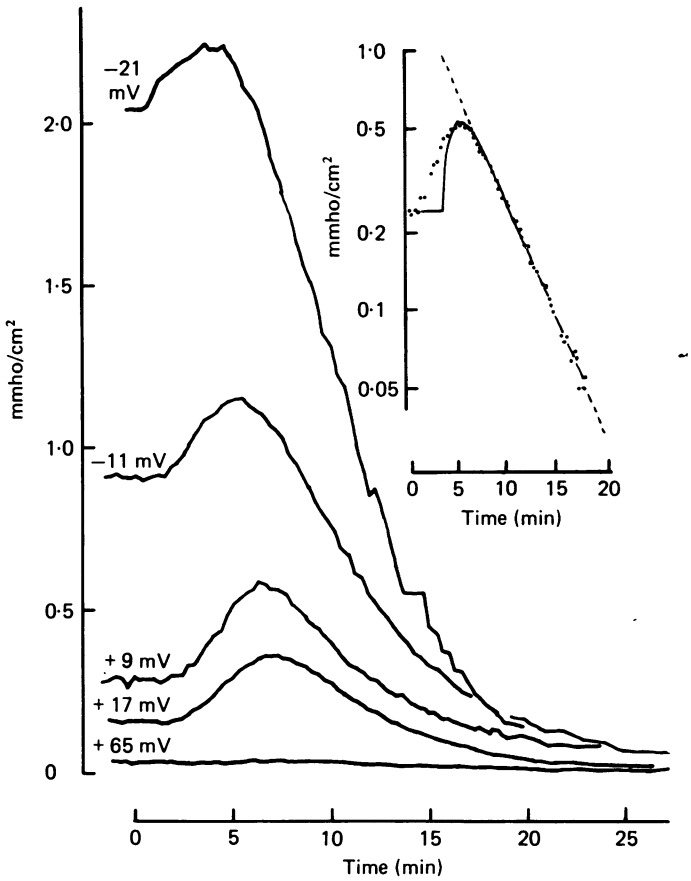


Fig. 6. Time course of conductance changes during formaldehyde treatment measured at five different clamp voltages. Three-electrode technique. Formaldehyde introduced at $t = 0$. Each curve connects conductance values measured once every 20 sec, at end of 500 msec voltage clamp pulse. Clamp voltage noted for each curve. Curves for +65 and +17 mV from same expt., diameter = 85 $\mu$m, $l$ = 1 mm. Curves for +9 and −11 mV from same expt., diameter = 75 $\mu$m, $l$ = 475 $\mu$m. Curve for −21 mV, diameter = 85 $\mu$m, $l$ = 500 $\mu$m. Inset: curve for +9 mV on logarithmic ordinate.

pulse voltages. Clamp pulses were imposed periodically throughout the formaldehyde treatment and the results obtained were converted to conductance values and plotted against time. Fig. 6 shows the changes in inward rectification occurring at five different pulse voltages in three experiments on fibres of similar diameters. Before exposure

to formaldehyde, the conductance measured at $-21$ mV was 2·05 mmho/cm$^2$. At less negative clamp voltages, the conductance was less, until at $+65$ mV, the conductance was only 0·04 mmho/cm$^2$.

After a delay of 1·5–3 min following the introduction of formaldehyde, the conductance measured at all voltages, except $+65$ mV, began to increase. This increase was soon overtaken by the subsequent decrease. The maximum conductance occurred later the more positive the clamp pulse voltage, from 4·1 min at $-21$ mV to 7·0 min at $+17$ mV. This staggering of the time of maximum conductance suggests that the peak value itself is not an adequate measure of the process underlying the increase in conductance. Rather, both the peak value and its time of occurrence appear to be determined for each voltage by a balance between two opposing processes: one which tends to increase and the other to decrease the conductance. A method was therefore sought to estimate, for each voltage, the amount of conductance increase which would have occurred if the decrease were not also occurring.

When the conductance minus its final value was plotted on a logarithmic scale against time, the eventual decrease was found to proceed exponentially, as shown for one curve in the inset of Fig. 6. In any one experiment, the time constant of decay was approximately equal for all voltages. Backward extrapolation of each decay line might therefore facilitate the analysis of the time course, but difficulty arises in assigning zero time. The first changes in conductance are not seen until at least 1·5 min after formaldehyde is introduced. Although this time provides a lower limit for the desired zero time, it is unsatisfactory for determining the initial magnitude of the extrapolated decay line, since the rise in concentration of formaledhyde at its site of action requires some finite time. In an attempt to make a better choice of zero time, the approach of the data to the extrapolated decay line was investigated. Aside from the first 2 or 3 min of conductance changes, each curve could be fitted by subtracting a second exponential term from the extrapolated decay, so that the conductance minus its final value was given for each voltage by an equation of the form

$$G = G_1 e^{-t/T_1} - G_2 e^{-t/T_2}, \tag{1a}$$

where $G_1$ is the value of the extrapolated decay line at the time chosen as zero, $T_1$ is the time constant of this exponential decay, and $G_2$ is the value at time zero of the second exponential term of time constant $T_2$. For the four curves of Fig. 6, the average value of $T_1 = 4{\cdot}6 \pm 0{\cdot}3$ (S.D.) min, while that of $T_2 = 2{\cdot}2 \pm 0{\cdot}14$ (S.D.) min. For each voltage, an equation of this form could describe the data at all times except the first few minutes. It was felt that little would be gained by the introduction of additional functions, so the early points were ignored, and a zero time was chosen for each curve such that the above equation with $t = 0$ gave the value of the conductance before treatment. In other words, zero time was chosen such that the parameter $G_2$ could be eliminated, and the conductance described by

$$G = G_1 e^{-t/T_1} - (G_1 - G_0) e^{-t/T_2}, \tag{1b}$$

where $G_0$ is the conductance before treatment. The continuous line in the inset of Fig. 6 has been drawn from eqn. (1*b*). $G_1$ appears as the value of the extrapolated decay line at the time the continuous curve rises from its initial value $G_0$.

The parameter $G_1$ was determined in this way for each curve of Fig. 6, and has been

plotted against clamp pulse voltage in Fig. 7. On the same axes are plotted the initial values of conductance as a function of voltage, for the same experiments. It can be seen from the figure that $G_1$ as a function of $V$ does not represent simply a proportionate increase in the inwardly rectifying conductance, but that it can be obtained by a shifting of the voltage dependence by approximately 25 mV in the depolarizing direction.

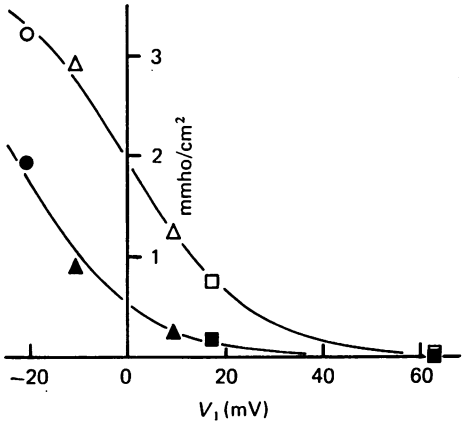


Fig. 7. Initial increase in inwardly rectifying conductance, obtained by extrapolation of logarithmic decay during formaldehyde treatment. Filled symbols: rectifying conductance before treatment. Open symbols: parameter $G_1$ from eqn. (1) (see inset of Fig. 6.) Three different symbols represent different fibres. Same experiments as Fig 6. Curves drawn from eqn. (7).

*Effect of different formaldehyde concentrations*

Two muscles were treated with formaldehyde at a concentration of 2 mM and two muscles at 20 mM, in constant-current experiments. Both the magnitude of the early increase and the completeness of the final abolition appeared to be independent of the concentration used. However, 120–150 min were required for completion in 2mM, compared with 30–40 min in 10 mM and less than 20 min in 20 mM formaldehyde. Thus the general shape of the time courses appeared to differ only by elongation or contraction of the time axis. In addition, the highest concentration caused an increase in the linear conductance during formaldehyde treatment. A similar increased leakiness occurs in fibres which have been for over 2 hr in 10 mM formaldehyde.

All other experiments were conducted with 10 mM formaldehyde, as a compromise between too slow and too harsh a treatment.

*Reversibility of changes in the inward rectifier*

In three muscles which had been treated with formaldehyde, the bathing solution was replaced by formaldehyde-free solution after the conductance had reached its low and constant state. After 60–90 min in formaldehyde-free solution, during which time no changes were apparent, a progressive increase began in the conductance to both inward and outward current, which continued for another 60–90 min until a new steady state was reached, in which inward rectification was re-established. However, the conductance to outward current was slightly greater and the conductance to inward slightly less than before treatment in all three experiments, suggesting an

increase in the linear conductance and an incomplete recovery of the inwardly rectifying conductance. In two of the three experiments, the muscles were subsequently retreated with formaldehyde. The time course of the retreatment resembled the initial treatment, except that the early increase in conductance was attenuated and the final value of the conductance was greater, confirming the suggestion that the conductance of the inward rectifier had not fully recovered, and that the linear conductance had increased during the course of the experiment.

The zero-current conductance before treatment was 0·70 and 1·54 mmho/cm$^2$ in these two experiments, and the linear conductance after the first treatment was 0·08 and 0·14 mmho/cm$^2$, respectively. Following the washing period the zero-current conductance was 0·41 and 1·60 mmho/cm$^2$. After the second formaldehyde treatment the linear conductance was 0·11 and 0·28 mmho/cm$^2$. Hence in these two experiments approximately 48% and 94% of the rectifying conductance recovered during the washing period.

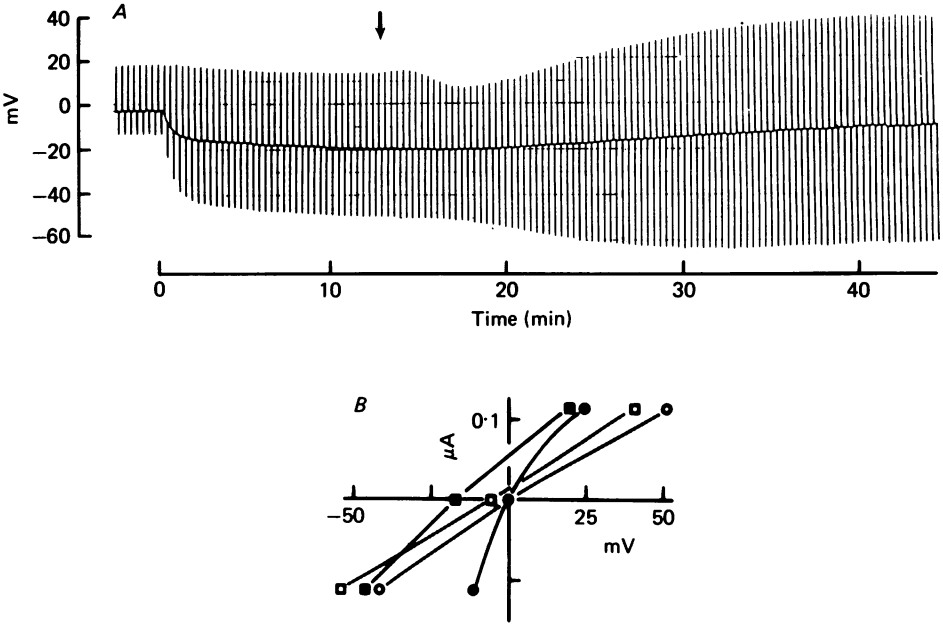


Fig. 8. Interaction of Rb and formaldehyde treatment. *A*, membrane potential measured during periodic application of 500 msec constant-current pulses ($1{\cdot}2 \times 10^{-7}$ A) of alternating polarity. Two-electrode technique. Normal K-containing fibre. At beginning of trace, muscle in K-containing solution C. At time 0, Rb-containing solution E introduced. At arrow, solution F containing Rb and formaldehyde introduced. *B*, current–voltage relations of same fibre. ●, solution C (K); ■, solution E (Rb); □, solution F (Rb and formaldehyde); ○, solution D (K and formaldehyde), obtained soon after sequence shown in *A*.

*Experiments with Rb and formaldehyde*

It has long been known that the resting membrane of skeletal muscle is much less permeable to Rb ions than to K ions, and that Rb ions interfere with the movement of K ions (see Sjodin, 1959). Adrian (1964) determined the current–voltage relations of fibres with various combinations of K and Rb inside and outside. He concluded

that Rb itself does not pass through the inwardly rectifying channel, and that even small concentrations of Rb interfere with the passage of K through that channel. The linear conductance and the delayed rectifier, by contrast, do not distinguish between K and Rb (Adrian, 1964).

In the present study, the interplay of Rb and formaldehyde on the inward rectifier was explored by examination of the effect of Rb after treatment with formaldehyde, and vice versa. In three fibres from different muscles which had been treated with formaldehyde, so that only a slight degree of rectification remained, substitution of Rb for most of the K in the bathing solution (solution F) abolished the residual rectification. This effect is illustrated by the open circles (K) and open squares (Rb) of Fig. 8*B*. In another two muscles in which inward rectification had been nearly abolished by Rb (solution E), addition of formaldehyde (solution F) produced a further decrease in conductance and completed the linearization of the current–voltage relation, as shown by the filled (untreated) and open (treated) squares in Fig. 8*B*. It appears, therefore, that either Rb or formaldehyde can abolish nearly all of the inwardly rectifying conductance, and that by their combined action the pathway can be effectively closed.

The changes in resting potential illustrated in Fig. 8 confirm this interpretation. When only one permeant ion is present, the resting potential simply reflects the ratio of ionic activities inside and outside the cell. In the case of a K-containing fibre in solution C

$$V_R = \frac{RT}{F} \ln \frac{f_{K,o}[K]_o}{f_{K,i}[K]_i}, \tag{2}$$

where $f_{K,o}$ and $f_{K,i}$ are extracellular and intracellular activity coefficients for K, $[K]_i$ the intracellular K concentration, and $[K]_c$ the concentration in solution C. The internal resistivity of a fibre does not change with formaldehyde treatment, so the intracellular activity of K apparently remains the same. Since the activity of K in the bathing solution also remains constant during formaldehyde treatment, no change in resting potential was to be expected, nor was a significant change observed, during the large changes in conductance occurring during the course of formaldehyde treatment of a K-containing fibre in K solution (Fig. 5).

On the other hand, when K is the only permeant internal ion but some of the external K has been replaced by Rb, the resting potential is given by

$$V_R = \frac{RT}{F} \ln \frac{f_{Rb,o}[Rb]_E (P_L + P_{Rb}) + f_{K,o}[K]_E (P_L + P_K^{Rb})}{f_{K,i}[K]_i (P_L + P_K^{Rb})}, \tag{3}$$

where the subscript E denotes solution E and $P_L$ is the permeability coefficient of the linear component, which is taken as being equal for K and Rb, since it was confirmed in these experiments that at voltages above 50 mV the membrane does not distinguish between these two ions. $P_K^{Rb}$ is the permeability coefficient at the resting potential of the inwardly rectifying pathway to K when Rb is the major external cation. An analogous coefficient for the permeability of the rectifying component to Rb has been included, since it may not be zero, and has been designated as $P_{Rb}$. It was not deemed necessary for clarity to include subscripts to denote that the latter two coefficients apply only to the rectifying component, nor to include a superscript on $P_{Rb}$ to denote that this coefficient refers to the permeability in Rb solution. By writing the permeability coefficients for either ion as a sum of two terms, the supposed independence and parallelism of the linear and the inwardly rectifying components is expressed.

The hyperpolarization which occurs on replacement of most of the external K by Rb (time 0, Fig. 8*A*) can be taken as evidence that Rb alone does not completely block the inwardly rectifying pathway, for if that were the case, $P_K^{Rb}$ and $P_{Rb}$ would be zero, and eqn. (3) would become

$$V_R = \frac{RT}{F} \ln \frac{f_{Rb,o}[Rb]_E + f_{K,o}[K]_E}{f_{K,i}[K]_i}. \tag{4}$$

Since the combined activity of Rb and K in the high Rb solution (E) is essentially equal to the activity of K in the K solution (C), eqns. (2) and (4) predict the same value for the resting potential, and no change would be expected to occur. The fact that a change in resting potential does occur at time 0 in Fig. 8*A* therefore indicates that Rb does not completely close the rectifying pathway. By comparing eqns. (2) and (3), it can be shown that the direction of the observed resting potential change indicates that the rectifying pathway is more permeable to K, even in the presence of Rb, than it is to Rb. In the final stages of formaldehyde treatment (Fig. 8*A*), the resting potential returned to within 2 mV of its value in K solution, showing that the membrane became much less able to distinguish between K and Rb, and thus confirming the near abolition of the inwardly rectifying pathway. In all five experiments, the membrane potential after formaldehyde treatment was 1–3 mV more negative in Rb than in K solution, suggesting that a small fraction of the inwardly rectifying conductance remained after treatment with both formaldehyde and Rb even though no significant alinearity could be detected. Thus the effects of Rb and formaldehyde are not additive, but would appear to combine in a factorial manner.

## DISCUSSION

The primary purpose of this work was to provide a more detailed description of the effects of formaldehyde upon the inward rectifier, so that treatment with formaldehyde might become an established tool in electrophysiological research of the muscle membrane. In addition, we hoped that an analysis of the actions of formaldehyde might provide clues about the mechanism of inward rectification.

From the first point of view, the almost complete abolition of inward rectification by formaldehyde may be considered as the principal result. The linear component of the K conductance which dominates the current–voltage relation in its positive reaches was found not to be reduced by formaldehyde; instead it tends to increase after prolonged exposure to formaldehyde. Whether this increase is due to a failure of the membrane to remain sealed around the micro-electrodes, or whether the membrane eventually undergoes an over-all increase in permeability could be decided by isotope flux measurements; but we have not pursued this question. When formaldehyde-treated muscles were used to study the delayed rectifier (Argibay & Hutter, 1973; Argibay *et al.* 1974) the experiments were usually completed within 2 hr of admission of the formaldehyde-containing solution, i.e. before the increase in the leak conductance became severe.

Several foreign cations including Rb suppress the conductance of K through the inward rectifier without altering the linear component. The fact that an agent so different as formaldehyde attacks the same conductance component, whilst leaving the linear component untouched, strongly supports the view that the inwardly rectifying and linear components are separate entities that lie in parallel to each other. The finding that formaldehyde in the early stages of its action increases the conductance of the inward rectifier but not the linear component is also evidence for this view; and it is of additional interest, since it points to the potentiality of the inwardly rectifying conductance to increase in magnitude at a given driving force.

The separability of the inward rectifier from the linear component is compatible with the possibility that the two components are located in different regions of the membrane. It has been suggested for instance, that the inward rectifier may reside in the transverse tubules (Hodgkin & Horowicz, 1960; Adrian, Chandler & Hodgkin, 1970*b*; Williams, 1976). Be that as it may, there are no grounds for supposing that formaldehyde acts by closing off the transverse tubule; for the low frequency

membrane capacity remains unaltered by treatment with formaldehyde. Direct evidence for the continued patency of the transverse tubules has been provided by the findings of Dr Sally Page that ferritin continues to enter transverse tubules of fibres treated with formaldehyde so as to cause the abolition of inward rectification (see Hutter, 1969). Actions of formaldehyde on the molecular scale must therefore be sought as explanations for its effect. However, before consideration can be given to the chemical possibilities, the mechanism of inward rectification requires further discussion.

According to present views, the current flowing through the inward rectifier is determined by (i) a practically instantaneous function of the driving voltage, (ii) a time-dependent scaling factor which diminishes at voltages more negative than $-100$ mV (Adrian *et al.* 1970*b*; Almers, 1972), and (iii) the concentration of $K^+$ in the transverse tubules (Adrian & Freygang, 1962*a*). In the present experiments the last two factors are not operative as the muscles were in isotonic $KCH_3SO_4$ solution and relatively small voltage pulses were normally used. We are therefore here concerned only with the instantaneous current–voltage relation and the effects of formaldehyde upon it. The peculiar feature of this instantaneous current–voltage relation is the negative slope resistance in the positive quadrant, which arises from the progressive fall in the K conductance with increasingly positive membrane potential.

Thus in the present experiments the rectifying conductance of untreated fibres can be expressed as

$$G_0 = \bar{G} \times f(V), \tag{5}$$

where $\bar{G}$ is a scaling factor, $V$ is the applied voltage and $f(V)$ is a function which decreases with increasing $V$ as in Figs. 3 and 7. Formaldehyde can be supposed to alter $\bar{G}$ and $f(V)$ in such a way that before formaldehyde treatment and during all but the first few minutes of such treatment, the conductance can be described by eqn. (1*b*), with the voltage dependence of $G_0$ and $G_1$ as shown in Fig. 7.

The double exponential nature of the time course can be explained by supposing that formaldehyde undergoes two different reactions, one of which increases and the other of which decreases the conductance, both reactions obeying first-order kinetics. These two reactions could be postulated to proceed either independently in parallel, or sequentially, and still give rise to the form of eqn. (1*b*). However, investigation of the kinetic equations describing such reactions reveals that the interpretation placed on the parameter $G_1$ differs in these two cases. In the case of parallel reactions, both the magnitude and the voltage dependence of $G_1$ as shown in Fig. 7 can be explained by assuming that one reaction increases the conductance by acting only on $f(V)$ of eqn. (5). Thus, over the course of formaldehyde treatment, this reaction would cause the voltage dependence of the conductance to undergo a shift from $f(V)$ to $f(V-\phi)$, where $\phi$ is the magnitude of the shift in the positive direction, equal to about 20 mV in these experiments (see Fig. 7). At the same time, the other reaction would produce a progressive decrease in the scaling factor $\bar{G}$. On the other hand, in the case of sequential reactions $G_1$ of eqn. (1*b*) is influenced by the time constants $T_1$ and $T_2$ in such a way that this model cannot be made to fit our data without introducing further arbitrary assumptions. Thus the simplest explanation of our results appears to be that one reaction increases the conductance by a positive shift in the voltage dependence while another simultaneous reaction decreases the conductance by eroding the scaling factor $\bar{G}$.

A well-known reaction of formaldehyde with protein is a condensation with free amino groups, which increases the negativity of the protein molecule. If such a reaction occurred on the inner surface of the muscle membrane, thus creating or altering an existing boundary potential, a positive shift in the voltage dependence $f(V)$ of the conductance would occur, such as has been postulated as the basis of the conductance-increasing effect of formaldehyde. A similar reaction could render a carrier or gating molecule ineffective, leading to a progressive decrease in $\bar{G}$ of eqn. (5). An alternative mechanism for this decrease could derive from cross-linking reactions, which could immobilize or otherwise inactivate a carrier or gating molecule.

Theoretical models of mechanisms capable of producing inward rectification have been developed by Adrian (1969) and by Ciani, Krasne, Miyasaki & Hagiwara (summarized in Hagiwara, Miyazaki, Krasne & Ciani, 1977). The mathematical descriptions of both these models contain parameters which can be related to the present findings.

Adrian (1969) has developed a model in which K ions cross the membrane in combination with a divalent negative carrier molecule whose concentration at the inner surface of the membrane, $[A]_a$, is buffered. The possibility of a boundary potential at the inner surface, $\psi_a$, has also been incorporated in the model. If his eqn. (15.1) is expressed as conductance, in the particular case where $[K]_i = [K]_o$, then

$$G = \alpha [A]_a [K]_o F \frac{\exp(VF/RT) - 1}{V\{\exp 2(V+\psi_a)F/RT + \exp(V+\psi_a)F/RT\}}, \tag{6}$$

where $\alpha$, which has the units of $\text{mole}^{-1}\ \text{sec}^{-1}\ \text{cm}^4$, is the rate constant for the formation of the K-carrier complex, and $[K]_o$, $F$, $R$ and $T$ have their usual significance. $V$ refers to the applied voltage, as before. This equation fits our data for values of $V$ positive to about $-20$ mV in the case of untreated fibres, and corresponds to the general form of eqn. (5).

Formaldehyde could thus be supposed to decrease the conductance of the inward rectifier by reacting with the carrier molecules or with the buffer system which controls the concentration of mobile carriers in the membrane, such that a progressive decrease in $[A]_a$ occurs, leading to a decrease in $\bar{G}$. Furthermore, the supposed change from $f(V)$ to $f(V-\phi)$ brought about by formaldehyde could be related to a change in the boundary potential $\psi_a$. A shortcoming of this approach is that a change in $\psi_a$ does not produce simply a shift in the voltage dependence, since $V$ appears twice in eqn. (6) without $\psi_a$.

In the model by Ciani *et al.* (Hagiwara *et al.* 1977), K apparently moves by electrodiffusion through a channel which is created by a voltage-dependent re-orientation of charged molecules in the membrane and which is stabilized by the binding of external K ions to the gating site. The mathematical formulation given by these authors was initially derived empirically from studies of the inward rectifier in starfish eggs (Hagiwara & Takahashi, 1974). The latter obtained

$$G = \bar{G} \frac{1}{1+\exp(V-V_h)/v}, \tag{7}$$

where $\bar{G}$ is the maximum conductance (at very negative $V$) for a given $[K]_o$, and $V_h$ and $v$ are parameters of the model. This equation lends itself well to our results, since formaldehyde can be supposed to decrease the value of $\bar{G}$ by one reaction, and to

alter the value of $V_h$ by the other. The two curves of Fig. 7 have been constructed from eqn. (7), with $\bar{G} = 4{\cdot}0$ mmho/cm$^2$, $v = 12{\cdot}5$ mV, and $V_h = -22{\cdot}4$ mV before formaldehyde treatment and $-0{\cdot}4$ mV after.

The models discussed above differ categorically in the predicted behaviour of the conductance at very negative voltages: eqn. (6) provides for a progressive increase in conductance at increasingly negative voltages, owing to the supposedly unlimited availability of carriers; eqn. (7), by contrast, implies saturation of the conductance once formation of channels is complete. As very negative voltages were usually avoided, most of our experiments do not contain evidence on the basis of which these models might be distinguished. However, in fibres 'detubulated' by glycerol treatment, 10–20% of the inwardly rectifying conductance remains intact (Williams, 1976), and very negative voltages, which would reprime the contractile apparatus in normal fibres, may be more readily imposed. In two such fibres, studied with the three-electrode voltage-clamp technique, conductance values 10 msec after the onset of a pulse were determined. Saturation was seen to occur at about $-100$ to $-120$ mV. In one of these fibres, conductance was determined at voltages covering the range from $-120$ to $+70$ mV, and eqn. (7) was found to fit the data well, with $\bar{G} = 0{\cdot}45$ mmho/cm$^2$, $V_h = -27$ mV, and $v = 20{\cdot}4$ mV.

This work was supported by an M.R.C. project grant. Thelma L. Williams was a British American Research Fellow of the American Heart Association and the British Heart Foundation. The technical assistance of Mrs Jean Black and Mr Ian Drake is gratefully acknowledged.

## REFERENCES

ADRIAN, R. H. (1964). The rubidium and potassium permeabilities of frog muscle membrane. *J. Physiol.* **175**, 134–159.

ADRIAN, R. H. (1969). Rectification in muscle membrane. *Prog. Biophys. biophys. Chem.* **19**, 341–369.

ADRIAN, R. H., CHANDLER, W. K. & HODGKIN, A. L. (1970*a*). Voltage-clamp experiments in striated muscle fibres. *J. Physiol.* **208**, 607–644.

ADRIAN, R. H., CHANDLER, W. K. & HODGKIN, A. L. (1970*b*). Slow changes in potassium permeability in skeletal muscle. *J. Physiol.* **208**, 645–668.

ADRIAN, R. H. & FREYGANG, W. H. (1962*a*). The potassium and chloride conductance of frog muscle membrane. *J. Physiol.* **163**, 61–103.

ADRIAN, R. H. & FREYGANG, W. H. (1962*b*). Potassium conductance of frog muscle membrane under controlled voltage. *J. Physiol.* **163**, 104–114.

ALMERS, W. (1972). The decline of potassium permeability during extreme hyperpolarization in frog skeletal muscle. *J. Physiol.* **225**, 57–83.

ARGIBAY, J. A. & HUTTER, O. F. (1973). Voltage-clamp experiments on the inactivation of the delayed potassium current in skeletal muscle fibres. *J. Physiol.* **232**, 41–42*P*.

ARGIBAY, J. A., HUTTER, O. F. & SLACK, J. R. (1974). Consecutive activation and inactivation of the delayed rectifier in skeletal muscle. *J. Physiol.* **237**, 46–47*P*.

COLE, K. S. & CURTIS, H. J. (1941). Membrane potential of the squid giant axon during current flow. *J. gen. Physiol.* **24**, 551–563.

HAGIWARA, S., MIYAZAKI, S., KRASNE, S. & CIANI, S. (1977). Anomalous permeabilities of the egg cell membrane of a starfish in $K^+$–$Tl^+$ mixtures. *J. gen. Physiol.* **70**, 269–281.

HAGIWARA, S. & TAKAHASHI, K. (1974). The anomalous rectification and cation selectivity of the membrane of a starfish egg cell. *J. Membrane Biol.* **18**, 61–80.

HODGKIN, A. L. & HOROWICZ, P. (1960). Effect of sudden changes in ionic concentration on the membrane potential of single muscle fibres. *J. Physiol.* **153**, 370–385.

HUTTER, O. F. (1969). Potassium conductance of skeletal muscle treated with formaldehyde. *Nature, Lond.* **224**, 1215–1217.

HUTTER, O. F. & NOBLE, D. (1960). The chloride conductance of frog skeletal muscle. *J. Physiol.* **151**, 89–102.

KATZ, B. (1949). Les constantes électriques de la membrane du muscle. *Archs Sci. physiol.* **3**, 285–299.

NAKAJIMA, S. & HODGKIN, A. L. (1972). The effect of diameter on the electrical constants of frog skeletal muscle. *J. Physiol.* **221**, 105–120.

SJODIN, R. (1959). Rubidium and cesium fluxes in muscle as related to the membrane potential. *J. gen. Physiol.* **42**, 983–1003.

STANFIELD, P. R. (1970). The differential effects of tetraethylammonium and zinc ions on the resting conductance of frog skeletal muscle. *J. Physiol.* **209**, 213–256.

WILLIAMS, THELMA L. (1976). The location of inward rectification in skeletal muscle. *J. Physiol.* **256**, 125*P*.