J. Membrane Biol. 94, 19–35 (1986)

The Journal of
Membrane Biology

Inwardly Rectifying Single-Channel and Whole Cell K^+ Currents in Rat Ventricular Myocytes

Ira R. Josephson* and Arthur M. Brown†
†Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, Texas 77030

Summary. The voltage-dependent properties of inwardly rectifying potassium channels were studied in adult and neonatal rat ventricular myocytes using patch voltage-clamp techniques. Inward rectification was pronounced in the single-channel current-voltage relation and outward currents were not detected at potentials positive to the calculated reversal potential for potassium (E_K). Single-channel currents having at least three different conductances were observed and the middle one was predominant. Its single-channel conductance was nonlinear ranging from 20 to 40 pS. Its open-time distribution was fit by a single exponential and the time constants decreased markedly with hyperpolarization from E_K. The distribution of the closed times required at least two exponentials for fitting, and their taus were related to the bursting behavior displayed at negative potentials. The steady-state probability of being open (P_o) for this channel was determined from the single-channel records; in symmetrical isotonic K solutions P_o was 0.73 at −60 mV, but fell to 0.18 at −100 mV. The smaller conductance was about one-half the usual value and the open times were greatly prolonged. The large conductance was about 50 percent greater than the usual value and the open times were very brief. The $P_o(V)$ relation, the kinetics and the conductance of the predominant channel account for most of the whole cell inwardly rectifying current. The kinetics suggest that an intrinsic K^+-dependent mechanism may control the gating, and the conductance of this channel. In the steady state, the opening and closing probabilities for the two smaller channels were not independent of each other, suggesting the possibility of a sub-conductance state or cooperativity between different channels.

Key Words inward rectifier · cardiac myocytes · patch clamp · voltage dependence · cooperativity · substates

Introduction

The inwardly rectifying potassium current in cardiac muscle referred to as I_{K_1} or K^+ background current (Noble & Tsien, 1968; Isenberg, 1976; Carmeliet, 1982) is important for an understanding of the electrogenesis of the cardiac action potential. As an example, its conspicuous absence at depolarized potentials minimizes the amount of inward current necessary to maintain the depolarization during the plateau (Hutter & Noble, 1960; Noble, 1965). This channel is present in many excitable membranes and has been studied extensively in skeletal muscle (Katz, 1949), and egg cells (Hagiwara et al., 1976). In heart, voltage-clamp results have been obtained mainly from multicellular preparations. Useful information has been produced but it is limited because of the complex membrane of these preparations. The most straightforward appoach is to study single channels and recently this has been done in isolated ventricular myocytes of guinea pig (Trube, Sackmann & Trautwein, 1981; Sakmann & Trube, 1984*a*,*b*), and rabbit (Kameyama et al., 1983) and isolated atrial myocytes of bullfrog (Momose et al., 1983). In the extensive studies of Sakmann and Traube (1984*a*,*b*) a number of different conductance states was observed although attention was concentrated on the most frequently encountered channel. These is other evidence of more than one type of inwardly rectifying K^+ channel in heart, such as the differences in the conductance and kinetics between inwardly rectifying K^+ channels of nodal and atrial or ventricular muscle (Sakmann et al., 1983). The extent of this heterogeneity is unknown, and a comparison between single-channel and macroscopic current measurements from the same preparation would therefore be important. The macroscopic measurements are difficult to evaluate in single adult ventricular myocytes because of the presence of a complex internal membrane system connected through T-tubules to the surface membrane (Lee et al., 1979; Brown, Lee & Powell, 1981). Spatial and temporal homogeneity of the applied voltage-clamp potential are limited and accumulation or depletion of ions provides an additional complication. Due to these problems, we have com-

* *Present address:* Department of Physiology and Biophysics, Wright State University School of Medicine, Dayton, Ohio 45435.

pared single-channel I_{K_1}'s with macroscopic measurements either from outside-out vesicles formed from adult myocytes or from single cultured neonatal rat ventricular myocytes. Before the 3rd to 5th day, the latter have no internal membrane system (Moses & Kasten, 1979). We found incomplete agreement between whole cell and averaged single-channel currents. One reason for the difference is that the I_{K_1} in rat ventricular myocytes is composed of several classes of single channels which differ in their probability of opening, kinetics and conductance. Another is that these channels do not function independently of each other. An abstract presenting part of these results has been given (Josephson & Brown, 1984).

Materials and Methods

Preparations

Single adult rat ventricular myocytes were prepared by a method similar to that described previously (Powell et al., 1980). In brief, adult rats were sacrificed by cervical dislocation. The chest was opened, the heart was rapidly removed and was washed twice in Krebs solution (4°C) (composition in mM: NaCl, 140; KCl, 5.4; $MgCl_2$, 1.2; $CaCl_2$, 3.6; glucose, 10; Hepes, 5; adjusted to pH 7.4 with NaOH). Retrograde perfusion through the aorta was performed at 37°C using a Langendorff apparatus. The elapsed time from excision of the heart to cannulation and perfusion was less than 1 min. The heart was perfused with nominally Ca^{2+}-free Krebs solution ($[Ca^{2+}]$ (10 to 20 μM) at a rate of 12 ml/min for 5 min. After this period, collagenase (Sigma Type II), at a concentration of 0.04%, was added to the Krebs solution and was recirculated through the heart for 10 to 20 min. The heart was then removed from the cannula, the atria were cut off and discarded, and the ventricles were finely minced into 1 to 2-mm pieces by a mechanical tissue chopper. Incubation in the collagenase-containing solution proceeded for an additional 5 to 15 min, during which time gentle agitation was provided using a 10-ml pipette. The resulting cell suspension was filtered through a 250-μm nylon mesh and centrifuged (22 × g). The cell pellet was resuspended in 20 ml of Krebs solution (0 Ca^{2+}) and recentrifuged. This washing procedure was repeated a second time to remove any residual collagenase. The final cell pellet was suspended in Krebs Ringer's containing either 20 μM or 1 mM $CaCl_2$ and was kept at room temperature (20 to 22°C).

Single neonatal rat myocytes were prepared by the following method. Hearts from 8 to 12 neonatal rats (1 to 3 days old) were removed under sterile conditions and pooled in Dulbecco's phosphate-buffered salt solution at 4°C. The atria were dissected free of the ventricles, which were then opened, washed free of blood, and minced in small (2 to 3 mm) pieces. Tissue digestion was accomplished by swirling the ventricular fragments in a Ca^{2+}- and Mg^{2+}-free Krebs solution containing 0.05% trypsin for 5 to 10-min intervals. The supernatant from the second and third digestion period was collected and centrifuged. The cell pellet was then resuspended in 30 ml of Dulbecco's salt solution and recentrifuged to remove any residual trypsin. Cell density was measured in the final cell suspension and was adjusted to give 10^5 to 5×10^5 cells/dish (Falcon 35 mm culture dish). This procedure yielded isolated single-cell cultures which could be maintained for 2 to 3 weeks in a moist air CO_2 incubator. The myocytes in this study were chosen during the first three days, since during this time they displayed a spherical, or slightly flattened appearance. At later times in culture the myocytes may extend one or more projections; such cells are generally not useful for whole-cell voltage-clamp experiments.

Patch and Whole-Cell Voltage Clamp

Patch and whole-cell voltage-clamp experiments were performed according to the method of Hamill et al. (1981) using a List patch clamp (EPC-5). The command signals were obtained from a WPI interval generator and pulse modules which were connected to buffered attenuators. Voltage steps were applied at a rate of 20 min^{-1}. Pipettes were fabricated from thin-wall glass (WPI) by pulling in two stages. The tips were fire-polished using a heated Pt wire while they were observed at 375× magnification. The resulting pipettes had tip openings of approximately 1 to 2 μm in diameter and resistances of 2 to 5 MΩ, when filled with 150 mM KCl solution. Cell-attached patches were formed by gently touching the surface of the cell with the tip of the pipette and then applying suction. Seal resistances formed in this manner ranged from 5 to 50 gigohms. Using the appropriate solution conditions whole-cell voltage clamp was achieved by rupturing the membrane within the cell-attached patch by applying an additional pulse of suction. Successful access to the cell interior was evaluated by several criteria:

1) An increase in the magnitude of, and time constant for decay of the capacitive current (*see* Hamill et al., 1981).
2) A shift of holding current level which, when nulled, gave a measure of the cell resting potential. (Note, however, that the validity of this measurement is critically dependent upon the maintenance of a large seal resistance after membrane rupture.)
3) The presence of rapidly activating Na currents and fast tail currents demonstrated that the patch pipette access resistance was not increased markedly after membrane rupture.

Outside-out vesicles were formed by gently lifting the pipette away from the cell after obtaining a whole-cell voltage-clamp configuration.

Experimental Solutions

The ventricular myocytes were bathed in a Tyrode solution with the following composition (in mM): NaCl, 140; KCL, 5.4; $MgCl_2$, 1.2; glucose, 10; Hepes, 5; pH 7.4 with Tris base. In some cases $CaCl_2$ (3.6 mM) was added to the Tyrode solution. To test for the effects of $[Na^+]_o$ either choline chloride or Tris chloride was substituted isosmotically for NaCl. Alternatively, myocytes were bathed in a isotonic potassium solution (150 mM KCl) to rule out effects possibly arising from other ionic species and to depolarize the myocytes to a known, constant potential (0 mV). The pipette solution contained 150 mM KCl (pH 7.4) for cell-attached patches, and 150 mM K^+-Aspartate (pH 7.2) for whole-cell and outside-out patches, unless noted otherwise.

All experiments were conducted at room temperature (20 to 22°C).

Recording and Analysis

Both whole-cell and patch currents were filtered at 5 kHz using a 4-pole Bessel filter and were recorded at 7.5 in/sec on analog tape. Selected experiments were subsequently filtered and then digitized on an LSI 11/23 minicomputer using sampling rates that were at least twice the Nuquist frequency (50 to 200 μsec/point). The single-channel data could be filtered further using a zero phase, nonringing digital filter. Whole-cell current relaxations were curve-fit using an algorithm based on the Marquardt method. A sum of exponentials model was computed using a fourth-order Runge-Kutta integration scheme. An unweighted chi-square error was minimized during the fit, and provided a criterion for evaluating the success of the fit, and for statistically comparing between different models. The latter could be accomplished by use of the F test, which incorporates the chi-squared errors, as well as the degrees of freedom of the models under consideration.

Single-channel currents from patch-clamp recordings were analyzed by several methods. A computer program was used which allowed for the removal of capacitive and leakage currents during a voltage step by subtraction of averaged traces showing no activity. The corrected currents from repetitive steps (usually 50 to 100 traces) to a given potential were averaged to reconstruct a "macroscopic" current from the individual single-channel recordings. Incomplete corrections, due to the variability of the capacitive currents, resulted in the deletion of some channel transitions during the first 3 to 5 msec, in some cases. The amplitudes and durations of individual channel events were analyzed by an interactive program that could also run automatically. The opening and closing transitions were identified in a filtered record using two threshold levels (one for detecting channel opening, the other for channel closure). The opening threshold was set at 4 to 6× the standard deviation of the baseline noise. Amplitudes were measured directly from data recorded at 5 kHz and the points defining half-maximum amplitude (hma) were identified. The opening threshold was then reset and the process was repeated until the hma points were unchanging. These were taken as the transition times for opening and closing. Each record displayed three results: the data filtered at 5 kHz referred to as unfiltered, the filtered data used to set the thresholds, and the idealized data. With this method the effective bandwidth was extended to a value between that used to set threshold and 5 kHz. In the Figure legends of the single-channel data the filter value refers to the lower one. The open-times, closed-times and amplitudes of the idealized events were binned for histogram plots of these parameters. A curve-fitting algorithm using a maximum likelihood estimation routine (Colquhoun & Sigworth, 1983) was used to fit sums of exponentials to the open- and closed-time data. For the determination of probability of opening (P_o) records were selected in which only one active channel was in the patch. Events shorter than two to three points (usually 200 to 300 μsec) were systematically excluded in the analysis, thus introducing a bias in the histograms of lifetimes.

For the analysis of longer-lived open and closed states single-channel data were recorded during long voltage steps (2 sec), digitized at 1 msec/point and analyzed at a 500-Hz bandwidth. Events shorter than several msec were excluded, thus introducing a bias towards longer open and closed times in the frequency histogram.

A computer program was developed which calculated the predicted whole-cell relaxation time constants from the experimentally determined rate constants of the three-state model as a function of voltage (*see* Colquhoun & Hawkes, 1981). An initial condition specifying whether the channel begins the step in the open or closed configuration is required.

Results

Whole-Cell and Multi-Channel Inwardly Rectifying Potassium Currents

The first aim of the study was to characterize the voltage- and time-dependence of the inwardly rectifying macroscopic K^+ currents in single ventricular cells. We selected cultured neonatal rat ventricular myocytes for some of these experiments since they offered several advantages as a preparation for the whole-cell voltage-clamp method. First, their small (~15 μ diameter) spherical shape and lack of T-tubules enable the voltage clamp to produce both temporal and spatial uniformity (*see* Hamill et al., 1981). Second, the absence of T-tubules (Moses & Kasten, 1979), eliminates the possibility that depletion of K^+ ion could account for the current relaxations (e.g. *see* Almers, 1972). This feature was of special importance for experiments using low (5.4 mM) potassium bathing solutions inasmuch as such conditions accentuate depletion phenomena. Preliminary admittance studies performed in our laboratory (Lacerda & Brown, *unpublished observation*) confirm the morphological findings and reveal a simple R/C behavior of the myocyte membrane.

Alternatively, adult rat ventricular myocytes were selected for recording ensemble (multi-channel) currents in the outside-out configuration. The adult mycotyes were used for outside-out patches since it was found that single inward-rectifier channel currents could be more easily recorded from them in the cell-attached mode, than from the neonatal cells. This allowed a comparison of the microscopic and multi-channel "macroscopic" currents from the same cell type.

It should be noted that the input resistances of these cells, recorded using the patch-clamp method, are significantly larger than those previously recorded for single ventricular cells using conventional microelectrode techniques (Powell et al., 1980; Isenberg & Klockner, 1982; Josephson et al., 1984*a*). In normal Tyrode solution (5.4 mM K^+) the input resistance of the neonatal myocytes ranged from 400 to 1,00 MΩ at the resting potential while the adult myocytes ranged from 100 to 300 MΩ. This finding probably reflects the high seal resistance formed between the patch pipette and cell membrane, which almost eliminates leakage current.

The first series of experiments was conducted

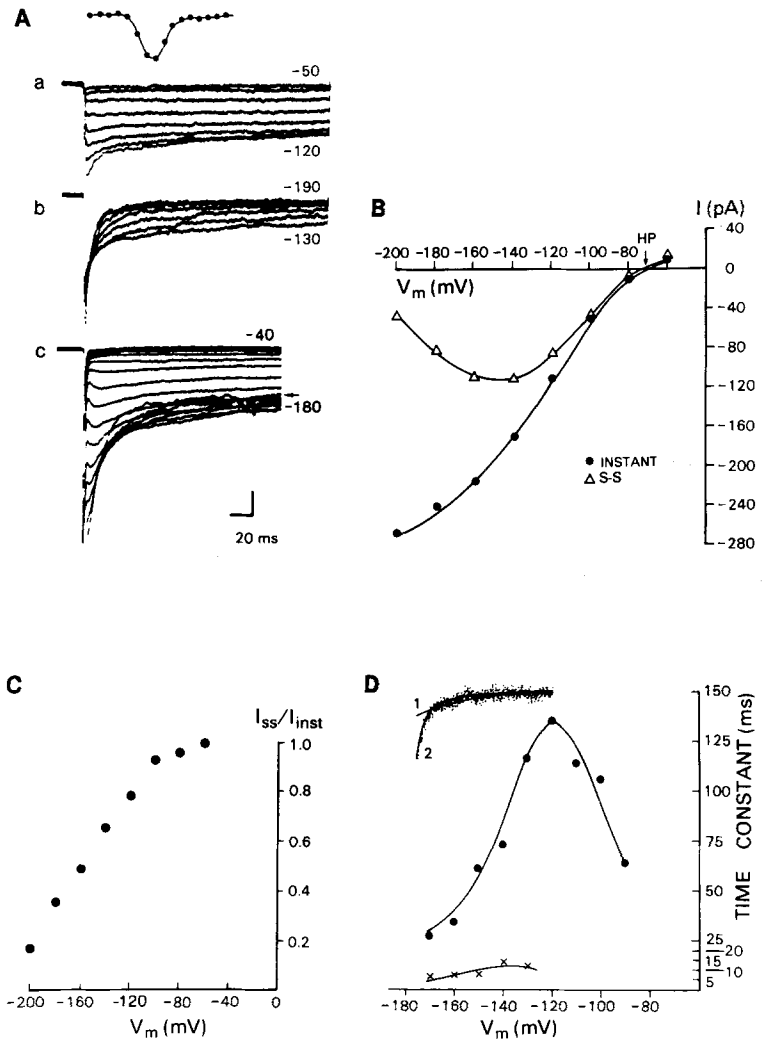

Fig. 1. Whole-cell inwardly rectifying K^+ current relaxations recorded from neonatal rat ventricular myocytes in external solution containing 5.4 mM KCl and 140 mM NaCl. (*A*) Current relaxations in response to voltage steps to −50 through −120 mV (in *a*) and to −130 through −190 mV (in *b*) in 10-mV increments from a holding potential (H.P.) of −40 mV. Filtered at 2 kHz (−3 dB) 4-pole Bessel filter. Inset: Time-expanded plot showing the capacity current recorded for a 20-mV, hyperpolarizing step. Digitized at 50 μsec/pt, 5 kHz filter. Part *c* shows a family of current traces recorded from an adult myocyte, for comparison. H.P. was −30 mV, voltage steps from −40 to −80 mV. Current calibration 80 pA (*a*,*b*), 200 pA (*c*). (*B*) Current-voltage plot for the instantaneous (I_{inst}) and steady-state (I_{ss}) K^+ currents; H.P. was −70 mV. (I_{inst} was measured isochronally at 1 msec, after the decay of the capacitive current, I_c (τ of I_c was <150 μsec at 5 kHz bandwidth); I_{ss} was measured at 150 msec. (*C*) The probability of being open of inward-rectifier channels as estimated by the ratio I_{ss}/I_{inst}. Data have been normalized, since the absolute P_o at the holding potential was not known. Data taken from part *B*. (*D*) Time constants for the fast (×) and slow (○) exponential relaxations obtained in solutions containing 150 mM NaCl and 5.4 mM KCl. Currents above −130 mV, which displayed an initial activation or only a small component of fast decay, were fit with a single exponential. Inset: an example of a current relaxation at −150 mV, fit with a single exponential (τ = 68 msec) and with a sum of two exponentials (τ_1 = 7.2 msec, τ_2 = 68 msec); 5 kHz filter

using neonatal rat myocytes bathed in a Tyrode solution including 140 mM Na^+ and 5.4 mM K^+. When exposed to this normal $[K^+]$ the myocytes have resting potentials of −70 to −80 mV (Josephson et al., 1984*b*). Under voltage clamp, a complicated pattern is observed in the current relaxations obtained during hyperpolarizing steps, as shown in a representative recording in Fig. 1*A* (part *a* and *b*). Hyperpolarizing voltage steps below −80 mV produced an instantaneous jump, followed by a slower increase (activation) in the inward K^+ current magnitude. The latter is also evident in Fig. 2. At more negative potentials activation became more rapid and finally could no longer be resolved, leaving only a time-dependent decay of the current. A similar pattern of inwardly rectifying current relaxations was recorded using adult ventricular myocytes (Fig. 1*A*, part *c*). Because the voltage control may have been adequate these experiments were not analyzed further.

A current-voltage plot of the "instantaneous" (measured isochronally at 1 msec) and the steady-state (measured at 150 msec) inward-rectifier current from a neonatal myocyte in which the holding potential was set at the resting potential again shows the instantaneous current increasing with hyperpolarization, whereas the steady-state current displays a pronounced negative resistance region below −140 mV (Fig. 1*B*). Similar relaxations of the inward rectifier current were recorded in a majority of the myocytes tested. In some cases, however, the time-dependent turn-off of the current with extreme hyperpolarization was not observed, most probably due to the contribution of a leakage current created during membrane rupture. Such results were discarded from the analysis.

The steady-state probability of opening (P_o) of the inward-rectifier channels at the test potential can be estimated by the ratio of the steady-state current to the instantaneous currents (I_{ss}/I_{inst}). This assumes a P_o of 1.0 at holding potentials near E_K which from our single-channel studies (*see later*) is approximately true. It should also be noted that this method may overestimate P_o if the channel rectifca-

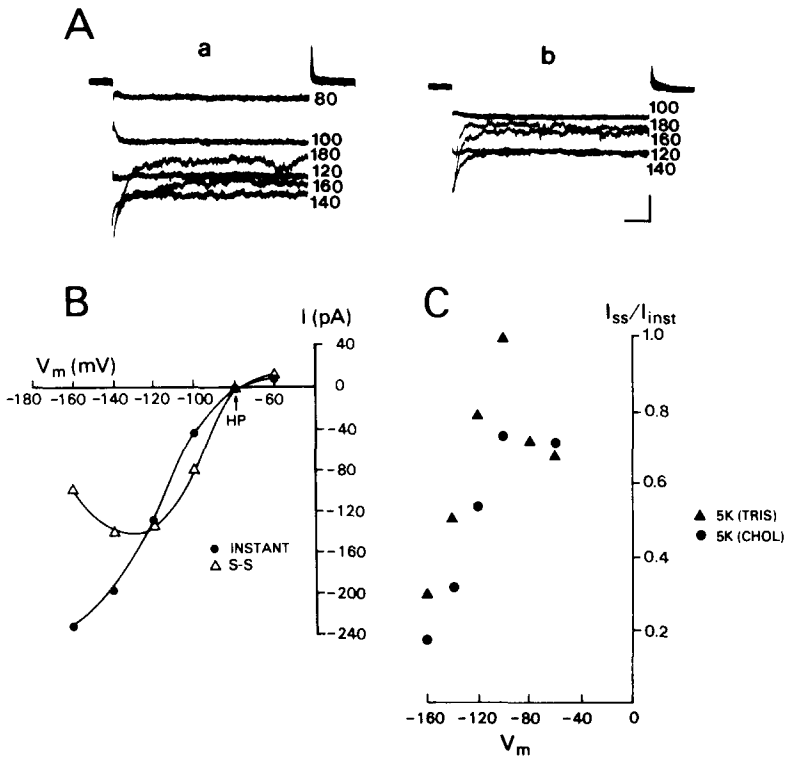

Fig. 2. Inwardly rectifying K^+ current relaxations recored from neonatal rat ventricular myocytes bathed in Tris-substituted or choline-substituted 0-Na^+ (K_o^+ = 5.4 mM) Tyrode solution. Calibration marker is 20 msec and 40 pA. Filtered at 2 kHz, 4-pole filter. Numbers next to current traces are the negative command potentials in millivolts. (*A*) *a*. Tris-substitution for NaCl. Holding potential was −70 mV. *b*. Choline-Cl substitution for NaCl. Holding potential was −80 mV. Recordings in *a* and *b* are from different cells. (*B*) Current-voltage relation in Tris-substituted 0-Na^+ solution. (*C*) I_{ss}/I_{inst} P_o as a function of voltage for Tris-(▲) and choline(●)-substituted solutions. Unlike the usage in Figs. 1 and 3, I_{inst} was the peak value of the current at any test potential

tion is instantaneous (i.e. faster than the voltage-clamp response time), because it may underestimate I_{inst}. The method also assumes that the single-channel conductance is invariant during the test pulse, which is true. Using this somewhat limited approach we find that P_o is highest near E_K and that is decreases with hyperpolarization.

To characterize further the processes underlying the current decay relaxations obtained in the normal Tyrode solutions, their time course was curve-fit with a sum of exponential functions. The best fits (lowest chi-squared errors) were obtained using a sum of two exponentials (*see* Materials and Methods). An example of a relaxation fit with a single, and with a sum of two exponentials, is shown in the inset of Fig. 1*D*. It can be seen that the initial, fast decay of the current required a two-exponential fit. The fast (tau 1) and slow (tau 2) time constants obtained from fitting a family of currents is plotted in Fig. 1*D*. The fast decay time constant ranged from 6.4 to 14.0 msec, and the slower time constant ranged from 27 to 135 msec. In this case, and with currents obtained from two other cells, both tau 1 and tau 2 displayed a biphasic voltage-dependent behavior, such that they become larger with moderate hyperpolarization but then become smaller again with strong hyperpolarization.

Since it has been reported that external Na^+ ion may act as a blocker of the inward-rectifier channel in frog skeletal muscle (Hille & Schwartz, 1978; Standen & Stanfield, 1979) and in invertebrate egg cell membrane (Ohmori, 1978; Fukushima, 1982) experiments were also conducted with myocytes bathed in zero-Na^+ solutions, using either TrisHCl (n = 3 experiments) or $Choline^+Cl^-$ (n = 3 experiments) as substitutes. As is shown by the representative results in Fig. 2*A*, the removal of Na^+ ion from the bathing solution did not abolish the time-dependent decay of the K^+ currents at hyperpolarized potentials, nor did it diminish the negative resistance region of the steady-state *I-V* curve that was observed in the Na^+-containing solutions (Fig. 2*B*). Consequently, as shown in Fig. 2*C*, P_o also decreased at negative potentials as it did in Na^+-containing solutions. An interesting finding obtained with both the Tris- and chlorine-substituted 0 Na^+ solutions was an increase in the magnitude of the time-dependent current following the initial instantaneous jump (defined on p. 22 as activation), and a decrease in the magnitude of the time-dependent decrease or decay (deactivation, p. 22) of the inward-rectifier current over the range of potentials from −80 to −120 mV. Because we assume that P_o approaches its maximum at full activation we normalized in these cases to the peak currents. In these experiments the removal of Na_o^+ also decreased the half-times for the relaxations of the inward-rectifier currents at hyperpolarized potentials. The above findings suggest that, in this system, Na^+ ion may act as a modulator of the kinetics of the inward-

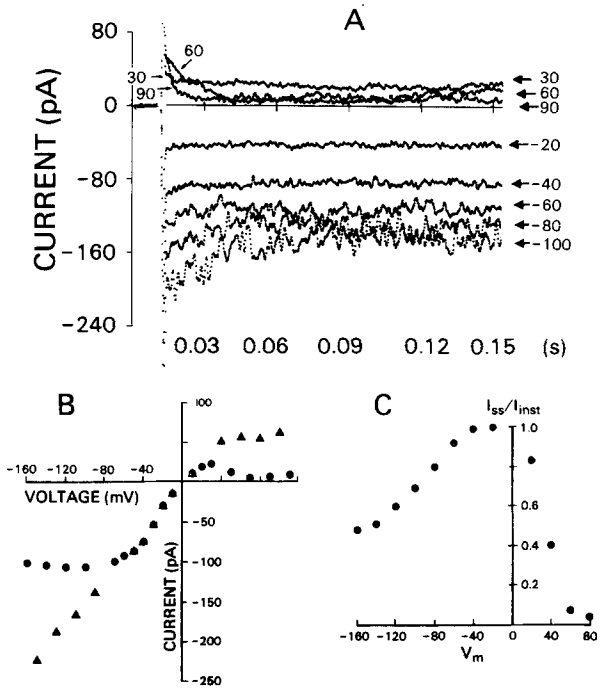

Fig. 3. Inwardly rectifying K^+ currents recorded from an isolated adult rat ventricular myocyte, using the outside-out patch-clamp method. The bath solution was 150 mM KCl; the patch pipette was filled with 150 mM K^+-aspartate. (*A*) Superimposed single-current traces for hyperpolarizing voltage steps from a holding potential of 0 to −100 mV (in 20-mV increments), and for depolarizing voltage steps from 0 to +90 mV (in 30-mV increments). Filtered at 1 kHz, 4-pole Bessel filter. Current-voltage relations (*B*) and P_o (*C*) were determined from the instantaneous (I_{inst}) and steady-state (I_{ss}) currents, as in Fig. 1.

rectifier channel (Hagiwara & Yoshii, 1979). Thus, barring the unlikely explanation that both Tris and choline are able to act as blockers of the inward-rectifier channel in this preparation, we conclude that the decline in conductance of the K^+ current at negative potentials results from an intrinsic change in the probability of opening of the inward-rectifier channel. Evidence supporting this hypothesis is presented in the next section.

In the following series of experiments the ventricular myocytes were bathed in a 150-mM K^+ solution in order to observed the inward-rectifying K^+ current in the absence of possible effects arising from the presence of other ionic species and in order to compare directly the macroscopic relaxations with the subsequent single-channel recordings. The holding potential was 0 mV (approximately equal to E_K) and hyperpolarizing and depolarizing voltage steps of 150-msec duration were applied, as illustrated in Fig. 3*A*. The current traces in this Figure were recorded from a multi-channel, outside-out vesicle, formed from an adult myocyte. We have assumed voltage control to be adequate in these vesicles because the currents are small and the waveforms give no indication of complications from membranes accessed by high series resistance. It can be seen that over this voltage range the currents show marked changes in their time dependence. Voltages steps to −20 and to −40 mV produce currents that appear to rise instantaneously (i.e. faster than 1 msec) and are essentially time independent or time invariant; i.e. they do not display activation or deactivation. At more negative potentials, a time-dependent decline of the current is observed, the magnitude of which increases with hyperpolarization.

Also noteworthy is the fact that the magnitude of the current noise, which is superimposed on the mean current, increases with hyperpolarization. Such behavior is consistent with a potential-dependent change in the probability of opening of single inward-rectifier channels, as will be described below.

The outward-current relaxations during voltage steps above +30 mV also display a time-dependent decline to their steady-state values, which becomes more rapid, and of greater magnitude at increasingly more positive potentials. Thus, even in high external K^+ solutions, the inwardly rectifying K^+ current is turned off in the steady state at depolarized potentials (i.e. at 60 to 100 mV positive to E_K). Relaxations were recorded in two other outside-out patches, exposed to 150 mM K_o^+ which confirmed the above findings.

The transient (isochronally measured at 1 msec) and steady-state (measured at 150 msec) current-voltage relations for the macroscopic inwardly rectifying currents recorded in 150 mM K^+ are presented graphically in Fig. 3*B*. It can be seen that the slope conductance in the hyperpolarizing direction is relatively constant over a wide range of potentials (averaging 1.5×10^{-9} Siemens). The steady-state current-voltage relation, however, shows a marked deviation from linearity at more negative potentials, resulting in a negative slope conductance. At potentials positive to E_K, there is a limited voltage region of steady-state outward current, which is maximal at ~30 mV positive to E_K. Further depolarization results in a virtual turn-off of the steady-state current resulting in a second negative slope region of the *I-V* curve.

The probability of opening (P_o) of the inward-rectifier channels (I_{ss}/I_{inst}), in 150 mM K_o^+ is approximately 1.0 between −40 and −20 mV from E_K and decreases with either hyperpolarization or depolarization (Fig. 3*C*). At the holding potential used in these experiments (0 mV), P_o was estimated to be

between 0.9 and 1.0. The macroscopically derived values for P_o will be compared with single-channel measurements of P_o subsequently.

A similar pattern of inward-rectifier current relaxations was also obtained in the whole-cell recording configuration from neonatal rat myocytes bathed in 150 mM KCl solutions. A time-dependent decline of the inward-rectifier current was clearly seen at potentials negative to −80 mV, and the steady-state current-voltage relation flattened although a negative resistance was not observed. In contrast to this behavior, in some of the whole cell neonatal currents studied in 150 mM K_o^+ solutions there was little time dependence, although there was a marked degree of inward rectification.

At present, the reason(s) for the diversity of the result in regard to the time dependence of the neonatal currents in 150 mM KCl solutions is not known, however, several possible explanations may be offered at this point in the text. First, an intracellular factor, which may be necessary to produce the channel block which underlies the time dependence of the current, may have been removed or altered under these experimental conditions. This possibility seems unlikely, since identical intracellular conditions were employed in recording the relaxations under lower K_o^+ conditions. Second, it is possible that the myocyte cultures contained heterogeneous cell types which, although ventricular in origin, display inward rectifier currents with differing time dependencies. Third, it is important to note that the high-potassium solutions used in these experiments tended to reduce the magnitude of, and slow the time course for the relaxations recorded in all cells tested. Thus, although there was a greater variability in the results under these conditions, a reduced time-dependent decline in the currents was the common finding using elevated K^+ solutions, as compared to lower K^+-containing solutions.

Macroscopic measurements were also made with myocytes exposed to bathing solutions containing 75 mM KCl and 75 mM NaCl. The results of these experiments (*not shown*) demonstrated that the magnitude of the current relaxations (I_{ss}/I_{inst}) were smaller than those obtained in 5.4 mM K_o^+, but larger than those in 150 mM K_o^+.

Single-Channel K^+ Currents

In an effort to gain more information about the nature of the time- and voltage-dependent relaxations of the whole-cell macroscopic K^+ currents, as well as to identify the elementary events underlying its conduction, patch-clamp recordings of single K^+-channel currents of the inward rectifier were undertaken.

Single-channel currents were recorded with patch pipettes filled with 150 mM KCl (buffered to pH 7.4) so as to be comparable with the whole cell voltage-clamp currents obtained under similar conditions. Initially, patches were made on cells that were bathed in normal Tyrode solution (or Tyrode minus Ca^{2+}) and had resting potentials of −70 to −80 mV. In later experiments the bathing solution was made identical to the 150 mM KCl patch pipette solution. Under those conditions the resting membrane potential was depolarized to 0 mV, the value of E_K. The latter group of experiments was performed to insure that the patch potential was known and would not change during the course of the experiment.

The following criteria and observations were used in establishing the identity of putative single inward-rectifying K^+ currents:

1) Inward-channel currents were observed only at potentials below the calculated E_K. Above E_K no outward currents were observed in 150 mM K_o^+. This characteristics of inward rectification rules out the possibility of Ca-activated K^+ current (Barret et al., 1982) or a Ca-activated leakage current (Reuter et al., 1982), both of which have been shown to conduct large outward currents.
2) The single-channel current was selective for K^+ ion and did not conduct other cations. Single-channel currents were never observed when pipettes were filled with isotonic CsCl. Furthermore, the single-channel conductance was reduced to approximately 15 pS in 15 mM K_o^+, thus varying with K_o^+ to the .43 power. In addition, lowering the K^+ concentration in the pipette to 15 mM (Na^+ substitution) shifted the appearance of single-channel currents to potentials below −60 mV, the calculated E_K.
3) The single-channel current was present (although transiently) in inside-out ripped-off patches, exposed to 1 mM EGTA; thus, it does not appear to require Ca_i^{2+} for its activation, nor for its open and closing transitions. Mg^{2+} ion was also not required, and was usually omitted from the pipette solution.
4) In some experiments patch solutions contained K^+-Aspartate instead of KCl to rule out any effects of Cl^- on single K^+-channel conductance or kinetics. No differences could be detected between the results obtained using these two solutions.

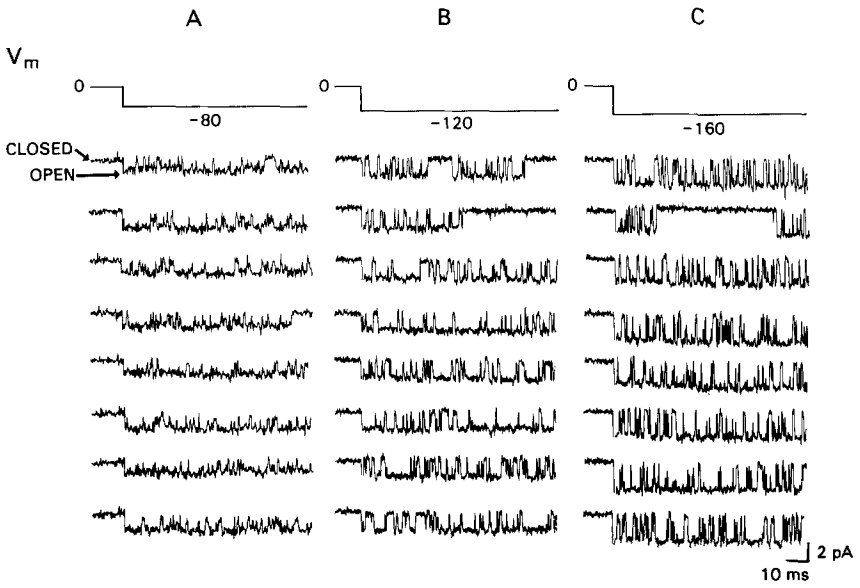

Fig. 4. Single-channel K^+ currents recorded from a cell-attached patch on a neonatal rat ventricular myocyte bathed in a 150-mM KCl solution. The patch pipette also contained 150 mM KCl. The trans-patch potential (V_m) was stepped from a holding potential of 0 mV to −80 (*A*), −120 (*B*) and −160 (*C*) mV. Eight representative traces are shown for each potential. The capacitive and leakage currents were digitally subtracted using an average of the current traces showing no single-channel currents (i.e., "failures"). The variability of the capacitive currents resulted in the depletion of some of the channel transitions during the first 3 to 5 msec of the step in some cases. The channel currents are inward, which is a downward direction in the Figure. Note the marked change in kinetics as the potential is made more negative. Filtered at 1 kHz, zero-phase digital filter

Neonatal Ventricular Myocytes

Single K^+-channel currents displaying inward rectification and voltage-dependent state transitions were recorded using cell-attached patches on neonatal rat ventricular mycoytes. Examples of corrected current traces recorded during voltage steps from zero mV to −80 (*A*), −120 (*B*) and −160 (*C*) are shown in Fig. 4. The single-channel conductance was approximately 20 pS with 150 mM K_o^+ and was unchanged during the relaxation that occurred in the whole cell records. A fast "flickering" to the nonconducting state can be observed at all three potentials. At the more negative potentials (i.e. −120 and −160 mV) a second, longer-lived closed state appeared occasionally. In addition, there was a tendency for the open times to decrease with hyperpolarization.

We had difficulty in obtaining patches with single inward-rectifier K^+ channels when using the neonatal myocytes. This was not the case in adult myocytes and therefore we have analyzed the K^+ channel behavior using the latter cells. The limited results are qualitatively similar in the two cells.

Adult Ventricular Myocytes

Early in the course of this study, it became clear that there were more than one type or class of inwardly rectifying K^+ channels in this preparation. Both the kinetics and the conductances of the elementary inwardly rectifying K^+ currents, when viewed across all of the cell-attached patches recorded, could be separated into one of three groups. Group I channels were recorded from patches having one or more large conductance channels displaying very brief open times. Recordings containing only Group I channels were very infrequent and not sufficient to provide a detailed analysis. Group II channels were recorded from patches containing one or more channels of relatively smaller conductance having considerably longer open times than the channels in Group I. Group III channels were a set having a conductance like Group II channels but in addition another conductance which was smaller and greatly prolonged.

Voltage Step Responses of Group II Channels

When symmetrical isotonic K^+ solutions bathed the patch, the holding potential was generally kept at zero mV, which was the zero holding current level, and the capacity and leadage currents were digitally subtracted from the single-channel current traces. The kinetic analysis of these experiments was restricted to three patches containing a single Group II (long open time) channel. This channel type had a

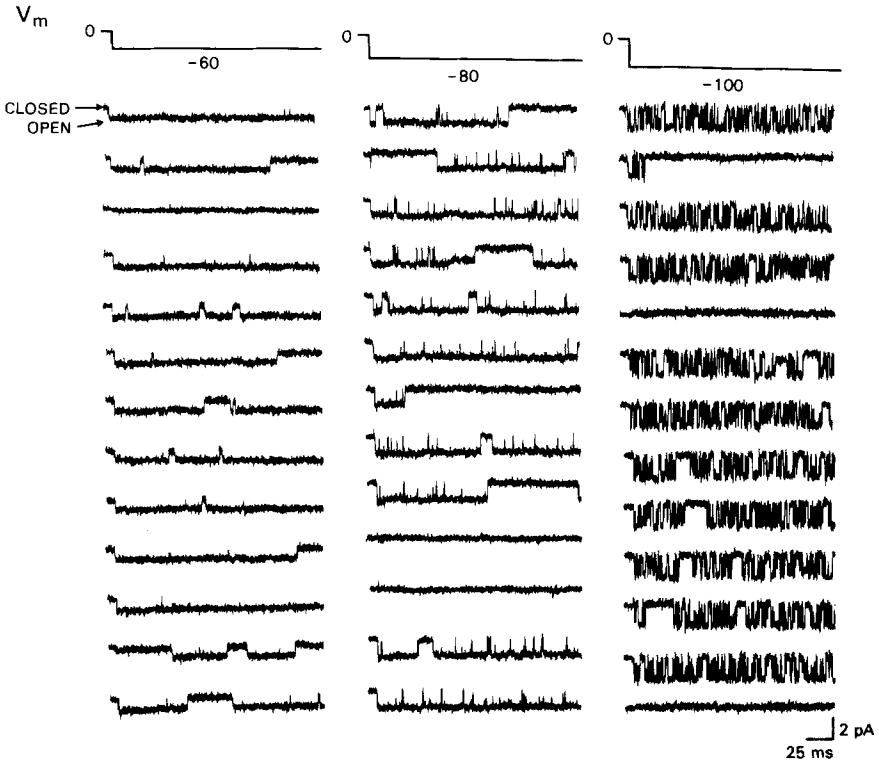

Fig. 5. Single-channel K^+ currents recorded from a cell-attached patch on an adult ventricular myocyte bathed in a 150-mM KCl solution. The patch pipette also contained 150 mM KCl. The trans-patch potential was stepped from a holding potential of 0 mV to −60, −80 and −100 mV. Thirteen representative current traces are shown for each potential. Filtered at 1 kHz

high probability of being open and might, therefore, be expected to be responsible for conducting most of the current in the whole-cell voltage-step experiments. Examples of typical single-channel currents recorded during repetitive voltage steps from 0 mV to −60, −80 and −100 mV are shown in Fig. 5. It is strikingly apparent that the kinetics of the transitions of the channel from the closed or nonconducting state, to the open, conducting state are markedly affected by the trans-patch potential. At the less negative potentials (e.g. −60 mV) the channel remains open for a much greater period of time than at more negative potentials (e.g. −100 mV), where a rapid bursting behavior predominates.

In order to characterize the amplitudes and kinetics of these single-channel currents the computer-based level detection program described in the Materials and Methods section was employed. The accuracy of this automatic program in identifying the transitions was checked by comparing the idealized single-channel current traces which were generated by the program, against the original data.

Single-Channel Conductance

The frequency distributions of the single-channel current amplitudes were fit with Gaussian curves; the mean value of the fit was taken as the current amplitude at that potential. The standard deviation of the Gaussian fit was approximately equal to the background current noise which caused small variations in the measured current amplitudes (*see* Fig. 6*A*). A plot of the current-voltage relationship for the single-channel amplitude in 150 mM K_o^+ is shown in Fig. 6*B*. It can be seen that the single-channel *I-V* appears nonlinear. The conductance between −100 and −80 mV was 40.8 pS, and between −60 to −20 mV was 20.8 pS. The averaged conductance between −100 and −20 mV was 26.7 pS. The single-channel currents became too small to be measured reliably above −20 mV and outward single-channel currents were not observed positive to E_K (0 to +150 mV).

Single-Channel Kinetics

Frequency histograms of the open times and closed times were generated for the three records containing only one channel at −60, −80 and −100 mV, and an example of each is shown in Fig. 7*A*. An accurate description of the longer-lived open and closed states was insured by analysis of long (2 sec) duration steps, as described in Materials and Methods. The distribution of the open times were fit by a single exponential at all three potentials, suggesting the presence of a single conducting state in these records. The average lifetime of the conducting state was 50.9 msec at −60 mV, 12.2 msec at −80 mV, and 3.5 msec at −100 mV, as shown in Fig. 7*B*. We compared the open time distributions in the first and second halves of these records and found no differences.

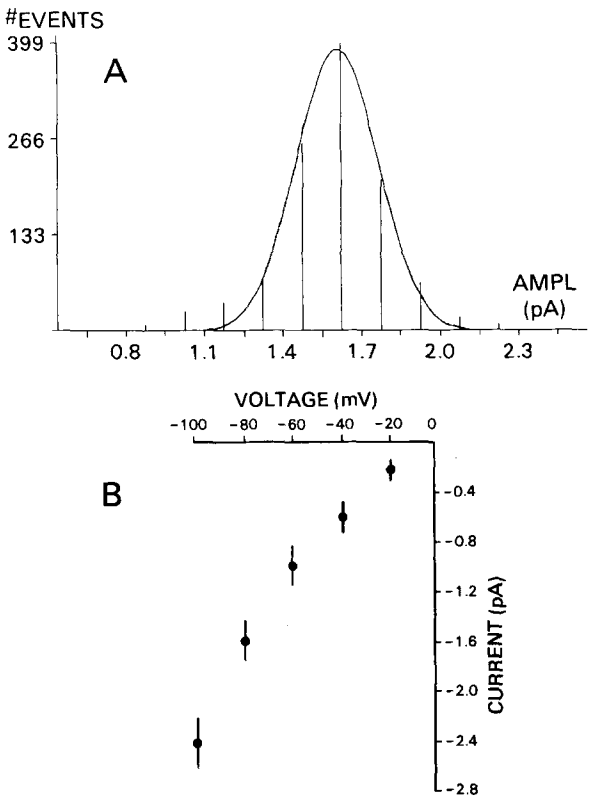

Fig. 6. (*A*) An example of a frequency distribution of the amplitudes of the single-channel K^+ currents obtained at a potential of −80 mV, fit with a Gaussian curve. Recording conditions the same as in Fig. 5. (*B*) Current-voltage plot for single K^+ channels. The single-channel conductance was nonlinear and ranged from 40 to 20 pS. The number of events at each potential was 131 (−20 mV), 212 (−40 mV), 532 (−60 mV), 786 (−80 mV), 974 (−100 mV). Means ± SD are shown

The average lifetime of the nonconducting state displayed a more complicated pattern than the open times. A minimum of two exponentials were needed to fit the distributions at all three potentials. It should be noted, however, that due to the quasi-stationarity of the measurements the relative proportions of the two components do not reflect those found in the steady state. At −60 mV the time constant of the population of short closed times (τ_f) was 2.9 msec as compared with 13.1 msec for the time constant of the longer closed times (τ_s). Hyperpolarization to −80 mV shortened τ_f (1.49 msec) but increased τ_s (27.9 msec). At −100 mV, τ_f returned to the same value as it has at −60 mV (2.9 msec), however τ_2 continued to increase at the more negative potential (62.9 msec). The frequent short closures at −100 mV produced a burst-like appearance of the current traces.

The averaged single-channel K^+ currents (from a Group II type channel) are compared with the outside-out (multi-channel) K^+ channel currents during voltage steps from a holding potential of 0 mV to −60, −80 and −100 mV in Fig. 8*A*. The averaged traces for −60 and −80 mV, when scaled by a constant N (120 channels), show a qualitative agreement to the multi-channel currents at the corresponding potentials thus tending to reinforce the idea that Group II channels are the major current pathways. There is a discrepancy, however, at −100 mV between the summed single-channel currents and the multi-channel current. As is shown in Fig. 8*B*, when scaled by the same number of channels as were used for the averages at −60, and −80 mV, the averaged channel currents at −100 mV predict a greater negative slope for the multi-channel steady-state *I-V* relation than was observed. One possible explanation for this apparent difference may be that a voltage-dependent, time-invariant leakage current contributes to the multi-channel current during extreme hyperpolarizations. Alternatively, there is a contribution from Group I channels. This explanation is considered more likely since it was found that although the P_o of the Group I channels was also reduced with hyperpolarization below E_K, their steady-state contribution to the whole cell currents ($P_o \cdot i$) would be proportionately greater at −100 mV. A third possibility for the discrepancy is that N, the number of active channels, increases with hyperpolarization. We consider this possibility unlikely, since the number of multiple current levels in patches containing more than one channel did not appear to increase with hyperpolarization.

In order to relate and compared the "microscopic" single-channel events with the "macroscopic" currents obtained from all activatable channels residing throughout the entire cell membrane, it is useful to determine the average behavior of a single channel; i.e., the probability that a given channel is open (P_o) at a given potential as a function of time. This was accomplished by summing the single-channel records point by point and obtaining the average value for each point at a given potential (as displayed in Fig. 8*A*). After division by i, this procedure gives the probability of the channel being in the open state as a function of time. The instantaneous P_o could not be determined by this method since, in some cases, the initial 3 to 5 msec following the voltage step could not always be analyzed due to imperfect subtraction of the capacity current.

The steady-state probability of opening as a function of voltage is shown in Fig. 8*C*. It can be seen that P_o was markedly decreased at more nega-

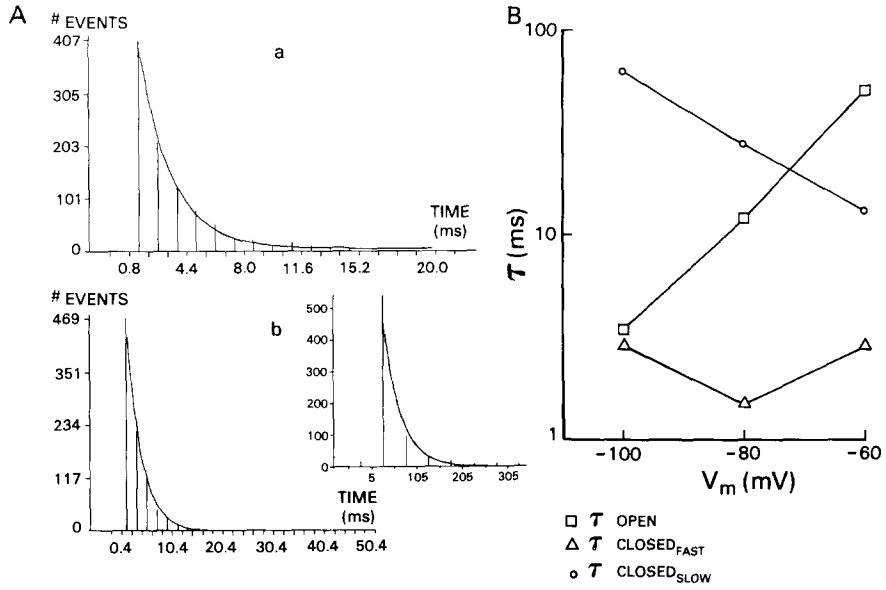

Fig. 7. Kinetics of single inwardly rectifying K^+ currents as a function of potential. (*A*) Example of the frequency distributions of the open (*a*) and closed (*b*) times at −100 mV. The histograms for the open times were fit with one exponential; histograms for the closed times required two exponentials (*see* Materials and Methods section for additional details). The total number of events was 987 in *a* and 973 in *b*. The bin-width for plotting was 1.2 msec in *a*, and 2.0 msec in *b*. The inset shows the distribution of longer closed times detected from a 2-sec duration step (500 Hz bandwidth). The total number of events was 745, and the bind-width was 50 msec. (*B*) A plot of the open and closed time constants as a function of potential (n = 3 experiments)

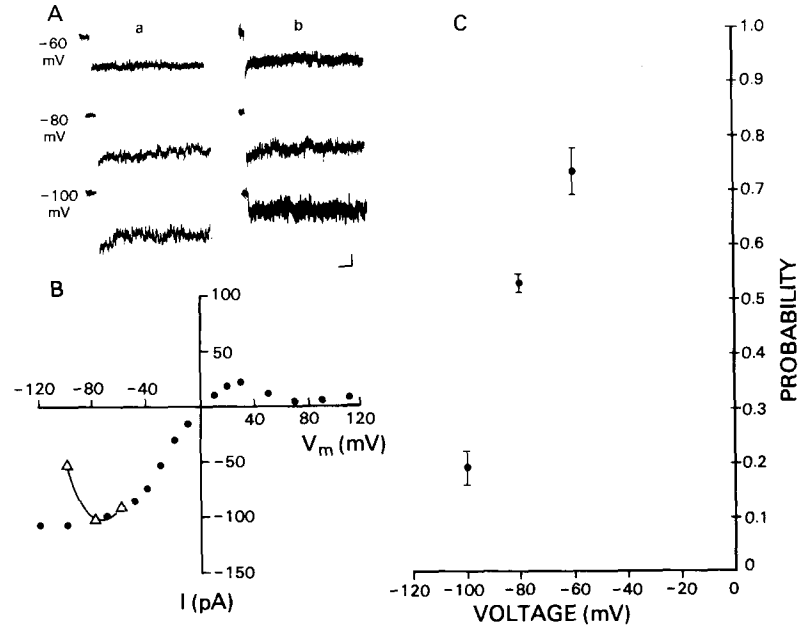

Fig. 8. (*A*). Comparison of multi-channel currents (left side) with the scaled, averaged single-channel K^+ currents (right side) at three potentials. Current calibration is 30 pA (*a*) and 0.27 pA (*b*); time calibration is 20 msec. (*B*) A plot of the multi-channel steady-state currents (●), and the scaled, averaged single-channel currents (△). The number of channels (120) was assumed to be constant at all three potentials, −60, −80 and −100 mV. (*C*) The steady-state probability of opening of single K^+ channels as a function of potential. For each measurement 50 to 100 single-channel traces (corrected for capacitive and leakage currents) at each potential were averaged and then scaled by the single-channel current amplitude at the same potential thus yielding the probability of opening as a function of time (n = 3)

tive potentials. This result accounts for the decrease in the macroscopic steady-state conductance, in spite of the increasingly larger single-channel conductance at negative potentials.

Group III Behavior

In many of the patches studied several differing current levels were observed, and an example of this

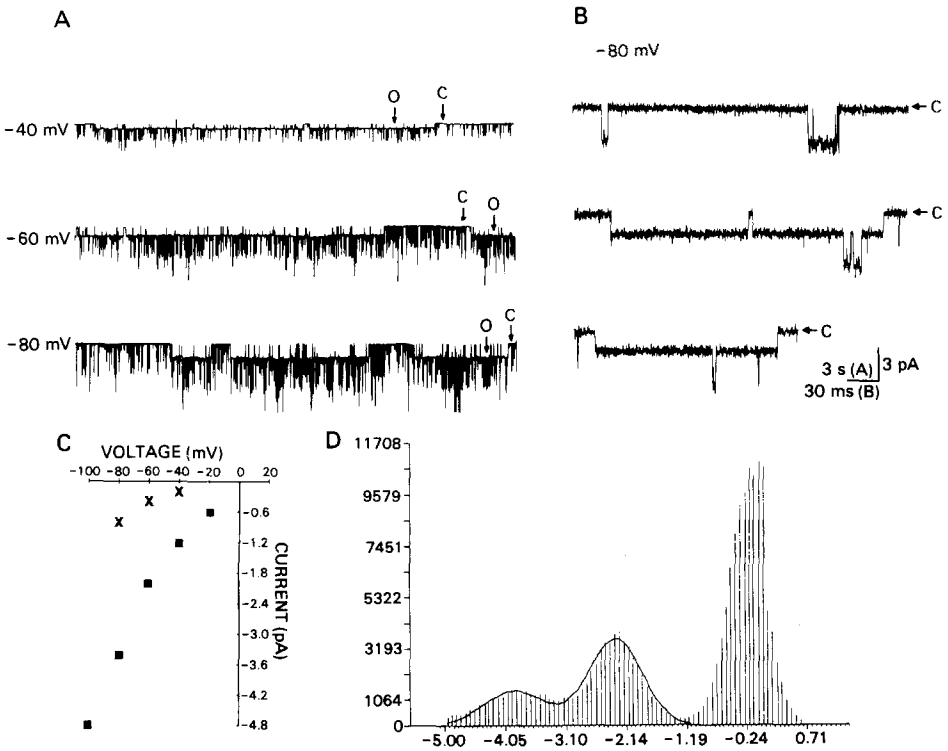

Fig. 9. Patch containing two distinct types of inwardly rectifying K^+ channel currents, recorded with a 150-mM K^+ pipette on an adult rat ventricular myocyte. (*A*) Time-compressed recordings at −40, −60, and −80 mV showing two types of single K^+ currents; one of smaller amplitude but long lifetime, and the other of larger amplitude but shorter lifetime. Filtered at 200 Hz. (*B*) An example of the two types of channel currents at −80 mV at a higher time resolution. Filtered at 1 kHz. Note the different time calibrations for *A* and *B*. (*C*) Current-voltage relation for the largest and smallest conductance levels of unitary amplitude. (*D*) An example of the frequency distribution of all amplitude points in a data record at −80 mV. The ordinate gives the number of points and the abscissa is the current amplitude, in picoamps. The distributions of the two conducting levels are fit with Gaussian curves with parameter values for mean ± SD and (amplitude) of −2.31 ± 0.42 (3781) and −3.89 ± 0.49 (1786). These values are corrected for any zero-current level offset before plotting, as in part *C*

type of behavior is shown in Fig. 9*A*,*B*. The magnitudes of the major current levels were not multiples of each other, thereby ruling out the possibility that these patches contained several identical channels. Both the larger and smaller amplitude current levels showed inward rectification as a function of potential, as well as the other criteria previously set forth for the inward-rectifying K^+ channel. The current-voltage relationships for the largest and the smallest unitary conductances are shown in Fig. 9*C*. The nonlinear conductance of the larger amplitude channel ranges from 25 to 68 pS, whereas the smaller amplitude channel ranges from 10 to 25 pS.

In addition to the differences in amplitude, the mean open times of these two conductance levels were dissimilar, differing by as much as two orders of magnitude. The mean open times for the long, small amplitude events and the brief, large amplitude events, at −60, −80, −100 mV were 330, 160 and 33 msec; and 5.2, 3.0 and 1.7 msec, respectively. The steady-state probability of opening for each conductance level was determined by histogramming all of the amplitude points in a data set (shown in Fig. 9*D*), and by then comparing the integral of the points lying under a Gaussian fit to the distribution at each conductance level to the total number of points in the record. With this method the P_o for the long, small amplitude and brief, large amplitude events was 0.842 and 0.112 at −60 mV, 0.237 and 0.130 at −80 mV, and 0.148 and 0.082 at −100 mV.

The most simple hypothesis to explain these bi-level events is that they represent two distinct and independent populations of inward-rectifier channels. Such a view was supported by the following evidence: (1) In most cases (>95%) the current level attained when the large and small levels overlapped in the time was simply the sum of the individual levels, i.e., when they occurred individually in the same record. (2) In addition, the probability of opening of the larger amplitude, shorter-lived level was found to the same whether or not the smaller amplitude, longer-lived level was in the

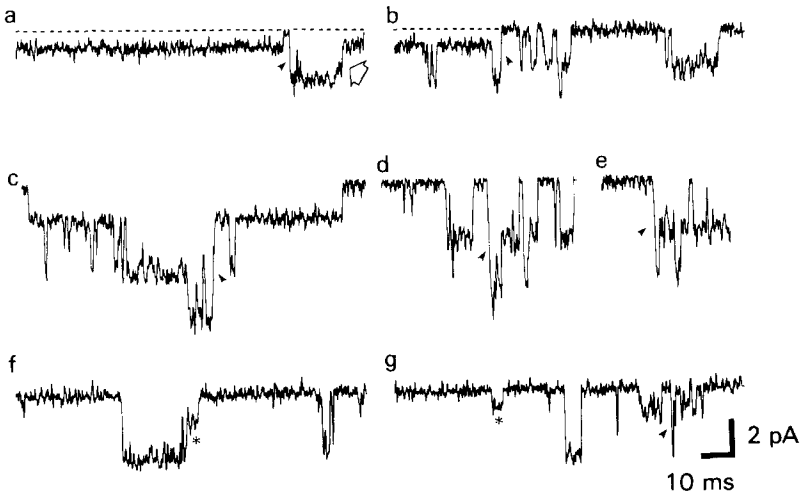

Fig. 10. Examples of simultaneous opening or closing of two conductance levels of the inward-rectifying K^+ channels (filled arrows). The open arrow in *a* points to a closing of a large channel and a simultaneous opening of a small channel. Membrane potentials were −60 mV in *a* and *b*, −80 mV in *c*, *d* and *e*. Plane *f* shows a transition from a higher to a lower conductance state, and panel *g* shows an unusually small conductance state (asterisks). Both *f* and *g* were recorded at −100 mV

open state, suggesting independent events. (3) As stated previously, patches containing only one class of rectifier channels were recorded (Group I, Group II).

However, transitions were observed, which were not consistent with the above hypothesis of the two types of independent channels. In some patches containing channels with two different conductances the frequency of simultaneous openings or closings was much greater than expected. An example is shown in Fig. 10*A–E* in which there were two channels of conductance ~40 pS and one ~23 pS. A continuous record of 10.8-sec length, sampled at 100 μsec per point was obtained at −80 mV (Fig. 10*C–E*) in which the large channels had 724 transitions and the small ones had 53 transitions. There were seven simultaneous on and three simultaneous off transitions. In this record no instances of simultaneous on-off or off-on transitions were observed although an example of the latter is shown by the open arrow in Fig. 10*A*.

The predicted number of simultaneous transitions were calculated as:

(frequency of opening of small level) × (frequency open of large level) =

$$\left(\frac{\text{frequency small}}{\text{total of \# of points/sec}}\right) \times \left(\frac{\text{frequency large}}{\text{total of \# of points/sec}}\right)$$

$$= \left(\frac{2.46/\text{sec}}{10^4 \text{ pts/sec}}\right) \times \left(\frac{33.54\cdot/\text{sec}}{10^4 \text{ pts/sec}}\right)$$

$$= 8.25 \times 10^{-7} \frac{\text{transitions}}{\text{pt.}}$$

and (8.25 × 10^{-7} transitions/pt) × (10.8 × 10^4 pts/record) = 0.09 "on" or "off" occurrences/record. Nonsimultaneous transitions greater than 300 μsec were clearly resolved in our experimental conditions and, given this, the predicted number of simultaneous events for the record was less than one, that is, one order of magnitude less than was observed (10). Similar results suggesting nonindependence of channel behavior were obtained with records of equal length at −60 and −100 mV. The predicted number of simultaneous transitions at −60 mV was 0.14 whereas five events of this nature were observed in the record (*see* Fig. 10*A,B*) and at −100 mV seven simultaneous transitions occurred although only 0.4 were predicted. It seems therefore that some cooperativity in channel function occurs.

It was also observed that a channel could suddenly pass into a subconductance state and two examples of this are shown in Fig. 10*F,G*. These occurrences were too infrequent to permit analysis and possible explanations are given in the discussion.

Discussion

The results of this paper demonstrate that, in the absence of external blocking cations, the inwardly rectifying K^+ current in rat ventricular myocytes can display a time-dependent relaxation resulting in a reduced steady-state conductance. The nature of the decrease in the macroscopic conductance with strong hyperpolarization was found to be caused by neither a decrease in the number of channels nor the single-channel conductance, but by a marked reduction in the probability of opening of single channels. The behavior of the predominant channel (Group II) could explain many but not all of the results observed in multi-channel records. This is because of

the occurrence of Group I and Group III behavior. Several subconductance states were also observed but the effect of bandwidth limitations in the recording system was not evaluated. These events might represent a reduction in single-channel conductance due to a relatively long-term change in the channel conformation, or could be due to an increased rate of open and close transitions appearing as a time-averaged decrease in conductance. A fuller treatment of bandwidth limitation on open and closed time distributions is given in Kunze et al. (1985) and Wilson and Brown (1985).

The kinetic information gathered from Group II records was evaluated using a simple three-state kinetic:

$$o \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} c_1 \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} c_2$$

where o represents the open conducting state, c_1 represents the short-lived closed state, and c_2 represents the long-lived closed state.

In addition to its simplicity, the three-state model was chosen for the following reasons:

1) There were two populations of closed times in the single-channel analysis. This is an oversimplification since the very prolonged closed state described by Sakmann and Trube (1984*b*) was not evaluated.
2) Only one open state was present in the single-channel records of the predominant channel.
3) Bursting behavior was observed in the single-channel currents which could not be explained by a two-state model.
4) A two-exponential relaxation was observed in the macroscopic relaxations.

The initial condition for the single-channel voltage steps was a holding potential of 0 mV (E_K). Estimates of P_o from macroscopic relaxations (150 mM K_o^+, $V_{hold} = E_K$) indicate that P_o is approximately 0.9 to 1.0 at E_K, in spite of the fact that there is no observable net current. Thus, the model assumes that channels are in the open configuration preceding the voltage step. Voltage steps to the test potentials (−60, −80 and −100 mV) brought P_o to lower values, hence, a current decline was observed. If the membrane potential were held at −100 mV (low P_o) and then stepped up to −60 mV (higher P_o) a current activation would be obtained. The model we have used does give this result if we rearrange the starting conditions (i.e. a low P_o) and assume a closed → closed → open state diagram.

Following Kameyama et al. (1983) the rate constants of the model (k_{12}, k_{21}, k_{23}, k_{32}) were evaluated as a function of the time constants extracted from the exponential fits of the open (τ_o) times and the fast and slow closed times (τ_f, τ_s), and from the steady-state probability of opening (P_o) as follows:

$$k_{12} = \frac{1}{\tau_o}$$

$$k_{21} = \left(\frac{1}{\tau_s} + \frac{1}{\tau_f}\right) - (1 - P_o)\tau_o / P_o \tau_f \tau_s$$

$$k_{23} = [(1 - P_o)\tau_o / (P_o)(\tau_f \tau_s)] - [1/\tau_f \tau_s k_{21}]$$

$$k_{32} = 1/\tau_f \tau_s (k_{21}).$$

A plot of the rate constants as a function of voltage is shown in Fig. 11*A*. A computer program was used which allowed the calculation of the current relaxations from these rate constants. An example of a calculated relaxation obtained using the rate constants at −100 mV is shown (Fig. 11*B*) superimposed with multi-channel relaxation shown in Fig. 8*A*. There is good agreement between the time courses but the steady-state current of the single-channel model was smaller as was the case in Fig. 8*A*. The difference could be produced by Group I channels since their P_o is voltage independent and their relative contribution is much larger at −100 mV. Thus a simple three-state Markovian sequential model (Kameyama et al., 1983; Sakmann & Trube, 1984*b*) can be used to describe Group II channel kinetics (*see below*) but it is clearly insufficient for the entire behavior of the whole-cell inward-rectifier current. This is not unexpected given the heterogeneity of I_{K_1} channels and the additional complications of cooperativity and conductance state changes. However, the question of heterogeneity of I_{K_1} channels in neonatal cells could not be examined because the data were insufficient.

Macroscopic currents showing a time-dependent activation phase (as in Figs. 1 and 2) were only obtained when the probability of opening of the inwardly rectifying K^+ channels (P_o) at the holding potential was less than P_o of the test or step potential. Therefore, this additional activation relaxation of the macroscopic current under these conditions reflects the time it takes for the channel population to reach the new, larger steady-state P_o value after the voltage step. In both 5.4 mM K_o (Figs. 1 and 2) as well as 150 mM K_o (Fig. 3) the steady-state P_o was at its highest value at approximately 20 to 40 mV negative to E_K (−90 mV in 5.4 mM K_o^+, and 0 mV in 150 mM K_o). Thus, activation was recorded because the membrane potential was stepped into a region of higher P_o. At more negative potentials P_o

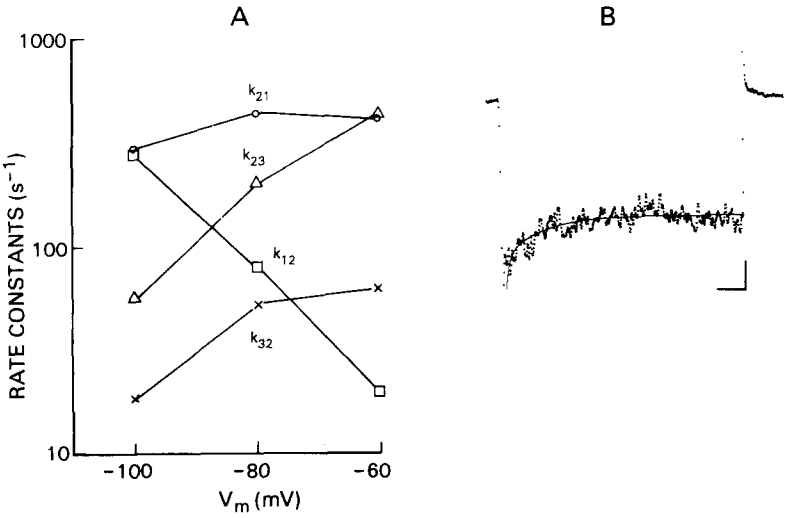

Fig. 11. (*A*) Rate constants for the three-state model calculated form time constants of the open and closed time histograms and from P_o as a function of potential. (*B*) The current relaxation predicted from the three-state model of the inward-rectifier channel is superimposed on a multi-channel recording at −100 mV; same conditions as in the single-channel experiments. The channel is assumed to be in the open state prior to the voltage step. The time constants calculated from the model are 1.66 and 22.0 msec. Calibrations are 25 pA, 20 msec

decreased to values below that at the holding potential, and a decline of the current was observed. Whether this is deactivation or inactivation is a moot point.

The whole cell current relaxations were found to be smaller and slower in the high [K^+] solutions. This dependence on external [K^+] may also help to explain why there appeared to be little time-dependent relaxation in some of the whole-cell recordings. Additional modulators, such as endogeneous intracellular factors which may act to regulate the kinetics or conductance of inward-rectifier channels, might be responsible for some of the variability observed. The absence of large, outward single-channel or macroscopic K^+ currents tends to rule out the possibility that ATP-sensitive K^+ channels (Trube & Hescheler, 1984) are contributing to the present results.

Single-channel currents contributing to the inward rectifier of several other types of excitable cells have recently been described (Fukushima, 1981, 1982; Ohmori, 1981; Momose et al., 1983; Sauvé et al., 1983). It is interesting to note that in the case of the egg cell membrane (Fukushima, 1981), skeletal muscle (Ohmori, 1981) and bullfrog atrium (Momose et al., 1983) open and close transitions of the single-channel currents are observed only after the addition of a blocking ion (e.g. Na^+, Cs^+, or Ba^{2+}) to the pipette solution. This finding is consistent with the absence of relaxations of the K^+ currents in the above-mentioned cells in pure K^+ solutions. In the present results, spontaneous open and close transitions of the single-channel currents were always observed in external solutions containing only K^+ ions. The possibility that Cl^- ion serves as a blocking ion for this inward-rectifying channel was ruled out by substituting aspartate ion for Cl^- in the external solutions.

In fact, in zero-Na^+ solutions the decline of the macroscopic current appears to develop more rapidly than in Na^+-containing solutions (at $K_o^+ = 5.4$ mM). One possible explanation for this result is that Na^+ ion may act as a partial agonist at a binding site in or near the channel which, when occupied, acts to stabilize the channel in an open or pre-open state. Thus, external Na^+ ion may compete with K^+ ion for this site, thereby slowing the kinetics of an endogenous, K^+-dependent channel closure. Further single-channel experimentation will be needed to resolve this question.

A fall in K^+ permeability has also been described in frog skeletal muscle under extreme hyperpolarization (Almers, 1972; Standen & Stanfield, 1979). However, a description of the mechanism for the decline of K^+ current in skeletal muscle fibers is complicated by the additional phenomenon of depletion of K^+ ion from narrow diameter diffusion-restricted t-tubule spaces (Almers, 1972). Nevertheless, it was possible to conclude that a change in K^+ permeability was responsible for the conductance decline at more negative potentials (Almers, 1972). This change in K^+ permeability was also demonstrated to be dependent on the external Na^+ ion concentration, and has been proposed to be caused by a potential-dependent block of the K^+ current by Na^+ ion.

In the present experiments a K^+ depletion phenomenon is not considered to be responsible for the current decline at hyperpolarizing potentials, for the following reasons: 1) Whole-cell voltage-clamp experiments were conducted on spherical neonatal rat ventricular myocytes which display no orga-

nized internal membrane systems. 2) Relaxations of the K^+ currents were observed in solutions containing up to 150 mM K^+; conditions which render ion depletion unlikely. 3) Measurements of single-channel currents were obtained from patches made on the surface membrane where there were no restricted spaces. Yet, the sums of the single-channel records also showed the reduced K^+ conductance at negative potentials. 4) The single-channel amplitude did not change as a function of time during a voltage step.

The present results may be compared with the findings reported by Sakmann and Trube (1984*a*) for single inward-rectifier K^+ channels recorded from adult guinea pig ventricular myocytes and by Kameyama et al. (1983) for rabbit ventricular myocytes. Although the properties of the single-channel inward-rectifier currents in the ventricular cells of these three species (rat, guinea pig and rabbit) clearly resemble each other, there exist certain differences which may result in functional alterations of the respective macroscopic currents.

1) Among the similarities, the rat, guinea pig and rabbit single inward-rectifier channels all show specificity for K^+ ions, and are blocked by Cs^{2+} or Ba^{2+} ion. The single-channel chord conductance for the predominant channel type in the rat (avg. 27 pS) is similar to that of the inward-rectifier channel in the guinea pig (28 pS) in 150 mM K_o^+, although in the rabbit it was somewhat larger (47 pS) probably due to the higher temperatures (31 to 36°C) used in that study. The density of inward-rectifier channels in adult rat myocytes is about 1 μm^{-2}, a value similar to that found in adult guinea pigs (Sakmann & Trube, 1984*a*). Outward single-channel currents were not recorded in any of these preparations, under these conditions, although they have been reported for rabbit inward-rectifier channels under conditions of lower external [K].
2) The opening and closing kinetics of the (Group II) inward-rectifier channel in the rat, in the absence of blocking ions, are substantially faster than those reported for the guinea pig (Sakmann & Trube, 1984*b*) or rabbit myocytes (Kameyama et al., 1983). This correlated well with the shorter time constants measured for the macroscopic relaxations in the rat myocytes, as compared to guinea pig (Josephson & Brown, *unpublished observation*). The closed time distributions were fit with two exponentials in rat and rabbit, while three were needed for guinea pig.
3) Sakmann and Trube (1984*b*) estimate a P_o at E_K of 0.3 from stationary steady-state single-channel data, but in contrast, they found a P_o of 0.62 from voltage-step experiments using patches with multiple channels. In the present study, these experiments were made using patches containing only one channel, thereby eliminating complications arising in the interpretation of P_o due to uncertainties in the estimation of the number of channels in the patch, or due to the possibility that P_o is affected by the number of channels in the patch. For rat, we estimate that P_o approaches 1.0 as V_m approaches E_K. This value is in agreement with estimates of P_o at E_K using macroscopic relaxation measurements. In rabbit, P_o at E_K was also found to be near unity from single-channel measurement. The results in rat and rabbit are consistent with the idea that the resting potential is determined mainly by the inward rectifier. The reason for the difference in guinea pig is unknown.
4) In the adult rat ventricle at least three classes of inward-rectifier channels require consideration whereas only one population was analyzed in the guinea pig and rabbit ventricular cells. Subconductance states were also observed in guinea pig and rabbit myocytes although the possibility of cooperativity was not addressed.

In summary, we have shown that three classes of inwardly rectifying K^+ channel currents contribute to the macroscopic current of adult rat ventricular myocytes. The predominant channel type (Group II) displaying long open times and steeply voltage-dependent kinetics, appears to be responsible for conduction of most of the macroscopic current, especially at potentials close to E_K. In addition, the time- and voltage-dependent decline in the probability of opening of Group II channels can account at least in part, for the relaxation kinetics of the whole-cell current. The small conductance channel recorded in Group III patches exhibited long open times lasting into hundreds of milliseconds. The larger conductance, group I channels, which display brief open times and a relatively small, voltage-insensitive probability of opening contribute a significant portion of the macroscopic current only at extreme hyperpolarization.

The authors would like to thank Mr. Brian Holt for preparing the isolated myocytes, Mr. Bill Little and Mr. Helmut Zucker for writing the computer programs used in the analysis, and Mr. Harold Henderson for preparing the Figures. We especially acknowledge the contribution of Dr. David Wilson in the modeling

of the channel, and Dr. Phil Palade for his careful suggestions on the manuscript.

References

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

Barrett, J.N., Magleby K.L., Pallotta, B.S. 1982. Properties of single calcium-activated potassium channels in cultured rat muscle. *J. Physiol. (London)* **331:**211–230

Brown, A.M., Lee, K.S., Powell, T. 1981. Voltage clamp and internal perfusion of single heart muscle cells. *J. Physiol. (London)* **381:**455–478

Carmeliet, E. 1982. Induction and removal of inward-going rectification in sheep cardiac Purkinje fibers. *J. Physiol. (London)* **327:**285–308

Colquhoun, D. Hawkes, A.G. 1981. On the stochastic properties of single channels. *Proc. R. Soc. London B.* **211:**205–235

Colquhoun, D., Sigworth, F. 1983. Single Channel Recording, Ch. 11. B. Sakmann and E. Neher, editors. Plenum, New York

Fukushima, Y. 1981. Single channel potassium currents of the anomolous rectifier. *Nature (London)* **294:**368–371

Fukushima, Y. 1982. Blocking kinetics of the anomalous potassium rectifier of tunicate eggs studied by single channel recordings. *J. Physiol. (London)* **331:**311–331

Hagiwara, S., Miyazaki, S., Rosenthal, N.P. 1976. Potassium current and the effects of cesium on this current during anomalous rectification of the egg cell membrane of a starfish. *J. Gen. Physiol.* **67:**621–638

Hagiwara, S., Yoshii, M. 1979. Effects of internal potassium and sodium on the anomalous rectification of the starfish egg as examined by internal perfusion. *J. Physiol. (London)* **292:**251–265

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. 1981. Improved patch-clamp techniques for high-resolution current recordings from cells and cell-free membrane patches. *Pfluegers Arch.* **391:**85–100

Hille, B., Schwartz, W. 1978. Potassium ions as multi-ion single-file pores. *J. Gen. Physiol.* **72:**409–442

Hutter, O.F., Noble, D. 1960. Rectifying properties of heart muscle. *Nature (London)* **188:**495

Isenberg, G. 1976. Cardiac Purkinje fibers: Cesium as a tool to block inward rectifying potassium currents. *Pfluegers Arch.* **365:**99–106

Isenberg, G., Klockner, V. 1982. Calcium currents of isolated bovine ventricular myocytes are fast and of larger amplitude. *Pfluegers Arch.* **395:**30–41

Josephson, I.R., Brown, A.M. 1984. Conductance and kinetics of elementary inwardly-rectifying K^+ currents in ventricular myocytes. *Biophys. J.* **45:**50a

Josephson, I.R., Sanchez-Chapula, J., Brown, A.M. 1984*a*. A comparison of calcium currents in rat and guinea pig single ventricular cells. *Circ. Res.* **54:**144–156

Josephson, I.R., Sanchez-Chapula, J., Brown, A.M. 1984*b*. Early outward current in rat single ventricular cells. *Circ. Res.* **54:**157–162

Kameyama, M., Kiyouse, T., Soejima, M. 1983. Single channel analysis of the inward rectifier K current in the rabbit ventricular cells. *Jpn. J. Phys.* **33:**1039–1056

Katz, B. 1949. Les constantes electriques de la membrane du muscle. *Arch. Sci. Physiol.* **3:**285–300

Kunze, D.L., Lacerda, A.E., Wilson, D.L., Brown, A.M. 1985. Reopening, waiting, and inactivating properties of single cardiac Na channels. *J. Gen. Physiol.* **86:**691–720

Lee, K.S., Weeks, T.A., Kass, R.L., Akaike, N., Brown, A.M. 1979. Sodium currents in single heart cells. *Nature (London)* 260–271

Momose, Y, Szabo, G., Giles, W. 1983. An inwardly rectifying K^+ current in bullfrog atrial cells. *Biophys. J.* **41:**311a

Moses, R.L., Kasten, F.H. 1979. T-tubules in cultured mammalian myocardial cells. *Cell Tissue Res.* **203:**173–180

Noble, D. 1965. Electrical properties of cardiac muscle attributable to inward going (anomalous) rectification. *J. Cell Comp. Physiol.* **66:**127–136

Noble, D., Tsien, R.W. 1968. The kinetics and rectifier properties of the slow potassium current in cardiac Purkinje fibers. *J. Physiol. (London)* **195:**185–214

Ohmori, H. 1978. Inactivation kinetics and steady-state current noise in the anomalous rectifier of tunicate egg cell membranes. *J. Physiol. (London)* **281:**77–99

Ohmori, H. 1981. Unitary current through sodium channel and anomalous rectifier channel estimated from transient current noise in the tunicate egg. *J. Physiol. (London)* **311:**289–305

Powell, T., Terrar, T., Twist, V.W. 1980. Electrical properties of individual cells isolated from adult rat ventricular myocardium. *J. Physiol. (London)* **302:**131–153

Reuter, H., Stevens, C.F., Tsien, R.W., Yellen, G. 1982. Properties of single calcium ion channels in cardiac cell culture. *Nature (London)* **297:**501–504

Sakmann, B., Noma, A., Trautwein, W. 1983. Acetylcholine activation of single muscarinic K^+ channels in isolated pacemaker cells of the mammalian heart. *Nature (London)* **303:**250–253

Sakmann, B., Trube, G. 1984*a*. Conductance properties of single inwardly-rectifying potassium channels in ventricular cells from guinea-pig heart. *J. Physiol. (London)* **347:**641–657

Sakmann, B., Trube, G. 1984*b*. Voltage-dependent inactivation of inward-rectifying single-channel currents in the guinea-pig heart cell membrane. *J. Physiol. (London)* **347:**659–683

Sauve, R., Roy, G., Payet, D. 1983. Single channel K^+ currents from HeLa cells. *J. Membrane Biol.* **74:**41–49

Standen, N.B., Stanfield, P.R. 1979. Potassium depletion and sodium block of potassium currents in frog sartorius muscle. *J. Physiol. (London)* **294:**497–520

Trube, G., Hescheler, J. 1984. Inwardly-rectifying channels in isolated patches of the heart cell membrane: ATP dependence and comparison with cell-attached patches. *Pfluegers. Arch.* **407:**178–184

Trube, G., Sakmann, B., Trautwein, W. 1981. Inward rectifying potassium currents recorded from isolated heart cells by the patch clamp method. *Pfluegers Arch.* **391:**R7

Wilson, D.L., Brown, A.M. 1985. Effects of limited internal resolution on single channel measurements with application to calcium channels. *IEEE Trans. Biomed. Eng.* **32:**786–797

Received 10 March 1986; revised 25 June 1986