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EFFECTS OF INTERNAL POTASSIUM AND SODIUM ON THE ANOMALOUS RECTIFICATION OF THE STARFISH EGG AS EXAMINED BY INTERNAL PERFUSION

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SUMMARY

1. The effects of alterations of the intracellular ionic composition on the properties of anomalous (or inward) rectification of the egg membrane of the starfish, *Mediaster aequalis*, were studied by using an intracellular perfusion technique. The following results were obtained, analysing the membrane current with the voltage-clamp technique.

2. The inward rectification of the K conductance depends only on the membrane potential, V, when the K equilibrium potential, V_K, is altered by changing the internal K^+ concentration at a fixed external K^+ concentration, while it depends on $V - V_K$ when V_K is altered by changing the external K^+ at a fixed internal K^+ concentration.

3. From the above the conclusion is reached that the gating of the K channel of the inward rectification depends on V and external but not internal K^+ concentration.

4. The conductance of the K channel at a given voltage is roughly proportional to the square root of $[K^+]_i$ when the latter is altered at a fixed external K^+ concentration.

5. Since the conductance is proportional to the square root of $[K^+]_o$ when this is altered at a fixed internal K^+ concentration, the final conclusion is that this conductance is proportional to the geometric mean of the external and internal K^+ concentrations.

6. Intracellular Na^+ ions are necessary for the activation of inward rectification; the K conductance increases sharply with internal Na^+ concentration, reaching saturation at 200 mM.

7. A similar potentiating effect is found for Li^+, although it is weaker. Rb^+, Cs^+ and organic cations such as arginine$^+$ do not have this effect.

INTRODUCTION

Since Katz (1949) described the anomalous or inward rectification of the K conductance in the membrane of frog skeletal muscle fibres, similar phenomena have been observed in a number of other tissues. The membrane of the immature starfish egg shows a very similar inward rectification (Hagiwara & Takahashi, 1974; Miyazaki, Ohmori & Sasaki, 1975; Hagiwara, Miyazaki & Rosenthal, 1976; Hagiwara, Miyazaki,

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Krasne & Ciani, 1977). The experimental results show: (1) that the rectification depends on $V - V_K$ rather than V alone, where V and V_K represent the membrane potential and the K equilibrium potential of the membrane, respectively, (2) that the K conductance at a given $V - V_K$ is approximately proportional to the square root of the external K^+ concentration, and (3) that, in the external bathing fluid, Na and Li ions, and organic monovalent cations such as NH_4^+, $Tris^+$, $choline^+$, $arginine^+$, etc. show no significant effect on the development of the inward rectification, whereas Rb^+, Cs^+ and Tl^+ show a blocking effect on the K current.

The present experiment was planned to observe the effects of alterations of the intracellular ionic composition on these properties. For this purpose, a modification of the intracellular perfusion technique developed for the tunicate egg by Takahashi & Yoshii (1978) was used. The membrane currents of the internally perfused egg of the starfish, *Mediaster aequalis*, were observed by means of the voltage-clamp technique.

METHODS

Materials. Immature eggs of the starfish, *Mediaster aequalis*, were used. These eggs are about 1 mm in diameter. Specimens were collected in Southern California during the months of January to March. Immature eggs were collected as described previously (Hagiwara, Ozawa & Sand, 1975).

Intracellular perfusion. The design of the perfusion apparatus and the procedure were similar to those used for the tunicate egg by Takahashi & Yoshii (1978; see Fig. 1 of their paper). Eggs were soaked first in an acidic saline (pH of the normal saline was reduced to 4·5 by adding HCl) for 5 min to remove the jelly coat of the cell, and then treated with collagenase (1 mg/ml.) in normal saline for 5–10 min to clean the egg surface. Glass funnels were made from Pyrex capillaries of 1·5 mm diameter and the edges of the opening were heat-polished. The diameter of the opening at the tip was 0·5 mm. A treated egg was introduced inside the funnel at its tip after coating the inner surface of the funnel at the tip with liquid silicon (curing agent of SYLGARD 184) to facilitate the contact between the egg and glass wall. The funnel was attached to the bottom of the upper chamber; this upper chamber and the interior of the funnel above the egg were filled with the external saline. The upper chamber system was brought above the lower chamber, which was filled with the internal solution, so that the opening of the funnel was immersed in the internal solution. The level of the upper chamber was adjusted so that the level of the solution in the upper chamber was slightly higher (about 1 mm) than that in the lower chamber. Establishment of contact between the egg and the funnel wall was examined by observing the resistance between the two chambers. When the resistance attained 0·5–1·0 MΩ, the upper chamber was temporarily lifted and the tip of the funnel was wiped with a piece of tissue paper to rupture the egg membrane and to remove excess amounts of liquid silicon. A fine needle was usually introduced inside the cell through the opening a few times to ensure the passage of the applied internal solution during perfusion. After this procedure the upper chamber was brought back to the original position. The solution of the lower chamber (internal solution) was perfused with a micropump (Minipulse 2, Gilson Medical Electronics, Middle Town, Wis.) at a rate of 2·8 ml./min. About 3 min was required for complete exchange of the solution in this chamber. As will be described later, it took a much longer time (40–60 min) to exchange the solution inside the egg. The major portion of the time was required for the solution to diffuse into the cell. In this respect, the present method might better be called dialysis (Kostyuk & Krishtal, 1977) rather than perfusion. It is, however, called perfusion, since similar techniques have been referred to as perfusion (Krishtal & Pidoplichko, 1975; Lee, Akaike & Brown, 1978; Takahashi & Yoshii, 1978).

Electrical recording. A few recordings were made from the intact egg, using a two-micropipette voltage clamp similar to that described by Hagiwara *et al.* (1975). Most other experiments involved internally perfused eggs, for which we used a voltage-clamp system similar to that used by Takahashi & Yoshii (1978), a brief description of which is given below. The potential and the current electrodes were placed in the external solution in the upper chamber, and the

reference electrode was in the internal solution in the lower chamber. All these electrodes were 3 M-KCl-filled Ag-AgCl glass pipettes with resistances less than 10 kΩ. The potential of the solution in the lower chamber was held at virtual ground by current passed through a fourth electrode (3 M-KCl, agar Ag-AgCl). The membrane potential was recorded differentially between the potential and reference electrodes. The membrane current was measured as a voltage drop across a 100 kΩ resistor inserted between the output of the feed-back amplifier and the current electrode of the upper chamber. No series resistance compensation was used. The size of the observed series resistance was less than 2·5 kΩ and this gave a potential error of less than 2·5 mV when the membrane current was maximal (1 μA). The rise time of voltage step was less than 1 msec.

TABLE 1. External solutions (mM)

	KCl	NaCl	$CaCl_2$	$MgCl_2$	PIPES	NaOH
Normal	10	460	10	50	5	7·5*
25 mM-K	25	445	10	50	5	7·5*
50 mM-K	50	420	10	50	5	7·5*

Internal solutions (mM)

Solution	KOH	NaOH	EGTA	Ca gluconate	PIPES	Glutamic acid	Glucose
A1	300	60	25	12·5	20	255*	315
A2	50	60	25	12·5	20	5*	815
B1	200	165	20	—	20	295*	300
B2	—	365	20	—	20	295*	300
C	200	200	25	12·5	20	295*	235
D	200	—	25	12·5	20	95*	635
E	200	100*†	25	12·5	20	195	435

* Used for titration to pH 7·0.
† Replaced with 100 mM-LiOH, RbOH, CsOH, or arginine.

Solutions. The composition of the solutions used is listed in Table 1. The external solution in most of the experiments contained 25 mM-K. Some experiments were performed in solutions with 50 mM-K and essentially the same results were obtained. The following points were common to all the internal solutions: (1) the major anion was glutamate⁻. In a few cases fluoride⁻ was used but this eliminated the inward rectification, (2) the total tonicity was kept equivalent to 0·5 M-NaCl by adding glucose, (3) the pH of the solution was buffered at 7·0 with 20 mM-PIPES (pKa_2 = 6·9). Internal solutions A contained 60 mM-Na and the Ca^{2+} concentration was buffered at 2×10^{-7} M with 25 mM-EGTA (ethyleneglycol bis (β-aminoethylether)-N, N′-tetraacetic acid) and 12·5 mM-Ca gluconate. Internal solutions B contained 20 mM-EGTA but no calcium salt; thereby the intracellular Ca^{2+} concentration should have been much lower than 2×10^{-7} M. Solutions with different K^+ concentrations were obtained by mixing A1 and A2 or B1 and B2. Solutions C and D were used to examine the effect of the internal Na^+ concentration at 200 mM-K. Solution E was used to compare the effects of internal Li^+, Rb^+, Cs^+ and arginine⁺ to Na^+. All experiments were performed at room temperature (21–22 °C).

Measurements of internal K^+ and Na^+ activities in the intact egg. The activity of the internal K^+ of the intact egg was observed by using a K^+-selective glass micropipette, filled with ion exchange resin (Corning 477317). The K^+ activity was observed as a potential difference between the K^+-selective electrode and a 3 M-KCl-filled glass micropipette, both intracellular (Walker, 1971; Steinhardt, Ludin & Mazia, 1971). To measure the intracellular Na^+ activity, the cell was cleaned as described above and washed with Na-free saline (Na^+ replaced with choline⁺), then trapped inside of the glass funnel using Na-free saline in both chambers. The procedure for preparing the egg for internal perfusion was then followed, up to and including the point of rupturing the membrane. The Na^+-selective electrode, made with NAS_{11-18} glass (Microelectrode Inc., Londonderry, N.H., U.S.A.; see Eisenman, Rudin & Casby, 1957), and a 3 M-KCl-filled glass micropipette were introduced inside the cell through the ruptured membrane. The opening was exposed to the air in this case. The saline inside the funnel was grounded.

RESULTS

The internal K concentration

The resting potential of the egg membrane, examined with 3 M-KCl-filled intracellular glass micropipette, changed with an almost perfect Nernst slope for changes in the external K^+ concentration, replacing NaCl in normal saline with equimolar KCl (Hagiwara & Takahashi, 1974). The intracellular K^+ concentration calculated from the Nernst equation ranged between 170 and 210 mM, assuming the activity coefficient of K^+ to be the same inside and outside the cell. The internal K^+ concentrations as measured with the intracellular K^+-selective electrode agreed closely. In one cell the calculated concentration was 189 mM and the observed value was 180 mM. In another cell the former was 193 mM and the latter was 200 mM. The evidence suggests that the membrane of the starfish egg is predominantly permeable to K^+ at rest and that the zero-current membrane potential, V_0, represents the K^+ equilibrium potential, V_K.

If the membrane potential was changed by passing current, the K conductance of the membrane showed an anomalous or inward rectification (Hagiwara & Takahashi, 1974; Miyazaki *et al.* 1975). When examined by the voltage-clamp technique, the rectification consisted of two components, one present immediately after the onset of the voltage change (instantaneous), and one time-dependent. The current–voltage relation of an intact cell at $[K^+]_o$ = 10, 25 and 50 mM measured after the current had reached a steady-state level is shown in Fig. 1*A*. Since V_0 is equal to V_K, the steady-state K conductance, G_K, can be calculated from $I_s/(V-V_0)$ where I_s and V are the steady-state current and the membrane potential during the voltage pulse. Values of G_K calculated from the data in Fig. 1*A* are plotted against V in Fig. 1*B*. As shown previously in detail (Hagiwara & Takahashi, 1974; Hagiwara *et al.* 1976), (1) G_K depends on $V-V_K$ rather than V alone and (2) the amplitude of G_K at a given $V-V_K$ increases with external K^+ concentration, being roughly proportional to the square root of $[K^+]_o$. These results were obtained with intracellular micropipettes and the physiological internal ionic composition.

The external K^+ concentration was kept at 25 mM in most of the intracellular perfusion experiments. The resting potential of the intact cell was from -48 to -54 mV with this external K^+ concentration (Fig. 1*A*). The actual intracellular K^+ concentration did not seem to change significantly in the first several minutes after the onset of perfusion. Therefore, the zero-current potential recorded between the inner and outer compartments of the perfusion chamber should not differ from these values more than expected due to the liquid junction potential between the applied internal solution and the interior of the egg. The junction potential was likely to be less than a few mV for the solutions used. The observed value of the zero-current potential was, however, significantly more positive, ranging between -15 and -10 mV. This was probably due to the leakage between the inner surface of the glass funnel and the outer surface of the egg. The results in Fig. 1*B* show that the K conductance decreased rapidly as the membrane potential became more positive than V_K. Therefore, a small leakage current would be enough to shift the resting membrane potential in the positive direction significantly away from V_K. The fact that the zero-current membrane potential observed in the perfused cell at

$[K]_o$ = 25 mM tended to be −10 to −15 mV rather than some value between these and −48 to −54 mV is a consequence of the negative slope conductance between these two membrane potential regions (Fig. 1*A*). The zero-current potential changed during perfusion and the amplitude as well as the sign of the change depended on the composition of perfusing solution. In most of the voltage-clamp experiments the membrane potential was held at the zero-current potential determined at the beginning of perfusion.

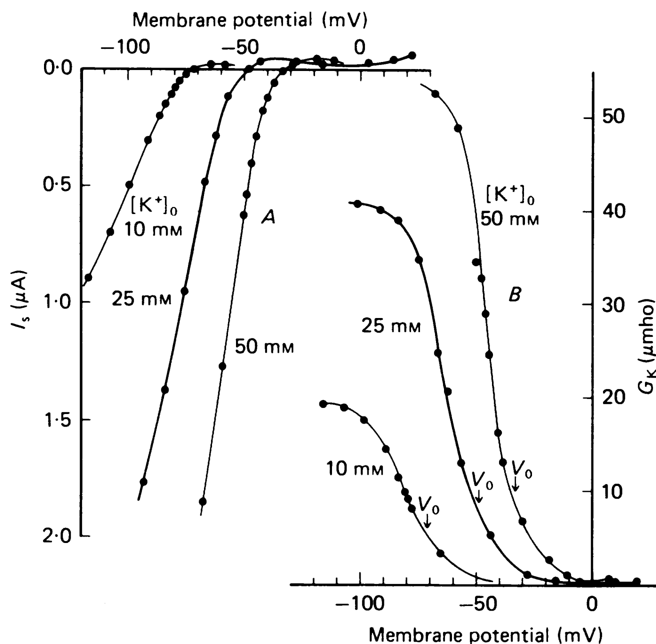

Fig. 1. *A*, steady-state current–voltage relations obtained by voltage clamp of an intact egg. Voltage clamp was performed with two intracellular micro-electrodes. The external K^+ concentration was altered from 10 to 25 and 50 mM by replacing NaCl in the normal external saline with KCl and is represented by the figures adjacent to the curves. The holding potential was the zero-current potential (−72, −48 and −34 mV for $[K^+]_o$ of 10, 25 and 50 mm respectively). *B*, steady-state K conductance $G_K = I_s/(V - V_0)$ is calculated from the data in *A* and plotted against *V*.

The membrane currents recorded during voltage clamp in a perfused cell are shown in Fig. 2*A*. After the initial capacitative current (not seen in records), the current changed from the initial instantaneous current, I_0, to a final steady value, I_s, with an approximately exponential time course. The time-dependent change was a decrease of the inward current for V = −41 or −51 mV whereas it was an increase for V = −60 mV. As shown previously (Hagiwara *et al.* 1976) the time-dependent change of the membrane current is due to the time-dependent increase in the K conductance. Therefore the K^+ equilibrium potential, V_K, can be obtained as a reversal potential of the time-dependent current. The initial instantaneous current, I_0, and the final steady value, I_s, are plotted against the membrane potential in Fig. 2*B*. It shows that V_K determined as the membrane potential at which the $I_s - V$ curve crosses the $I_0 - V$ curve was −53 mV. The value is very close to the

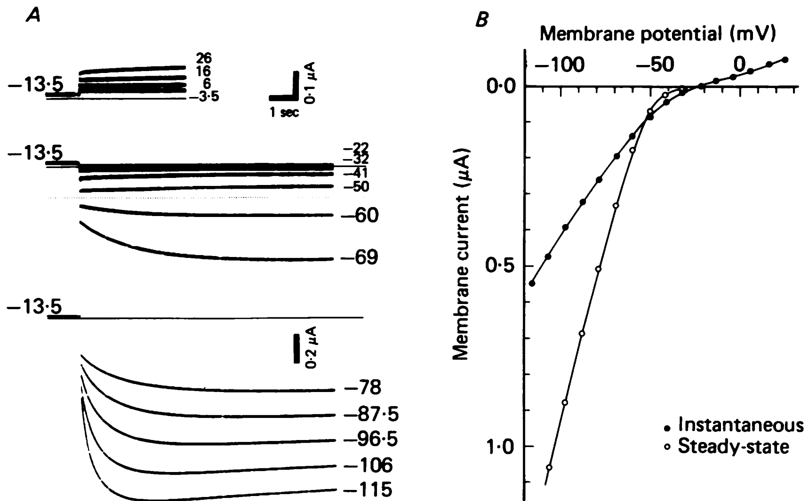

Fig. 2. A, membrane currents obtained during voltage clamp. The cell had been perfused with 200 mM-K solution A for 35 min. The holding potential was $-13\cdot5$ mV. The membrane potential during the voltage pulse is listed beside each current trace. $[K^+]_o = 25$ mM. The dotted line indicates that the level of V_K lies between -50 and -60 mV. B, current–voltage relations for the instantaneous current, I_0, and the steady-state current, I_s, obtained from the data of A. When the inward current started to decline after it had reached a peak, the peak amplitude was taken as the value of the steady-state current (for -106 and -115 mV).

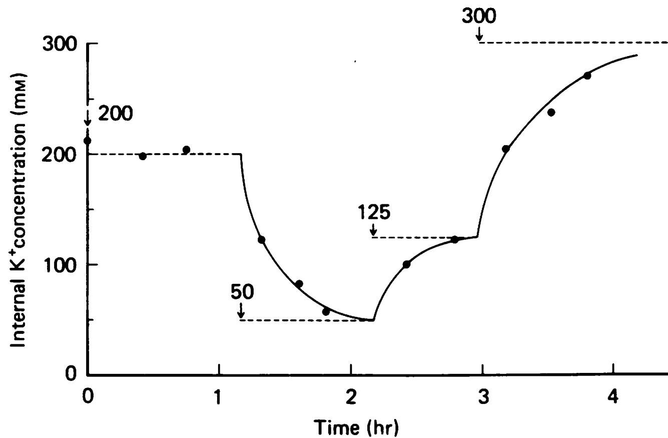

Fig. 3. Time course of internal K^+ concentration during intracellular perfusion. V_K was obtained as the membrane potential at which I_0 was equal to I_s and then $[K^+]_i$ was calculated from the Nernst equation at $[K^+]_o = 25$ mM. The internal solutions were solution A. An arrow indicates the moment of change of the internal solution.

potential calculated from the Nernst equation with $[K^+]_o = 25$ mM and $[K^+]_i = 200$ mM.

The effects of the internal K^+ concentration when the K^+ concentration of the perfusing solution was changed from an initial value of 200 mM to 50, 125 and 300 mM in succession (solution A) are shown in Fig. 3. V_K was measured approximately every 20 min by the method described above and the value of $[K^+]_i$ was then

calculated from the Nernst equation. The data show that $[K^+]_i$ changed with an approximately exponential time course after the change in the K^+ concentration of the applied solution and reached the concentration of the applied solution in 40–60 min. The final value of the $[K^+]_i$ thus determined was independent of the Na^+ concentration of the perfusing solution. Most experiments were carried out with the internal Ca^{2+} concentration buffered at 0·2 μM, but in some experiments the internal solution contained only 20 mM-EGTA (solution B), i.e. the internal Ca^{2+} concentration should be much lower than 0·2 μM. In both cases the final value of $[K^+]_i$ obtained by the above method agreed with the K^+ concentration of the applied solution. Therefore, it seemed safe to conclude that V_K obtained by the present method represented the actual V_K. This, at the same time, provided us assurance that the intracellular K^+ concentration was controlled by the present technique after perfusion for 40–60 min.

K conductance and K concentration

As described already, the leakage could not be abolished with the present perfusion technique. The current resulting from the leakage can be described as $G_L\,(V-V_L)$ where G_L and V_L are the leakage conductance and its e.m.f. respectively. They are assumed independent of the membrane potential and time. The steady-state current I_s at V is written as:

$$I_s = G_K\,(V-V_K)+G_L\,(V-V_L). \tag{1}$$

When the membrane current at $V = V_K$ is denoted by I^*,

$$I^* = G_L\,(V_K-V_L). \tag{2}$$

Then, the apparent steady-state K conductance G_K^* is given by

$$G_K^* = \frac{I_s-I^*}{V-V_K} = G_K+G_L. \tag{3}$$

The value of G_K^* can readily be calculated from the observed values of V_K and I^* in the current–voltage relation. The results in Fig. 4*a* were calculated from the steady-state current–voltage relation obtained from the cell perfused with 200 mM-K solution A for 35 min. The S-shaped curve was similar to that of an unperfused cell. In the latter case, the conductance was almost negligible for values of V between -10 and $+20$ mV compared with that for V_K at $[K^+]_o = 25$ mM. The value of G_K^* decreased as V became more positive and reached a potential-independent value (1·8 μmho in Fig. 4*a*) for the range between -10 and $+20$ mV. Therefore, the amplitude of G_K^* in this range can be considered roughly equal to G_L. The amplitude of G_L thus determined gradually increased during perfusion over 3–4 hr. Whenever it exceeded 5 μmho the subsequent data were discarded.

Although the relationship between the steady-state K conductance and the membrane potential was sigmoidal in the internally perfused cell, it rose less steeply when compared with those obtained with intracellular micropipettes in an unperfused cell (see Fig. 1*B*). This tendency became more marked with time during perfusion. The data in curve *b* in Fig. 4 were obtained from the same cell as that of curve *a* but after an additional 20 min of perfusion with the same internal solution;

b is slightly broader than *a*. This broadening tended to occur faster when the K^+ concentration of the perfusing solution was low. The data in curve *c* in Fig. 4 were obtained 10 min after the K^+ concentration of the perfusing solution was switched from 200 mM to 20 mM. At this time the observed V_K indicated that the actual internal K^+ concentration was about 100 mM. The cell was perfused continually with 20 mM-K solution for 50 min and then with 100 mM-K solution for 40 min. The

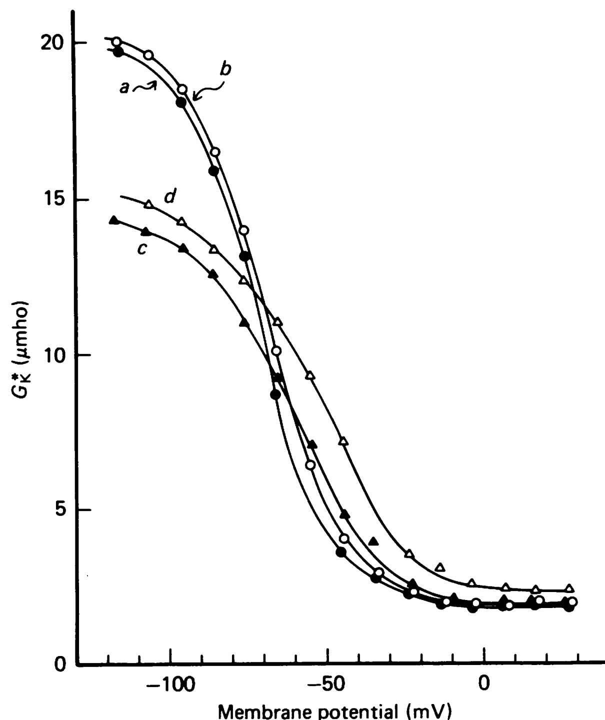

Fig. 4. Relation between the apparent K conductance, G_K^* and V obtained with the same cell. $[K^+]_o$ = 25 mM. $[K^+]_i$ = 200 mM in *a* and *b* and 100 mM in *c* and *d*. For further explanation see text.

data in curve *d* were then obtained. The observed V_K indicated that the internal K^+ had reached 100 mM. The steady-state K conductance, G_K, obtained by subtracting G_L from G_K^* was the same for *c* and *d* at large negative membrane potentials. However, curve *d* is substantially broader than curve *c*. One of the aims of the present work was to distinguish whether the inward rectification depended on $V - V_K$ or V alone when V_K was altered by changing the internal K^+ concentration. A comparison of curve *a* with curve *d* gives an impression that the $G_K - V$ relation shifted along the V axis with V_K, when V_K was altered by changing the internal K^+. This, however, did not seem to be the case for the following reasons: (1) the shape of this curve changed with time even when the internal K^+ was fixed. (2) once the broadening effect reached a saturation level as the result of a prolonged perfusion with a low K solution (about 1 hr of perfusion at 20–60 mM-K) the shape of $G_K - V$ relation became almost independent of internal K^+. Therefore, the change cannot be considered to be a shift of the curve with V_K. To avoid any confusion, the effect of internal K^+ concentration

upon G_K was examined after the broadening reached a saturation level. The broadening was similar in a Ca-free solution (containing EGTA) and a Ca-buffered solution ($[Ca^{2+}]_i = 0{\cdot}2\ \mu M$). The Na^+ concentration of the internal solution did not alter the time course of the broadening. The broadening process was associated with two additional changes in the membrane current: (*a*) the time constant for the development of the time-dependent K current at a given V increased and (*b*) the

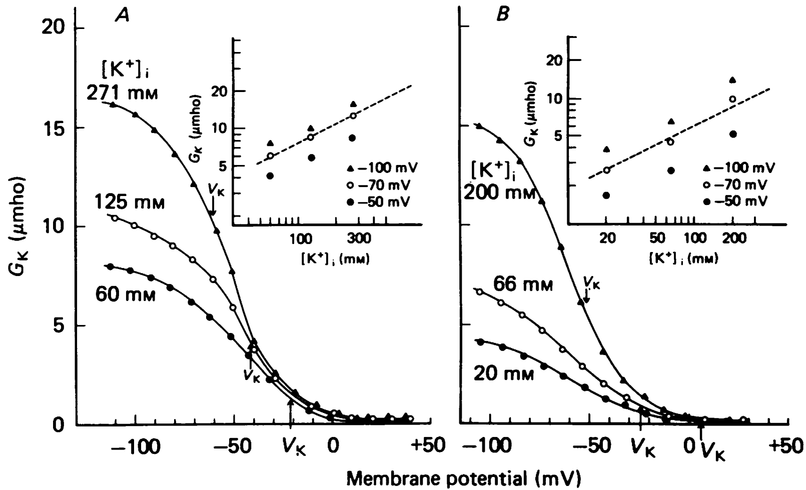

Fig. 5. Relationship between G_K and V obtained after the broadening effect of the G_K–V relation had reached saturation. *A*, in solution A and *B*, in solution B. The external K^+ concentration was 25 mM. Insert: G_K values at three different voltages are plotted against $[K^+]_i$ on log–log scale. A dashed line in each insert indicates a slope of one half. The internal K^+ concentration listed by each curve was calculated from the Nernst equation at $[K^+]_o = 25$ mM and V_K obtained as the membrane potential at $I_o = I_s$ except curve marked 20 mM in *B*. In the latter case 20 mM represents the K^+ concentration of the perfusing solution. Since the cell was perfused with this solution for more than 60 min the actual internal K^+ concentration should have been very close to 20 mM.

decline or 'inactivation' of the inward current observed at large negative voltages became less marked. Both effects reached saturation at the time when the broadening effect of $G_K - V$ relation reached saturation. At present we have no adequate explanation for these three parallel effects of perfusion. Some conditions other than K^+ in the perfusing solution seem to be responsible, since the broadening occurred at 200 mM-K which corresponded to the normal internal K^+ concentration. The following two questions have to be solved in the next season: (1) what are those conditions? and (2) why does the low K^+ concentration accelerate the process?

The $G_K - V$ relations obtained at different internal K^+ concentrations after the broadening process had reached saturation are shown in Fig. 5. The values of G_K were obtained by subtracting G_L from G_K^*. The data in Fig. 5*A* were obtained with solution A in which the Na^+ concentration was kept at 60 mM and Ca^{2+} concentration was buffered at $0{\cdot}2\ \mu M$. The data in Fig. 5*B* were obtained with solution B which contained 20 mM-EGTA and no Ca^{2+}. The Na^+ concentration was not constant but

was always kept above 165 mM. As will be described below, G_K at a given voltage increased with the internal Na^+ concentration when observed at a constant internal K^+ concentration. This effect, however, saturated almost completely at 165 mM-Na. The inset of each figure gives a log–log plot of G_K at -50, -70 and -100 mV against internal K^+ concentration. The relations obtained at the three different membrane potentials are almost linear and are parallel to each other. The line drawn in each inset has a slope of 1/2. The result, therefore, indicates that the $G_K - V$ relations obtained at different internal K^+ concentrations should coincide when G_K is normalized by dividing the square root of $[K^+]_i$.

These findings lead to the following two conclusions. (1) The inward rectification depends on V alone rather than $V - V_K$ when the internal K^+ is altered at a fixed external K^+ concentration and (2) G_K at a given V is roughly proportional to the square root of $[K^+]_i$ when external K^+ is fixed. As seen in Fig. 5, $G_K - V$ relations obtained at different internal K^+ concentrations tended to deviate from these general conclusions when V was more positive than -50 mV. In this potential range the value of G_K at a higher internal K^+ was slightly smaller than expected from that when this concentration was lower and from the above proportionality factor. In some extreme cases, G_K at a lower internal K^+ became slightly greater than that obtained at a higher concentration at V between -30 and 0 mV. In other words, the activation of the inward rectification tended to become partly dependent on $V - V_K$ rather than on V. A possible explanation for this deviation will be discussed later.

Effect of the internal Na

As mentioned already, the internal K^+ concentration of the unperfused cell was close to 200 mM. The internal Na^+ activity measured with a Na^+-selective glass electrode indicates that internal Na^+ of the intact cell was 10–15 mM if the activity coefficient was the same inside and outside the cell. To determine the effects of internal Na^+ concentration on the K conductance, the Na^+ concentration of the perfusing solution was varied while the K^+ concentration was kept at 200 mM. The curve marked Na-free (8 min) in Fig. 6*A* represents the relation between G_K^* and V obtained 8 min after the onset of perfusion with a Na-free solution (solution D). Even though the K^+ concentration of the solution was kept at 200 mM, G_K decreased as the perfusion proceeded and after 40 min the amplitude of G_K at a given potential was approximately a quarter of the value found at 8 min. This result shows that the removal of the internal Na^+ reduced the inward rectification. The value of G_K was restored when the internal Na^+ concentration was increased. The curves marked 5, 20, 66·7 and 200 mM-$[Na^+]_i$ were obtained from the same cell at the end of perfusion with the indicated solution. The cell was perfused for 50 min with each solution, and so the internal Na^+ concentration should have become equal to that of the perfusion solution. The amplitude of G_K at a given V increased sharply with internal Na^+ concentration and almost reached saturation at 200 mM. The amplitudes of G_K at $V = -70$ and -100 mV were calculated and $1/G_K$ is plotted against $1/[Na^+]_i$ in Fig. 6*B*. Either relation can be considered a straight line. This indicates that G_K can be expressed as a function of $[Na^+]_i$ as:

$$G_K = G_{K\max} \cdot \frac{[Na^+]_i}{K_{Na} + [Na^+]_i}. \qquad (4)$$

where $G_{K\max}$ represents G_K at the saturation level. The apparent dissociation constant K_{Na} was 35 mM for $V = -100$ mV and 37 mM for $V = -70$ mV. According to eqn. (4) G_K should vanish when $[Na^+]_i = 0$. The amplitude of G_K after 40 min perfusion with Na-free solution corresponded to that of G_K at $[Na^+]_i$ = 2–3 mM, and it was likely that this much Na^+ remained after 40 min perfusion. In one experiment the cell was perfused continually with Na-free solution for 2 hr and this eliminated

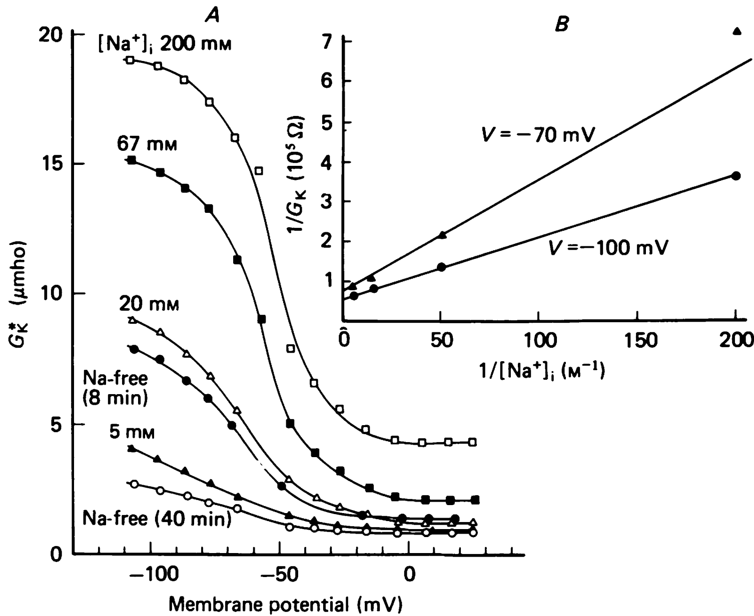

Fig. 6. *A*, relations between the apparent K conductance G_K^* and the membrane potential at different Na^+ concentrations of the perfusion solution (cited next to each curve). The cell was perfused with the Na-free solution D for 40 min and thereafter with 5, 20, 67 and 200 mM-Na solution C for 50 min in each. Each curve was obtained from the data at the end of 50 min perfusion in the respective solution. Curves marked Na-free (8 min) and Na-free (40 min) were obtained 8 and 40 min after perfusion with the Na-free solution D, respectively. The K^+ concentration of the solution was 200 mM throughout. The external K^+ concentration was 25 mM. *B*, relations between the reciprocal of the K conductance $G_K = (G_K^* - G_L)$ and $1/[Na^+]_i$ at $V = -70$ mV and -100 mV. Straight lines were drawn with $K_{Na} = 35$ mM at $V = -100$ mV and 37 mM at $V = -70$ mV. $[Na^+]_i$ is that of the perfusate.

the inward rectification. The perfusion of a solution containing 30 mM-Na^+ restored the rectification in 25 min. Thus, one can conclude that internal Na^+ is required for the activation of the K channel of inward rectification.

Although both the instantaneous and the time-dependent conductances increased with internal Na^+ concentration, the value of V_K obtained as the membrane potential at which the instantaneous and steady-state current–voltage relations crossed was not altered. This indicates that Na^+ did not carry charges during inward rectification. In contrast to the remarkable effect of internal Na^+, the effect of total removal of the external Na^+ produced only a small reduction in G_K. In a few intact eggs, G_K was

measured by voltage clamp with two intracellular glass pipettes (S. Hagiwara & S. Miyazaki, unpublished observation). The total replacement of the 460 mM-NaCl in the normal external saline with choline chloride or Tris-OH·HCl reduced G_K by at most 10%. In these experiments the pH was adjusted to 7·5 by 10 mM Tris-HCl instead of PIPES-NaOH. The actual internal Na^+ activity observed with the Na^+-selective glass electrode indicates that the internal Na^+ was 10–15 mM if the activity

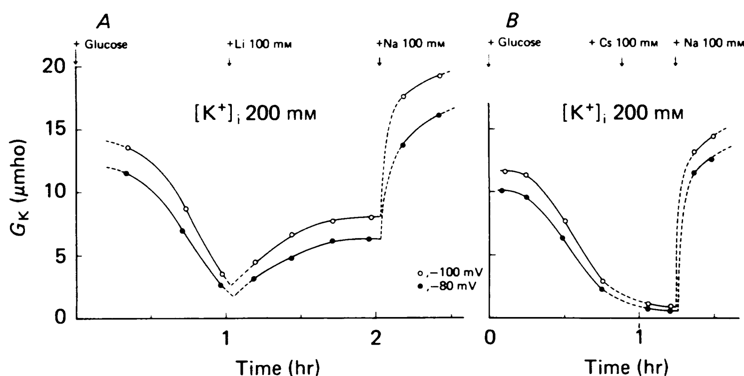

Fig. 7. Time courses of G_K values at $V = -100$ mV and -80 mV during internal perfusion. The K^+ concentration of the perfusing solution was kept at 200 mM throughout and $[K^+]_o = 25$ mM. An arrow indicates the moment of application of new solution. *A*, Na-free solution D, 100 mM Li solution E and then 100 mM-Na solution E. *B*, Na-free solution D, 100 mM-Cs solution E and then 100 mM-Na solution E.

coefficient was equal. Although the normal K^+ and Na^+ concentrations inside the cell were about 200 and 10–15 mM respectively, the perfusion of the cell with a solution containing 200 mM-K^+ and 15 mM-Na^+ often reduced the amplitude of G_K at any given membrane potential. This suggests that factors in the perfusing solution other than the K^+ and Na^+ concentrations also contribute to the amplitude of the inward rectification. This, however, does not influence our conclusions since the effects of K^+ and Na^+ were observed under the same conditions.

Effect of other monovalent cations

The effects of the internal Na^+ concentration were observed by replacing glucose in the perfusing solution with an isosmolar Na glutamate. This changed the ionic strength of the solution. The effect was, however, not due to the change in the ionic strength since a similar replacement with glutamate salts of organic cations such as arginine$^+$ did not show any restoring effect. At pH 7·0 arginine glutamate is almost completely dissociated.

Among other alkali cations tested, Li^+ had a small but significant restoring effect. In Fig. 7*A* the amplitudes of G_K at -100 mV and -80 mV are plotted against time from the onset of perfusion. The K^+ concentration of the perfusing solution was kept at 200 mM throughout. The cell was initially perfused with Na-free solution D (200 mM-K) and G_K decreased with time. When the solution was switched to one containing 100 mM-Li (solution E), G_K began to increase and attained a final steady-state amplitude after 1 hr of perfusion. The amplitude of G_K rapidly increased upon

the subsequent switch of the perfusing solution to one containing 100 mM-Na (solution E) and within 25 min had increased by a factor of 2. This indicates that Li^+ substituted for Na^+, but was much less effective. The effect of the internal Cs^+ is shown in Fig. 7*B*. A very similar result was obtained with Rb^+. These results show that neither Cs^+ nor Rb^+ had any restoring effect on the K conductance. Low concentrations of Cs^+ in the external medium block the K current during inward rectification (Hagiwara & Takahashi, 1974; Hagiwara, *et al.* 1976). The absence of a restoring effect with Cs^+ was not due to Cs^+ ions which might have leaked into the external solution since subsequent intracellular perfusion with 100 mM-Na (solution E) immediately restored the K conductance.

DISCUSSION

One of the remarkable properties of the inward rectification of the K conductance is that the rectification depends on $V - V_K$ rather than V alone. Since all of the previous experiments were performed at a fixed internal K^+ concentration the results can be interpreted to mean that the gating of the channel depends on the membrane potential and the external K^+ concentration rather than $V - V_K$. Two models based on such an interpretation have been proposed. Armstrong (1975) suggested that the gating of the channel depends on the occupancy of the external K ions at a certain site in the channel and that this site is located close to the inner end of the channel so that the probability of occupancy depends not only on the external K^+ concentration but also on V. Another model has been proposed by Ciani, Krasne, Miyazaki & Hagiwara (1978). The inward rectification of the K current is assumed to be due to a voltage-dependent orientation of charged molecules in the membrane followed by a voltage independent binding of external K^+ ions to the gating site of the oriented molecule. In these models the gating of the channel depends on the membrane potential and the external K^+ concentration. If an appropriate additional assumption is made, the gating in either model becomes dependent on $V - V_K$ when the external K^+ is altered at a fixed internal concentration, since V_K changes with the external K^+ concentration. The value of V_K changes also when the internal K^+ is altered at a fixed external concentration. The present experimental data suggest that the gating depends only on V, rather than on $V - V_K$, when V_K is altered by changing internal K^+ at a fixed external concentration. This result agrees with the prediction of the above models. The final conclusion is that the gating of the K channel of the inward rectification depends on the membrane potential and the external K^+ concentration, but not on the internal K^+ concentration.

Voltage-clamp analysis of the membrane current during the inward rectification shows that the rectification has instantaneous and time-dependent components (Hagiwara *et al.* 1976). The small but significant instantaneous rectification has been interpreted in either of the following two ways. The instantaneous current–voltage relation may represent the character of the single K channel, i.e. the single channel has a slight but significant inward rectification. Alternatively there may be another gating process, but its time constant is too fast to observe by the present methods. The experimental results show that this time constant should be smaller than 1–2 msec at the membrane potential at which the time constant of the time-dependent K

current is 0·5 sec. The relatively large leakage made it difficult to measure the actual instantaneous K current accurately. The measurements suggest that it is, at least in part, dependent on $V-V_K$ even when V_K is altered by changing the internal K^+ concentration. This may not be unreasonable if the instantaneous rectification represents the property of the single channel conductance. The instantaneous K conductance should be analysed more accurately with an improved method. As pointed out already G_K-V curves obtained at different internal K^+ concentrations differ only by a constant factor for large negative membrane potentials, i.e. curves coincide when normalized by the square root of $[K^+]_i$ (Fig. 5). However, G_K-V curves deviate from this principle for more positive membrane potentials, i.e. when V becomes close to or more positive than V_K. The normalized G_K at a smaller internal K^+ concentration tends to become greater than that at a greater internal concentration. In other words a slight $V-V_K$ dependence appears at these membrane potentials. Such a deviation would appear, e.g. if the single channel conductance has a slight inward rectification which depends on $V-V_K$.

The steady-state K conductance at a given $V-V_K$ is proportional to the square root of $[K^+]_o$, when external K^+ is altered in the intact cell (Hagiwara & Takahashi, 1974). This is true even when the amplitude of $V-V_K$ is much greater than RT/F (= 25 mV). If one assumes that the number of open K channels at a given $V-V_K$ is independent of external K^+ concentration the above result indicates that the single channel conductance is proportional to the square root of $[K^+]_o$ when the internal K^+ concentration is fixed. Recently Ohmori (1978) observed the single K channel conductance of the inward rectification in a tunicate egg by means of current noise analysis. He concluded that the single channel conductance is actually proportional to the square root of $[K^+]_o$ in an intact egg. The present data indicate that G_K at a given large negative V is proportional also to the square root of $[K^+]_i$ when external K^+ is fixed. The final conclusion drawn from these results is that the single channel conductance of the inward rectification is proportional to the geometric mean of internal and external K^+ concentration. The possibility of such ion channel conductance has been predicted theoretically by Ciani *et al.* (1978).

An unexpected result in the present study is the effect of the internal Na^+ upon the K conductance. The experimental results indicate that it is necessary for Na^+ to bind to the channel or the channel forming molecule to produce a permeable K channel. Thus the internal Na^+ controls the K permeability of the resting membrane although it carries no significant charge through the K channel. Among other monovalent cations only Li^+ has a similar effect although it is much less efficient. The finding may explain some phenomena observed during development. During maturation of a starfish egg the K permeability decreases (Miyazaki *et al.* 1975) and this may be due to the change in the Na^+ activity inside the egg. In fact, the activity of the internal Na^+ decreases during maturation in a *Xenopus* egg (Palmer, Century & Civan, 1978). The properties of the inward rectification of the starfish egg are very similar to those of the skeletal muscle fibre of a frog. It would be interesting to determine whether or not internal Na^+ also controls the inward rectification in frog muscle fibre.

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