Outward Currents through the Inwardly Rectifying Potassium Channel of Guinea-Pig Ventricular Cells

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Abstract Currents through the inwardly rectifying K channel were studied under whole-cell clamp of collagenase-treated single ventricular cells of guinea-pigs. The inwardly rectifying K channel was fully activated by hyperpolarizing the membrane from the equilibrium potential for K^+ (E_K) by 30–40 mV. Following depolarization above E_K , a decaying outward current was elicited. Prolongation of the hyperpolarizing prepulse increased the amplitude of the decaying outward current, with a time course similar to the increase of the inward current during the prepulse. Time-dependent changes in both outward and inward currents could be fitted with a single exponential function and were attributed to deactivation and activation of the inwardly rectifying K channel. The instantaneous current-voltage relation was almost linear, indicating that the conductance of the channel is ohmic and that the rectification of the steady-state current was due to the kinetic properties of the inwardly rectifying K channel. The activation kinetics of the channel was measured at different concentrations of K^+ in both the external and internal solutions. The time constant and the steady-state activation were not a function of the absolute membrane potential value, but were dependent on the driving force.

Key words: inwardly rectifying K channel, internal perfusion, single ventricular cell, whole cell clamp.

In the heart cells, the inwardly rectifying K channel is the major source of the K conductance to hold the resting membrane potential close to the equilibrium potential for K^+ (E_K). The conductance associated with this channel is markedly

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reduced at the potential range of the plateau of the action potential, which allows the action potential duration to last for several hundred milliseconds. In spite of its physiologically important functions, studies of the outward current component of the inwardly rectifying K channel are few (HAGIWARA *et al.*, 1976; HESTRIN, 1981; LEECH and STANFIELD, 1981), simply because of the difficulty of observing the outward current.

We reported previously that a decaying outward current through the inwardly rectifying K channel was elicited on stepping the membrane potential to values more positive than E_K , which was shifted in the positive direction by perfusing the cells with Cs-rich internal solution, and that its decay was accelerated with larger depolarization (MATSUDA and NOMA, 1984). To exclude the effect, if any, of internal Cs^+ on the channel in these experiments (OHMORI, 1984), in this work we changed E_K by increasing the external K^+ concentration ($[K^+]_o$) or decreasing the internal K^+ concentration ($[K^+]_i$) using substitutes other than Cs^+ and so extended the findings on the outward currents through the inwardly rectifying K channel. The results suggest that unitary conductance of the inwardly rectifying K channel is ohmic and that gating kinetics depends on $[K^+]_i$ as well as on $[K^+]_o$. This finding is different from results of the previous studies in different tissues that the gating kinetics of the inward rectifier depends on $[K^+]_o$, but not on $[K^+]_i$ (HAGIWARA and YOSHII, 1979; HESTRIN, 1981; LEECH and STANFIELD, 1981). Parts of this work have appeared elsewhere (MATSUDA *et al.*, 1987).

MATERIALS AND METHODS

Preparations of isolated single ventricular cells. Guinea-pigs of 300–600 g were anesthetized with pentobarbital sodium (40–60 mg/kg). Under artificial respiration, we opened the chest and cannulated the aorta *in situ* to perfuse the coronary artery with a hydrostatic pressure of approximately 65 cmH₂O, and isolated the heart. The blood was washed out by coronary perfusion with Tyrode's solution equilibrated with a 100% O₂ gas. The composition of the Tyrode's solution was (in mM): NaCl, 143.9; KCl, 5.4; CaCl₂, 1.8; MgCl₂, 0.5; glucose, 5.5; NaH₂PO₄, 0.33; and 5 mM HEPES-NaOH buffer (pH = 7.4). After the heart was perfused with about 100 ml of Ca-free Tyrode's solution, the solution was switched to a Ca-free Tyrode's solution containing 0.04 mg/ml collagenase (Sigma type I), which was recirculated with a peristaltic pump for 40 min. Thereafter, 100 ml of a high-K storage solution (see below; ISENBERG and KLÖCKNER, 1982) was perfused to wash out collagenase. The composition of the high-K storage solution was (in mM): taurine, 10; glutamic acid, 70; KCl, 25; KH₂PO₄, 10; MgCl₂, 3; glucose, 11; ethyleneglycol-bis(β -aminoethylether)N,N'-tetraacetic acid (EGTA), 0.5; and HEPES, 10; and pH adjusted to 7.4 with KOH. After washing out collagenase, the ventricles were chopped in the high-K storage solution and the cell suspension was filtered with a 100 μ m stainless steel mesh net to separate single cells. The isolated cells were stored in the high-K solution at 4 C for the later use. The temperature of

all perfusates was kept at 36–37 °C during coronary perfusion.

Electrodes. Patch electrodes were fabricated from capillaries of high melting temperature glass and the inner tip diameter was 3–5 μm . The electrode tip was heat-polished and was filled with the internal solution. The electrode resistance ranged between 1 and 3 M Ω .

Solutions. The compositions of the test solutions are listed in Table 1. Initially, we filled the electrode with the internal solution containing 1 mM CaCl_2 instead of 5 mM EGTA, a procedure which makes the "G Ω seal" easier to obtain. After the "G Ω seal" was established, the internal solution was changed to a given one containing EGTA and then the cell membrane at the tip of the electrode was ruptured. The method for exchanging the solution in the patch electrode was the same as described elsewhere (SOEJIMA and NOMA, 1984). To remove current flow through channels other than the K channel, external Na^+ was replaced with Tris $^+$, and Cd^{2+} was added to the external solution. In the standard experiment 40 mM K^+ external solution and 150 mM K^+ internal solution were used. The temperature of solutions in the chamber was kept at 14–15 °C to slow the kinetics and to facilitate analysis.

Recording techniques. The single-pipette whole-cell clamp technique is similar to those described by HAMILL *et al.* 1981. Compensation of the series resistance at the tip of the patch electrode was described previously (OHARA *et al.*, 1983). When

Table 1. Composition of solutions (in mM).

	External solution							V_j (mV)
	Tris Cl	KCl	MgCl $_2$	CaCl $_2$	CdCl $_2$	Glucose		
40 K $^+$ solution	114	40	0.5	1.8	0.1	5.5		+2
20 K $^+$ solution	134	20	0.5	1.8	0.1	5.5		+3
80 K $^+$ solution	74	80	0.5	1.8	0.1	5.5		0
0 K $^+$ solution	154	0	0.5	1.8	0.1	5.5		+4
	Internal solution							V_j (mV)
	K-aspartate	KCl	EGTA	K $_2$ -ATP	HEPES	MgCl $_2$ *	Glucose	
150 K $^+$ solution	120	20	5	5	5	1	0	–13
75 K $^+$ solution	45	20	5	5	5	1	150	–10
50 K $^+$ solution	20	20	5	5	5	1	200	–9.5

The pH of external solutions was adjusted to 7.4 (with Tris buffer) and the pH of the internal solutions was adjusted to 7.3 with 5 mM HEPES-KOH. V_j was the liquid junction potential between the solution and the Tyrode solution. The sign was positive when the side of Tyrode solution was negative. Correction of the membrane potentials was made as described by HAGIWARA and OHMORI (1982). * Free Mg^{2+} concentration calculated from the apparent dissociation constants (FABIATO and FABIATO, 1979) is 15 μM .

the tip of the electrode was in the Tyrode's solution, the command D.C. potential was adjusted to make the current through the electrode zero, and this voltage was taken as the reference potential. The resistance of the electrode was measured from the amplitude of the current produced by 1 mV voltage pulse. After the electrode tip was attached to the cell surface, a negative pressure of 30–70 cm H₂O was applied to interior of the electrode, and the "GΩ seal" was usually established within 1 to 2 min. After changing the internal solution containing 1 mM CaCl₂, to the other containing 5 mM EGTA, the membrane at the tip of the electrode was ruptured by applying further brief suction, and the repute was detected by a sudden increase of the capacitive current. The membrane potential was held around the zero-current potential and voltage steps were applied every 6 s, unless otherwise indicated. Current and voltage were recorded on the magnetic tape (TEAC, R-210), and they were analyzed using a computer (NEC, PC-98XA).

RESULTS

The inwardly rectifying K channel current

Figure 1 shows a typical experiment to observe outward current through the inwardly rectifying K channel. The uppermost trace of the left column represents the voltage step and traces in A and B represent the current records in response to it, with 150 mM K⁺ internal solution at 40 mM [K⁺]_o and 0 mM [K⁺]_o, respectively. The membrane potential was held at −30 mV, which is close to E_K predicted from the transmembrane K⁺ concentrations (150 mM [K⁺]_i; and 40 mM [K⁺]_o), −32 mV. In the presence of 40 mM [K⁺]_o, the inwardly rectifying K channel was activated by a hyperpolarizing prepulse of 40 mV. A subsequent test pulse to a value 15 mV positive to the zero-current potential elicited a decaying outward current (Fig. 1A). Since external Na⁺ was replaced with Tris⁺ and Ca current was blocked by Cd²⁺, neither Na nor Ca channels are responsible for this outward current. The decay of the outward current can be fitted with a single exponential function (Fig. 1D), with a time constant (τ) of 4.09 ms. At 0 mM [K⁺]_o, both the inward and outward current disappeared, leaving capacitive currents proportional to the value of voltage step (Fig. 1B), suggesting that the decaying outward current flows through the inwardly rectifying K channel. Figure 1C shows the difference current obtained by subtracting the current in Fig. 1B from the one in Fig. 1A. The time constant of the decay measured from this difference current was identical to that obtained from the original record in Fig. 1A (Fig. 1E), suggesting that the separation of the ionic current from the capacitive current is satisfactory.

To show that the decaying current flows through the inwardly rectifying K channel, the outward current was elicited after the inwardly rectifying K channel was activated to different degrees by varying the duration of the preceding hyperpolarizing pulse (Fig. 2). The amplitude of the outward current increased in parallel with the activation of the inward current and reached a steady level 20 ms after the onset of the hyperpolarizing pulse when the inward current attained a

Fig. 1. Outward current through the inwardly rectifying K channel. The uppermost trace in the left column shows the voltage step. The figure indicates a voltage deviation from the holding potential set at around the zero-current potential. A and B: the current traces obtained at 40 mM $[K^+]_o$ and 0 mM $[K^+]_o$; $[K^+]_i$, 150 mM. C: the difference current obtained by subtracting the current in B from that in A. D and E: the difference between the actual current and its steady-state value during depolarization plotted against time on a semi-logarithmic plot. The decay of the outward current can be fitted with a single exponential function having a time constant (τ) of 4.09 ms both before (D) and after (E) subtraction of the capacitive currents.

plateau level. The time course of the development of the outward current was fitted with a single exponential function having a time constant of 4.6 ms (Fig. 2B), which agreed well with the time constant of 4.4 ms in the current activation during the prepulse (Fig. 2C). This result supports the view that the outward current flows through the inwardly rectifying K channel, which was activated during the preceding hyperpolarization.

A possible alternative explanation for this decaying outward current is the contribution of the transient outward current, which is widely observed in other tissues (for review see CARMELIET and VEREECKE, 1979). The transient outward

Fig. 2. Correlation between the time-dependent increase of the inward current during hyperpolarization and the decaying outward current during depolarization. A: the upper panel indicates the potential protocol where the hyperpolarizing prepulse was prolonged progressively, and the lower panel indicates the superimposed current traces. They correspond, from left to right, to hyperpolarizing pulses of duration 1, 2, 3, 4, 5, 6, 8, 10, 15, 20, and 30 ms, respectively. The capacitive currents were subtracted. The figure in the pulse protocol indicates a voltage deviation from the holding potential. $[K^+]_o$, 40 mM; $[K^+]_i$, 150 mM. B: the instantaneous outward current was obtained by extrapolating the best fitted curve to the onset of the depolarizing pulse and subtracted from its maximum value. The resulting value was plotted against the duration of the preceding pulse on a semi-logarithmic plot. A line giving a time constant of 4.6 ms was fitted by eye. C: the semi-logarithmic plot of the inward current during the 30 ms hyperpolarizing pulse. The increase of the inward current can be fitted with a single exponential function having a time constant of 4.4 ms.

current, however, is observed much less frequently in quinea-pig ventricular cells than in rat ventricular cells. It is inactivated at around -40 mV and recovers slowly. The recovery time constant from inactivation of the transient outward current measured in rabbit atrioventricular node cells was 3.58 s at the holding potential of -70 mV (NAKAYAMA and IRISAWA, 1985). To exclude the possibility of the contribution of transient outward current, the recovery process of the outward current under investigation was examined (Fig. 3). A pair of depolarizing pulses to $+6$ mV was applied from the holding potential of -99 mV with various interpulse intervals. The current elicited with an interval of 30 ms showed an amplitude almost equal to that elicited by the first pulse. The decay of the transient outward current was fitted with two exponential components (NAKAYAMA and IRISAWA, 1985). The

Fig. 3. Fast recovery of the outward current. The pulse protocol is shown in the upper panel and the current records are superimposed in the lower panel. Note that the decaying outward current apparently recovered during hyperpolarization of 30 ms. The figures indicate the membrane potentials and the interval between a pair of depolarizing pulses. $[K^+]_o$, 40 mM; $[K^+]_i$, 150 mM.

time constant of the fast component was much slower than that of the outward current shown in the present study, which gives another argument against the contribution of the transient outward current.

Properties of outward current through inwardly rectifying K channels at different external K^+ concentrations

The results presented so far strongly suggest that the outward current under investigation flows through the inwardly rectifying K channel. Based on these results, we measured the instantaneous current-voltage relation and activation kinetics to know the mechanism of the inward rectification. We also studied the effects of the external and internal K^+ on the conductance and activation kinetics.

a) Current-voltage relationship. Outward current was elicited by depolarizing the membrane to various voltages after activating the inwardly rectifying K channel with a hyperpolarizing pulse. The amplitude of the instantaneous current was measured after subtraction of the capacitive currents by extrapolating the best fitted curve to the onset of the test pulse (Fig. 1E). Figure 4 shows the instantaneous and steady-state current-voltage curves at $[K^+]_o$ of 0, 40, and 80 mM, and a fixed $[K^+]_i$ of 150 mM. By changing $[K^+]_o$ from 40 to 20 mM (not shown in Fig. 4) and to 80 mM, the zero-current potential changed an average from -26.0 ± 2.0 ($n=6$) to -42.3 ± 2.5 mV ($n=6$) and to -10.0 ± 2.6 mV ($n=6$), respectively. Such behavior

Fig. 4. The instantaneous (open symbols) and steady-state (filled symbols) current-voltage curves at 0, 40, and 80 mM external K^+ concentrations. The amplitude of instantaneous currents was measured by extrapolating the best fitted curve to the onset of the test pulse, after subtraction of the capacitive currents. The steady level was measured near the end of the test pulse of 30 ms. The slope fitted to the instantaneous current by eye gives 95 nS (40 mM $[K^+]_o$) and 132 nS (80 mM $[K^+]_o$). $[K^+]_i$, 150 mM.

of the zero-current potential as a K electrode shows that it can be considered equal to E_K . While the steady state current-voltage curve showed inward rectification at the voltages positive to E_K , almost linear instantaneous current-voltage curves were obtained in 15 out of 21 cells in the standard experiments. This finding suggests that the current-voltage relation of the inwardly rectifying K channel is linear and that the rectification of the steady-state current is due to time-dependent closure of the channel on depolarization.

The slope conductance was increased by 1.32 ± 0.09 times ($n=5$) when $[K^+]_o$ was doubled. This result agrees well to the previous studies that the conductance was roughly proportional to the square root of $[K^+]_o$ (HAGIWARA and TAKAHASHI, 1974).

b) Time constant (τ). The time constant of the inward or outward current change was examined at $[K^+]_o$ of 20, 40, and 80 mM, and plotted against the voltage step (ΔV) from the zero-current potential (Fig. 5). The time constant decreased exponentially as the voltage step was increased (e -fold decline for a 28.7 mV hyperpolarization and for a 19.3 mV depolarization) and the time constant-voltage relation was bell-shaped with the peak (8–9 ms) at around E_K . The relations between the average of time constant and voltage were almost superimposable ($n=6$, for each $[K^+]_o$), in the range below E_K (HAGIWARA *et al.*, 1976; LEECH and STANFIELD, 1981) and also above E_K , if plotted the time constant against driving force.

c) The steady-state activation curve. The steady-state activation curve was measured by applying a test pulse of fixed amplitude following a prepulse of varying

Fig. 5. The time constant-voltage relation at $[K^+]_o$ of 20, 40, and 80 mM. The average of 6 experiments at each $[K^+]_o$ is plotted against the voltage step (ΔV) from the zero-current potential. Open circles, 20 mM; filled circles, 40 mM; triangles, 80 mM. $[K^+]_i$, 150 mM. The slope of the line fitted for the data at 40 mM $[K^+]_o$ was e -fold change/28.7 mV and e -fold/19.3 mV for the positive and negative slope, respectively.

Fig. 6. The steady-state activation curve of the inwardly rectifying K channel. A: the amplitude of the first pulse (V_1) was changed, while the second pulse (V_2) was fixed to $\Delta V = -40$ mV, where the channel was assumed to be fully activated. B: the average of 6 experiments of the steady-state activation at 20 (open circles), 40 (filled circles), and 80 mM (triangles). $[K^+]_o$ is plotted against V_1 expressed as ΔV . The vertical bars indicate the standard deviation of the data obtained at 40 mM $[K^+]_o$. The smooth curve is drawn using Eq. (2). $[K^+]_i$, 150 mM.

amplitude (Fig. 6). The difference between the instantaneous current and the steady-state current during the test pulse reflects the difference between the degree of steady-state activation at the prepulse voltage (V_1) and that at the test pulse voltage (V_2). The time-dependent increase in the inward current of hyperpolarization is larger with the prepulse of $\Delta V = +15$ mV (filled circle) than with the prepulse of $\Delta V = -15$ mV (open circle). It is evident that the inwardly rectifying K channel is deactivated more extensively during a conditioning pulse of $\Delta V = +15$ mV than of

$\Delta V = -15$ mV. The amplitude of the time-dependent change during V_2 (I) was obtained by extrapolating the best fitted curve to the onset of V_2 . It reached a maximum value at $\Delta V \geq +40$ mV. The steady-state activation is assumed to be zero at $\Delta V \geq +40$ mV (see Fig. 2 of MATSUDA *et al.*, 1987) and complete at $\Delta V = -40$ mV. The steady-state degree of activation, y , at potential $= \Delta V$ may then be defined as:

$$y(\Delta V) = 1 - I/I_{\max} \quad (1)$$

where $I_{\max}$ is the maximum amplitude of I .

In Fig. 6B, y is plotted against V_1 . The relations obtained at $[K^+]_o$ of 20, 40, and 80 mM are all fitted with a common theoretical curve given by the following equation:

$$y(\Delta V) = [1 + \exp((\Delta V - V_{\text{half}})/v)]^{-1} \quad (2)$$

where v is the slope factor, and V_{half} indicates the voltage of half activation. As evident in Fig. 6B, the steady-state activation curve at different $[K]_o$ was almost superimposable, if plotted against ΔV . For simplicity, only the curve fitted to the data at 40 mM $[K^+]_o$ is shown with the standard deviations. It has a slope factor of 7.98 mV and a half activation voltage of $E_K - 1.2$ mV.

The effect of the internal K concentration

Previous studies showed that the gating kinetics and conductance of the inwardly rectifying K channel did not depend on driving force when E_K was altered by changing $[K^+]_i$ (HAGIWARA and YOSHII, 1979; HESTRIN, 1981; LEECH and STANFIELD, 1981). When $[K^+]_i$ was reduced from 150 to 75 mM and further to 50 mM in this study, however, different results were obtained. Figure 7 shows the current-voltage curves determined with 150 mM $[K^+]_i$ and 75 mM $[K^+]_i$ at 40 mM $[K^+]_o$. The instantaneous current-voltage curve was linear at each K^+ concentration. When $[K^+]_i$ was increased from 50 to 75 mM, the slope conductance increased by 1.62 ± 0.19 times ($n=5$). The zero-current potential was -26.5 ± 2.2 ($n=9$), -17.8 ± 2.4 ($n=5$), and -11.1 ± 2.2 mV ($n=4$) with $[K^+]_i$ of 150, 75, and 50 mM, respectively. The change of the zero-current potential is smaller than predicted from the Nernst equation (17 mV by halving $[K^+]_i$; and 27 mV as a result of reducing $[K^+]_i$ by two-thirds), suggesting that the efficiency of the internal perfusion was limited.

The time constants of the current change at a constant voltage in different $[K^+]_i$ were plotted against ΔV (Fig. 8), which were superimposable over the potential range negative to E_K . At the voltage positive to E_K the deactivation time became slower in 75 mM $[K^+]_i$.

The steady-state activation-voltage relations measured using $[K^+]_i$ of 50, 75, and 150 mM, are plotted against ΔV (Fig. 9). The curves with 150 mM $[K^+]_i$ and 75 mM $[K^+]_i$ were almost superimposable, and it shifted more positive direction with 50 mM $[K^+]_i$. We therefore concluded that the kinetics of the cardiac inwardly

Fig. 7. Instantaneous (filled symbols) and steady state (open symbols) current-voltage curves determined with 150 mM K^+ (triangles) and 75 mM K^+ (circles) internal solutions. The slope conductance was 223 nS with 150 mM $[K^+]_i$ and 151 nS with 75 mM $[K^+]_i$. $[K^+]_o$, 40 mM.

rectifying K channel is dependent on the driving force even when E_K is shifted by changing $[K^+]_i$.

DISCUSSION

In a previous paper (MATSUDA *et al.*, 1987), we showed that the unitary conductance of the inwardly rectifying K channel is ohmic in the absence of intracellular Mg^{2+} and that the channel closes on depolarization because of voltage-dependent gating kinetics. We also found that internal Mg^{2+} blocks the outward current without affecting the inward current. We thus concluded that the inward rectification results from the gating kinetics of the inward rectifier K channel and the voltage-dependent block by internal Mg^{2+} . Single channel recording enables precise analysis of the Mg block, but not investigation of the intrinsic gating kinetics, because the outward single channel currents, which can be seen only when the cell is opened to low Mg solution, become prolonged with time (MATSUDA, 1988). This prolongation is attributed to the washing out of a soluble gating component. In the whole-cell clamp experiments, where the washing out of the intracellular material should be much more limited than in the open cell-attached configuration of single channel recordings, the time constant of relaxation of the outward current was relatively stable during the experiment. Limited efficiency of the internal perfusion, however, made it difficult to lower internal Mg^{2+} enough to study the intrinsic gating kinetics (see below). Thus the activation kinetics of the

Fig. 8. A: current changes by reducing $[K^+]_i$ from 150 to 75 mM. Top trace represents voltage step and bottom trace current in response to it. The decay of the outward current is shown on a semi-logarithmic plot in the right of each panel. $[K^+]_o$, 40 mM. B: the time constant-voltage relation with $[K^+]_i$ of 75 mM (filled circles) and 150 mM (filled triangles). The average of 5 experiments at each $[K^+]_i$ is plotted against the voltage step (ΔV) from the zero-current potential. The time constant of the inward and outward current decreased exponentially as the voltage step was increased. The time constant-voltage relation was superimposable in the inward direction, while the slope was less steep with 75 mM $[K^+]_i$ than with 150 mM $[K^+]_i$ in the outward direction. $[K^+]_o$, 40 mM.

channel described here may be more or less influenced by the remaining Mg^{2+} and not completely "intrinsic." The activation kinetics of the cardiac inwardly rectifying K channel has been studied mainly on the inward currents, in the cell-attached configuration of single-channel current recordings (KURACHI, 1985) and in the whole-cell clamp experiments (TOURNEUR *et al.*, 1987).

Several findings support the idea that the outward current under investigation flows through the inwardly rectifying K channel. 1) The amplitude of the instantaneous current was on the line extrapolated from the measurement of the inward current at different $[K^+]_o$ and $[K^+]_i$. 2) The peak outward current developed in parallel with the increase of the inward current during the preceding hyperpolarization (Fig. 2). 3) The current was suppressed by omitting K^+ from the external solution, by applying Ba^{2+} in the external solution, or by TEA^+ on either side of the membrane (not shown). These conditions are known to depress the

Fig. 9. The steady-state activation curves determined using 50 mM- K^+ (open squares, $n=4$), 75 mM- K^+ (filled circles, $n=5$), and 150 mM- K^+ (triangles, $n=9$) internal solutions. The degree of activation was measured with the same method as in Fig. 6. The curve was fitted to the data with 150 mM $[K^+]_i$. Slope factor, 8.9 mV; half activation voltage, $E_K - 5.7$ mV. $[K^+]_o$, 40 mM.

inwardly rectifying K current. 4) The outward current could be elicited by voltage steps from a relatively low potential and at a high frequency (Fig. 3), excluding the contribution of the transient outward current. 5) The time constants of decay during depolarization and those of activation during hyperpolarization were well explained by a common bell-shaped curve. 6) The single channel analysis disclosed an outward single-channel current through the inwardly rectifying K channel and the average current showed a time-dependent decrease similar to that obtained with the whole-cell current recording (MATSUDA *et al.*, 1987).

The decay of the outward current was fitted with a single exponential at voltages below $E_K = +30$ mV. Above this voltage, it required two exponentials for fitting. The time constant of the fast component was less than 1 ms. We ascribe this to incomplete removal of the capacitive current and limit the voltage range for analysis to $E_K < +30$ mV. The instantaneous current-voltage relations were linear in 15 out of 21 cells and the remaining cells showed inward rectification. In the present experiments, free Mg^{2+} concentration of the pipette solution calculated using the apparent dissociation constants of EGTA was $15 \mu M$. The actual intracellular Mg^{2+} concentration is predicted to be higher than this value considering the physiological intracellular free Mg^{2+} concentration ranging from 0.5 to 3.5 mM (HESS *et al.*, 1982; GUPTA *et al.*, 1984; BLATTER and MCGUIGAN, 1986) and limited efficiency of the internal perfusion. Recent single-channel current study has shown that the half-saturation concentration of the Mg^{2+} block was $1.7 \mu M$ at $E_K + 70$ mV (MATSUDA, 1988). Thus if we could have measured the instantaneous current-voltage curves at more positive potentials, most of them might have shown the inward rectification owing to the voltage-dependent Mg^{2+} block. We cannot tell at the present whether

the effects of internal Mg^{2+} on the measurement of the steady-state activation curves and the time constants were small or not in the voltage range examined. In spite of the uncertainty about the influence of Mg^{2+} , the present work gives the first quantitative analysis on the outward current through the inwardly rectifying K^+ channels.

The time constant of the activation or deactivation of the channel decreased exponentially as the voltage step from E_K was increased and the time constant-voltage relation was bell-shaped with the peak at around E_K . The decrease in the time constant with larger depolarization was reported in the egg cell membrane (HAGIWARA *et al.*, 1976) and the frog skeletal muscle (HESTRIN, 1981). Other workers suggested that the time constant becomes larger at more positive potentials in the frog skeletal muscle (LEECH and STANFIELD, 1981).

The present results showed that the gating of the channel depends on the driving force rather than the membrane potential alone. This notion holds in the potential range above E_K , and in contrast with previous reports, when E_K is altered with changing internal K^+ at a fixed external concentration. No outward current flows in the absence of external K^+ , suggesting that there is a binding site accessible to external K^+ but not to internal K^+ and that the occupation of this site by K^+ is essential for the channel to open. Our finding may indicate the presence of an additional binding site to which both external K^+ and internal K^+ have access to affect the gating kinetics.

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