J. Membrane Biol. 158, 265–274 (1997)

The Journal of
Membrane
Biology

Characterization of Single Inward Rectifier Potassium Channels from Embryonic *Xenopus laevis* Myocytes

R. Chauhan-Patel, A.E. Spruce
Department of Pharmacology, The Medical School, University of Birmingham, Birmingham B15 2TT, UK

Received: 16 September 1996/Revised: 14 March 1997

Abstract. Single inward rectifier K^+ channels were studied in *Xenopus laevis* embryonic myocytes. We have characterized in detail the channel which is most frequently observed (Kir) although we routinely observe three other smaller current levels with the properties of inward rectifier K^+ channels ($Kir_{(0.3)}$, $Kir_{(0.5)}$ and $Kir_{(0.7)}$). For Kir, slope conductances of inward currents were 10.3, 20.3, and 27.9 pS, in 60, 120 and 200 mM $[K^+]_o$ respectively. Extracellular Ba^{2+} blocked the normally high channel activity in a concentration-dependent manner (K_A = 7.8 μM, −90 mV). In whole-cell recordings of inward rectifier K^+ current, marked voltage dependence of Ba^{2+} block over the physiological range of potentials was observed. We also examined current rectification. Following step depolarizations to voltages positive to E_K, outward currents through Kir channels were not observed even when the cytoplasmic face of excised patches were exposed to Mg^{2+}-free solution at pH 9.1. This was probably also true for $Kir_{(0.3)}$, $Kir_{(0.5)}$ and $Kir_{(0.7)}$ channels. We then examined the possibility of modulation of Kir channel activity and found neither ATP nor GTP-γS had any effect on Kir channel activity when added to the solution perfusing the cytoplasmic face of a patch. Kinetic analysis revealed Kir channels with a single open state (mean dwell time 72 msec) and two closed states (time constants 1.4, 79 msec). These results suggest that the native Kir channels of *Xenopus* myocytes have similar properties to the cloned strong inward rectifier K^+ channels, in terms of conductance, kinetics and barium block but does show some differences in the effects of modulators of channel activity. Furthermore, skeletal muscle may contain either different inward rectifier channels or a single-channel type which can exist in stable subconductance states.

Correspondence to: A.E. Spruce

Key words: Inward rectifier K^+ channel — *Xenopus*, — Myocyte — Blockers

Introduction

Anomalous (inward) rectification was first described by Katz (1949) in frog skeletal muscle. This referred to a K^+ current which activated upon hyperpolarization (to voltages more negative than E_K). Much work has since been carried out to characterize this current (reviews: Hagiwara & Jaffe, 1979; Hagiwara, 1983). The limited amount of information on the properties of single inward rectifier (Kir) channels present in skeletal muscle has come from recordings from mammalian myotubes (Ohmori, Yoshida & Hagiwara, 1981; Matsuda & Stanfield, 1989) as well as a recent report on these channels in *Xenopus* myocytes (Hancock, Moody-Corbett & Virgo, 1996). There has been a large resurgence of interest in Kir channels since the cloning of structurally similar, but physiologically distinct, strongly (Kir2.1/IRK1: Kubo et al., 1993*a*; Kir3.1/GIRK1: Kubo et al., 1993*b*) and weakly rectifying (Kir1.1/ROMK1: Ho et al., 1993) K^+ channels. Kir2.1 mRNA is present in skeletal muscle and almost certainly forms a channel which contributes to the inwardly rectifying K^+ conductance. Nevertheless, it is important to confirm that the properties of the heterologously expressed cloned channels correlate closely with those of the native Kir channels.

We have characterized single Kir channels (using either the cell-attached or inside-out patch configuration) in embryonic skeletal muscle cells of *Xenopus laevis*. We find that there are a number of distinct channels of varying conductance with inward rectifier characteristics. That with the largest conductance has been characterized most fully. It is present at high density and is very sensitive to block by μM concentrations of Ba^{2+}. Attempts to remove rectification were unsuccessful for

Table. Composition of solutions that are exposed to the cytoplasmic face of an excised patch

	Aspartic acid	KCl	KOH	$MgCl_2$	EGTA	EDTA	HEPES	CHES	pH
Control	80	20	100	2	5	0	10	0	7.4
Mg-free	80	20	110	0	5	5	10	0	7.4
Mg-free, pH 9.1	80	20	130	0	5	5	0	10	9.1

All concentrations are in mM.

all Kir channel types. Kinetic analysis was also carried out on patches containing only one channel.

Materials and Methods

Preparation

All experiments used myocytes isolated from *Xenopus laevis* embryos. These were obtained as described previously (Spruce & Moody, 1992). Briefly, adult females were injected with 1,000 i.u. human chorionic gonadotrophin and oocytes extruded the following day into 1 × MBS. A portion of a testis removed from a male (anaesthetized in MS-222 immediately prior to killing by brain destruction) was macerated in 1× MBS and injected over the eggs. After 5 min the dish was flooded with dechlorinated tap water and left for 20–30 min until the animal poles had rotated. Subsequently, but before gastrula, the jelly coats were removed using a 2% cysteine solution and the embryos rinsed with 0.1× MBS.

Embryonic dissections were performed either between stages 15 to 28 (Nieuwkoop & Faber, 1967) and left in culture overnight or between stages 29 to 40 and used on the same day. The protocol is as follows. The dorsal third of an embryo was excised and placed in Danilchik's solution containing 0.1% papain. The developing somites were isolated and transferred to Ca/Mg-free solution and after 15–20 min when the cells had dissociated they were aspirated using a pulled Pasteur pipette and gently plated on to tissue culture plastic (Falcon 3001) in Danilchik's solution. The cells were left for varying periods of time before patch clamp recordings were made.

Solutions

1× MBS (Gurdon, 1977) contained (in mM) 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 10 Hepes, pH 7.4 (with NaOH). Cysteine solution contained 2% cysteine titrated to pH 7.8 using NaOH. Danilchik's solution (Keller et al., 1985) contained (in mM) 53 NaCl, 15 $NaHCO_3$, 4.5 K.gluconate, 1 $MgSO_4$, 1 $CaCl_2$, 27 Na Isethionate, pH 8.3 (with Na_2CO_3). In single-channel experiments, the patch pipette (extracellular) solution was based upon the following composition (in mM): 60 KCl, 60 NaCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 Hepes, pH 7.4. In some experiments, either 120 or 200 mM KCl was used and NaCl omitted. The effects of Ba^{2+} upon channel activity was tested by adding 0.1 μM–1 mM $BaCl_2$ to the patch pipette solution. For excised, inside-out patch clamp recordings, the composition of the bath (≡intracellular) solution is given in the Table (control). For some excised patch recordings, various modifications were made as described in the Table and either applied to the cytoplasmic face of a patch via an adjacent spritz electrode (to which the patch electrode was moved—to within 25 μm) or exchanged for the control bath solution. In addition, for some experiments, 10 μM GTP-γS or 1 mM ATP were added to the control solution. For cell-attached patch experiments, the bath and pipette solutions both contained 120 mM K^+ producing a patch potential equal to the resting membrane potential of around 0 mV (Ernsberger & Spitzer, 1995).

For whole-cell recordings, the patch pipette solution contained: 80 K.Asp, 20 KCl, 2 $MgCl_2$, 3 Glucose, 2 Theophylline, 2 Na_2 ATP, 2 EGTA, 0.1 cAMP, 10 HEPES, pH 7.4 with KOH 5 mm TEACl was added to this solution to reduce the contribution from voltage-activated K^+ currents. During an experiment, the myocyte cultures were maintained in the 120 mM K^+ extracellular solution (*see above*). Recordings were also taken while spritzing the same solution with added $BaCl_2$ on to the cell. Corrections were not made for the junction potential between K.Asp and the extracellular solution (−9 mV; from Spruce & Moody, 1992).

Electrical Recording

Single-channel currents were measured using the patch-clamp technique in cell-attached and excised, inside-out modes. Electrodes were made from borosilicate glass and coated with sylgard. In the bath solution, their resistances were between 8 and 25 MΩ. Giga-ohm seals were routinely established on the embryonic myocytes. Currents were measured using a LIST L/M-PC amplifier, filtered at 1 kHz (8-pole Bessel) and acquired at 1–4 kHz with pCLAMP software (Axon Instruments). Where possible, current amplitudes were measured by generating amplitude histograms and fitting Gaussian distributions using a nonlinear, least squares curve-fitting method (pCLAMP). For records which were contaminated by smaller conductance channels, definite peaks in the histograms were obscured and current levels were measured using cursors and a number of values averaged. The open channel probability (P_{open}) was calculated as follows: Cursors were set at each current level and transitions between levels were defined by a digitized point crossing a point halfway between each cursor. When smaller conductance channels were present (or, rarely, when subconductance levels were reached), current transitions which were not transient were only counted if they were full amplitude. Thus, a distribution of times spent at each level is measured and P_{open} can be calculated from:

$$P_{\text{open}} = \frac{\sum_{j=1}^{N} j \cdot t_j}{T \cdot N} \tag{1}$$

where j is the number of channels open simultaneously, t_j is the time spent at level j, T is the total recording time and N is the number of channels in the patch (= maximum number of overlapping openings).

Open and closed time analysis was performed only on one patch with a single, full amplitude open current level. Transitions between levels were defined as described above and histograms were generated of the times spent at each level. Single or double exponential fits to the data were again obtained by a nonlinear least squares curve-fitting routine.

Whole-cell patch clamp recordings were carried out using an Axopatch 200A (Axon Instruments) amplifier. Current was filtered at 2 kHz and sampled at 2 kHz with pCLAMP software (Axon). Following achievement of the whole-cell configuration, series resistances of 3–5 MΩ were measured and electronic compensation was routinely applied (80%). Recordings were made before and after spritzing of a solution containing $BaCl_2$ on to the cell from an electrode positioned to within 100 μm. Leak subtraction was carried out digitally by extrapolation of the current response to a 10-mV hyperpolarizing step from +30 mV (the holding potential).

The experiments were carried out at room temperature (19–24°C). Unless otherwise indicated, results are expressed as mean ± SE.

Results

Characterization of Inwardly Rectifying K^+ Channels

In whole-cell recordings from myocytes isolated from *Xenopus laevis* embryos inward rectifier potassium current appears at about Stage 15 (Spruce & Moody, 1992). All cells contain this current after 8 hr and its density increases over 1–2 days in culture from Stage 15. Here, we characterize the properties of inward rectifier potassium channels which are likely to constitute a major proportion of the whole-cell current in the embryonic myocytes. Cell-attached and excised, inside-out patch clamp recordings were made between 17 and 50 hr after Stage 15. Under our standard conditions (excised patch; 120 mM K^+, 2 mM Mg^{2+} on either side of the membrane) most patches (85%; 135/158) contained inward rectifier K^+ channels. Although we find channels of different conductances (*see later*) we have characterized most fully the largest of these, which we notify simply as Kir. Figure 1 illustrates channel activity for the Kir channel. As can be seen, channel activity at negative voltages is characterized by a high open probability and long openings. An important test of the identity of this channel is to establish that currents rectify strongly around E_K (≈0 mV under our conditions). The presence of other channel types in the myocytes complicated the demonstration of this feature. At sustained positive voltages, similar channel activity was usually not seen (Fig. 1) although brief channel openings were occasionally observed (Fig. 5). We believe, however, that these are openings of voltage-activated K^+ channels (K_v-type) which have been described previously in these cells (Ernsberger & Spitzer, 1995). Furthermore, sustained channel activity at positive voltages was also observed in a few patches (*data not shown*) but this activity was always associated with openings at negative voltages characteristic of ATP-sensitive K^+ channels (Honoré & Lazdunski, 1995). A more rigorous test of rectification was carried out and will be described later.

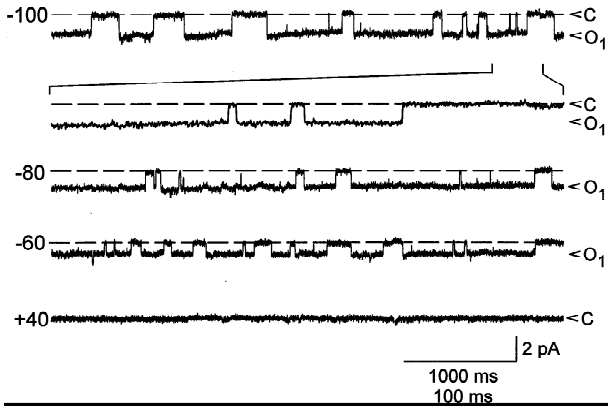

Fig. 1. Kir channel activity in excised patches. Unitary currents from an excised inside-out patch are shown under control conditions (120 mM K^+, 2 mM Mg^{2+} exposed to both sides of the membrane patch). The entire recording period is shown at four different voltages (indicated to the left of the traces). The dashed lines represent the closed level for the Kir channel, and the open level is indicated on the right. A portion of the recording at −100 mV is plotted on an expanded time scale (note different label for time scale bar). Filter 1 kHz; sample rate 4 kHz.

Mean current-voltage relations for the Kir channel, measured using 60, 120 and 200 mM $[K^+]_o$, are shown in Fig. 2*A*. Slope conductances were determined by regression fits to current-voltage relations generated from individual patches. The mean values are: 10.3 ± 0.8 pS (n = 6, 60 mM $[K^+]_o$); 20.3 ± 1.3 pS (n = 10, 120 mM $[K^+]_o$); 27.9 ± 2.4 pS (n = 5, 200 mM $[K^+]_o$. These values are plotted against extracellular K^+ concentration in Fig. 2*B*. The line is drawn according to a relation which assumes that conductance is dependent upon external K^+ concentration, to the power of 0.62; a value which is obtained from a mathematical fit to slope conductance measurements from individual patches. This compares favorably with values measured from macroscopic currents of 0.47 for Kir2.1 (Kubo et al., 1993*a*) and 0.55 for Kir current measured in a leukemia cell line (Wischmeyer, Lentes & Karschin, 1995).

Channel Block by Extracellular Ba^{2+}

Barium (Ba^{2+}) ions block inward rectifier K^+ current in skeletal muscle with high potency (Standen & Stanfield, 1978; Spruce & Moody, 1992). Ohmori et al. (1981) have described Ba^+ block of single Kir channels in cultured myotubes, although channel closures were not observed in the absence of blocking ions. In contrast, the present results and those of Matsuda & Stanfield (1989) show that channel blockers are not required to induce gating of the muscle Kir channel. The effect of external Ba^{2+} upon channel activity in *Xenopus* myocytes is il-

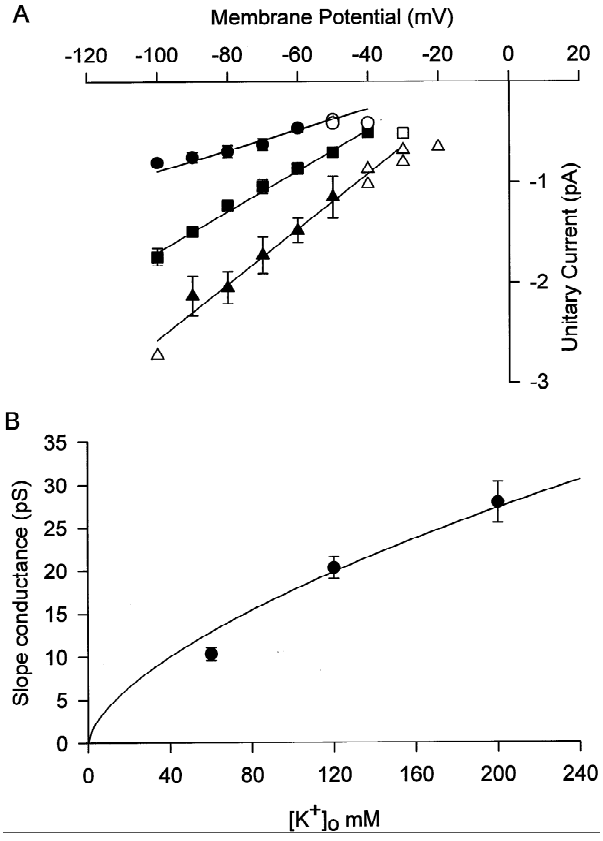

Fig. 2. K^+ dependence of current through Kir channels. (*A*) Current-voltage relations are shown for three different $[K^+]_o$: 60 mM (circles), 120 mM (squares), and 200 mM (triangles). Filled symbols represent mean values (n = 3–15) and open symbols are individual values. For each $[K^+]_o$, the lines are calculated using the average values of slope and intercept generated by regression fits to individual current-voltage relations. (*B*) Dependence of slope conductance upon extracellular $[K^+]$. Means and standard errors of slope conductances measured in individual patches are plotted for each K^+ concentration (n = 5–10). The line is a least squares fit to the individual values using the equation, $g = [K^+]_o^b$, from which a value of 0.62 is obtained.

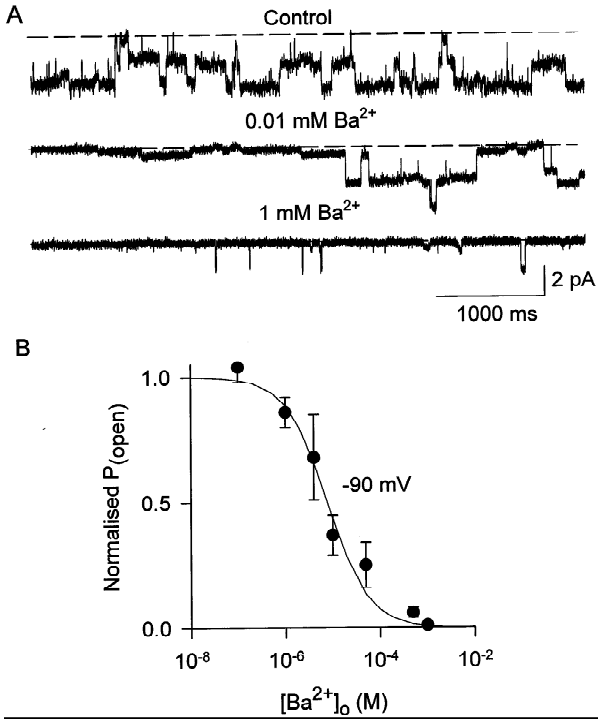

Fig. 3. Ba^+ block of Kir channel activity. (*A*) The traces plot current recordings at −90 mV from different patches using the indicated pipette $[Ba^{2+}]$. In addition to Kir channels, the activity of channels with smaller current amplitudes is evident, particularly in 0.01 mM $Ba^{2+} \cdot [K^+]_o$ = $[K^+]_i$ = 120 mM; Filter 1 kHz; Sample rate 4 kHz. (*B*) The effect of extracellular Ba^{2+} on normalized $P_{(open)}$, at −90 mV. Standard error bars are shown (n = 3–7). The line is obtained by a least squares fit to Eq. (2).

lustrated in Fig. 3*A*. Openings are virtually completely abolished in 0.5 or 1 mM Ba^{2+}, agreeing with data from whole-cell recordings (Spruce & Moody, 1992). Because Ba^{2+} is added to the pipette solution and this solution could not be exchanged during a recording, quantitative analysis of the block relies on calculation of the normalized channel opening probability: $P_{open}(Ba)$/mean(P_{open}(Control)). The dependence of the normalized P_{open} upon $[Ba^{2+}]_o$ at −90 is shown (Fig. 3*B*). The line represents a fit to the data points using a derivation of the Langmuir equation:

$$P_{open} = 1/(1 + [Ba^{2+}]_o/K_A) \quad (2)$$

which assumes 1:1 binding of Ba^{2+} to the channel with equilibrium constant, K_A. A value for K_A of 7.8 μM at −90 mV is obtained. Hagiwara et al. (1978) studied the block of inward rectifier K^+ current (I_{IR}) by Ba^{2+} ions in starfish eggs and deduced that K_A varied with voltage according to the following (simplified) equation:

$$K_A(V) = K_A(0).\exp(V/k) \quad (3)$$

where $K_A(0)$ is the equilibrium constant at 0 mV and k describes the degree of voltage dependency. Using the values assigned to these parameters by Hagiwara et al. (1978) and also those given by Standen and Stanfield (1978) for Ba^{2+} block of I_{IR} in adult frog muscle, K_A at −90 mV is calculated to be around 4–5 μM, close to the value determined in this single-channel study. It was found, however, by analysis of the block of unitary currents, that, at −50 mV, K_A was estimated to be 11.2 μM, indicating only a slight degree of voltage dependency. The above studies would predict a ninefold difference in K_A between −90 and −50 mV. The lack of variation in K_A may result from the indirect method of estimating the effect of Ba^{2+} using normalized P_{open} measurements. Therefore, experiments to examine the effect of Ba^{2+} block of whole-cell currents were carried out.

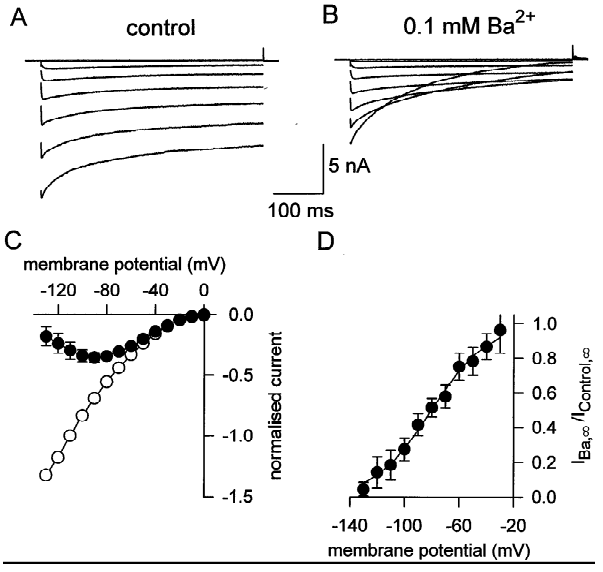

Fig. 4. Effect of Ba^{2+} on whole-cell inward rectifier K^+ current. (*A*) The set of current traces are recordings from a myocyte exposed to a bath solution containing 120 mM K^+. The recording pipette solution contains 5 mM TEA^{2+}. The cell was held at +30 mV and step hyperpolarizations were applied lasting for 450 msec. The traces shown are those recorded at 10, −10, −30, −50, −70, −90, −110 and −130 mV. (*B*) A recording from the same cell as in (*A*) while the bath solution with added 0.1 mM $BaCl_2$ was spritzed on to the cell. The traces were recorded at the same potentials as in (*A*). Filter 2 kHz; sample rate, 2 kHz. (*C*) Mean current normalized to the value at −110 mV is plotted against voltage. Open symbols: control recordings (n = 3–5). Closed symbols: 0.1 mM $BaCl_2$ (n = 5 for all values). Where standard error bars are not seen, they are smaller than the size of the symbol. (*D*) The fractional current which remains in Ba^{2+} is averaged for all cells at each voltage and plotted against voltage (n = 3–5). Standard error bars are shown. Because the full extent of Ba^{2+} block did not seem to be achieved by the end of the voltage pulse, particularly at more extreme voltages, the steady-state block was estimated by fitting exponentials to the declining current traces (control as well as Ba^{2+} recordings) and using the asymptote as the final current value ($I_{Ba,\infty}$,$I_{control,\infty}$). The line is a fit to an equation described in the text using a least squares procedure.

To simulate the single-channel conditions, I_{IR} was measured using 120 mM extracellular and intracellular K^+ and the degree of block analyzed over a voltage range which includes −50 and −90 mV. Figure 4*A* and *B* illustrate the effect of 0.1 mM extracellular Ba^{2+} applied from a spritzing electrode upon current activated by hyperpolarization in an individual myocyte. The holding potential is +30 mV. The declining currents traces in Fig. 4*B* illustrate that the block is not instantaneous but increases during the voltage step (Standen & Stanfield, 1978). K^+ current through voltage-activated channels (I_K) was blocked using 5 mM TEA^+ in the recording pipette solution. In test recordings (1 mM extracellular Ba^{2+} was used to block I_{IR}), little I_K remained following step depolarizations when TEA^+ was present although complete block was not observed even when internal TEA^+ concentration was increased up to 40 mM (*data not shown*). Nevertheless, four arguments suggest that there is little error in assessing the voltage dependence of Ba^{2+} block of I_{IR} under these conditions: (i) At sustained positive voltages in single-channel recordings, the open probability of voltage-activated channels was negligible (e.g., Figs. 1 and 5); (ii) Using the same voltage protocol as in Fig. 4 with internal TEA^+ and 1 mM external Ba^{2+}, the current-voltage relations for the non-leak-subtracted recordings did not show outward rectification, indicating an insignificant amount of I_K. (iii) When inactivated I_K is allowed to recover at hyperpolarized voltages, no current is seen upon subsequent depolarization in the presence of intracellular TEA^+ (*see* tail currents in Fig. 4*A*); (iv) Evaluation of the effect of voltage upon Ba^{2+} block relies on measurement of the block largely over a voltage range where I_K is not activated. The normalized current-voltage relations in Fig. 4*C* shows the effect of 0.1 mM Ba^{2+} on mean I_{IR} remaining at the end of the voltage step. It is clear that the blocking effect of Ba^{2+} is increased at more negative voltages. This was quantified by measuring, for each cell, the fractional current which remains in Ba^{2+} at each voltage (Fig. 4*D*). The line in Fig. 4*D* is drawn using Eq. (2) following substitution of K_A by the relationship given in Eq. (3), assuming k is 20.6 mV. This compares very favorably with values of 16.8 mV from Standen and Stanfield (1978) and 19.5 mV from Hagiwara et al. (1978). Therefore, the block of I_{IR} by Ba^{2+} in embryonic myocytes is indeed voltage dependent.

Investigation of Modulation of Channel Activity

In excised patches using 120 mM $[K^+]_o$, P_{open} of Kir channels at −90 mV was 0.67 ± 0.02 (n = 42; patches with too many channels are excluded). We examined whether this high channel activity could be modulated. Channel activity was not dependent on voltage (P_{open} at −50 mV: 0.72 ± 0.04; n = 15; $P > 0.7$), agreeing with previous results demonstrating a lack of intrinsic voltage-dependent gating for steady-state currents (Standen & Stanfield, 1979; Matsuda & Stanfield, 1989). Further, we have not found evidence for modulation of Kir channel function by cytoplasmic factors. Thus, in cell-attached patches, channel behavior appeared to be very similar to that in excised patches (Fig. 5; the brief openings seen at +40 mV probably reflect activity of K_v channels—*see above*). Indeed, P_{open} at −90 mV was not significantly different (0.65 ± 0.04; n = 11; $P > 0.6$). In contrast, rundown of channel activity in excised patches has been described for Kir2.1 channels expressed in oocytes (Kubo et al., 1993*a*; Fakler et al., 1994) as well as for Kir channels in a tumor mast cell line (Wischmeyer et al., 1995). Potential modulators of channel behavior were tested but an intensive investigation of these factors

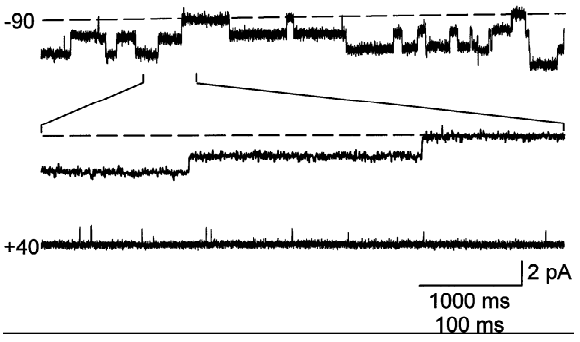

Fig. 5. Kir channel activity in cell-attached patches. The pipette and bath solutions both contain 120 mM K^+ and the patch potential is assumed to be close to the potential applied via the pipette (given to the left of the traces; *see* Materials and Methods). An extract of the recording at −90 mV is shown (note different label for time scale bar). For traces at −90 mV, the dashed lines indicate closed levels and channels open downwards. At +40 mV, only brief channel openings (upward deflections) are seen. Filter 1 kHz; Sample rate 4 kHz.

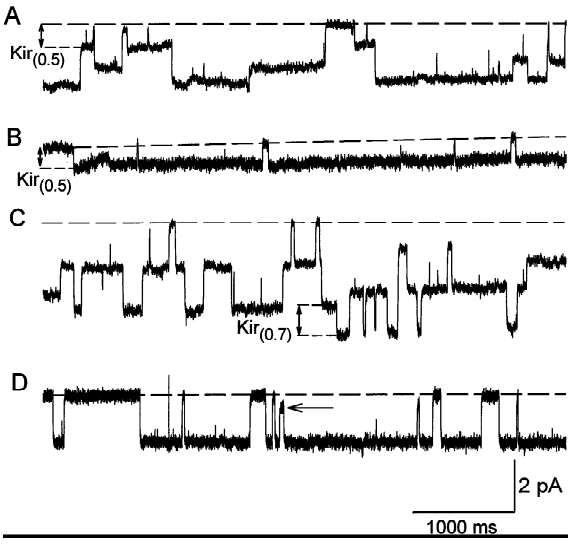

Fig. 6. Other Kir channel types. Channel openings of smaller current amplitudes than the Kir channel are seen frequently. Because of the large range of current amplitudes, they were categorized according to their fractional size, *f*, in comparison to the mean Kir current amplitude at −90 mV (−1.5 pA). Three classes were defined by this method ($0.2 < f < 0.4$; $0.4 < f < 0.6$; $0.6 < f < 0.8$), although more could not be excluded. They were named according to the mean values: $Kir_{(0.3)}$; $Kir_{(0.5)}$; $Kir_{(0.7)}$. (*A*) Patch containing one Kir channel and also a second channel assigned to the $Kir_{(0.5)}$ category. Filter 1 kHz; Sample rate 2 kHz. (*B*) A rare example of the activity of a smaller conductance Kir channel in isolation in a patch. In this case, $Ker_{(0.5)}$. Filter 1 kHz; Sample rate 4 kHz. (*C*) Two Kir channels and one assigned to the category of $Kir_{(0.7)}$ are operating in this patch. Filter 1 kHz; Sample rate 2 kHz. (*D*) A rare example of a subconductance state of the Kir channel (arrowed). Filter 1 kHz; Sample rate 4 kHz. All recordings are made at −90 mV and are from excised, inside-out patches using $[K^+]_o = [K^+]_i = 120$ mM.

was not carried out. The cytoplasmic face of excised patches was exposed to either 1 mM ATP or 10 μM GTP-γS. Channel activity was not affected: P_{open}(ATP) $= 0.67 \pm 0.08$ ($n = 4$; −90 mV); P_{open}(GTP-γS) $= 0.74 \pm 0.08$ ($n = 4$; −90 mV) (*data not shown*). These compounds were also found to be ineffective in modulating the activity of single Kir2.1 channels in excised oocyte patches (Kubo et al., 1993*a*) but, in a later study, using macropatches, an ATPase-dependent process was suggested to restore Kir2.1 channel activity following rundown (Fakler et al., 1994).

Other Inward Rectifier Channel Species

The data so far have described the properties of a Kir channel that is present most abundantly in the patches and also conducts the largest current. We have identified at least three other smaller current levels which display features of inward rectifier K^+ channels (long duration openings at negative voltages and no outward currents at positive voltages). The smaller current levels were categorized by their current amplitudes at −90 mV and named according to the fraction of the amplitude of the largest Kir channel; $Kir_{(0.3)}$, $Kir_{(0.5)}$ and $Kir_{(0.7)}$ (*see* legend to Fig. 6). The activity of $Kir_{(0.5)}$ is shown in Fig. 6*A* and *B* and that of $Kir_{(0.7)}$ in Fig. 6*C* (Fig. 3 shows the activity of $Kir_{(0.3)}$). All Kir channel subtypes were present in substantial numbers of patches ($Kir_{(0.3)}$: 45%; $Kir_{(0.5)}$: 27%; $Kir_{(0.7)}$: 7.8%). A recent study describes only two Kir channel types in *Xenopus* myocytes, however (Hancock et al., 1996), as was also found in mammalian myotubes (Matsuda & Stanfield, 1989). Virtually all of the smaller current transitions exhibited the features of channels which are gated independently, i.e., the current level was not a transitory state between open and closed levels of a Kir channel (usual recording period ≈5 sec). For example, Fig. 6*C* shows two Kir channels (same amplitude as recordings containing only one Kir channel—Fig. 8) plus one $Kir_{(0.7)}$ channel. It is possible, however, that the channel behavior could be explained by the Kir channel entering very stable subconductance states. A few recordings ($n = 4$) showed the Kir channel closing to an obvious subconductance level (Fig. 6*D*), but these rare events were much briefer than the usual sublevel open duration (lasting up to 2–3 sec) and were reached only from the full open state. Kir channels in mammalian myotubes exhibit frequent subconductance levels (Matsuda & Stanfield, 1989) but again they are much more clearly defined as such by their brief durations.

Rectification in Native Kir Channels

Investigations of the mechanism underlying inward rectification in cloned Kir channels have concluded that

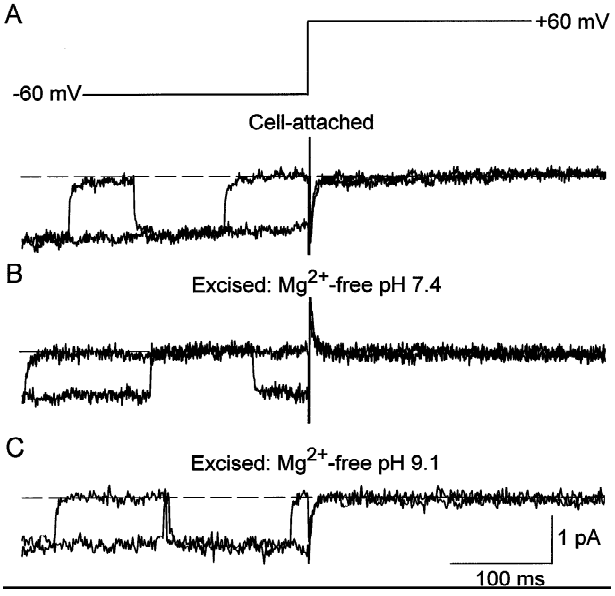

Fig. 7. Investigation of Kir channel rectification. Repeated step voltage pulses (schematic voltage record in (*A*)) were applied to excised patches under three different conditions and two overlying traces are shown in each case (extracellular $[K^+] = 120$ mM). (*A*) Cell-attached patch. The bath solution contains K^+ at 120 mM. (*B*) Excised inside-out patch exposed to Mg^{2+}-free solution (*see* Materials and Methods for composition). $[K^+]_i = 130$ mM (calculated $E_K = -2$ mV). (*C*) Excised inside-out patch exposed to Mg^{2+}-free solution at pH = 9.1. $[K^+]_i = 150$ mM (calculated $E_K = -6$ mV). All traces have been leak subtracted (by digital means; assuming linear leak) and the dashed line indicates the closed level. Channel openings are seen as downward deflections at −60 mV. Note that when the channel is open immediately before the step, no opening is seen at the positive voltage. Capacity currents could not be subtracted because of the high level of channel activity. Scale bars in (*C*) apply to all traces. Filter 1 kHz; Sample rate 2 kHz in (*A*) and (*B*) and 1 kHz in (*C*).

rectification can be fully explained by channel block by intracellular Mg^{2+} and polyamines (Lopatin, Makhina & Nichols, 1994; Fakler *et al.*, 1995). We have examined rectification of the native skeletal muscle Kir channels using step depolarizations (Fig. 7). Outward currents at +60 mV which could be correlated with openings of any of Kir channels at −60 mV were never seen. In many patches the opening of voltage-activated K^+ channels complicated this analysis. Nevertheless, strong rectification of Kir channels was confirmed by comparing, in turn, all non-leak subtracted traces with each other. A change in the number of open Kir channels immediately before the step was never associated with any change in the current level immediately afterwards. Each part of Fig. 7 illustrates this feature using only two overlying leak subtracted traces for ease of comparison. A number of different recording conditions were used for these experiments: Cell-attached patches (Fig. 7*A*); Excised, inside-out patches exposed to Mg^{2+}-free solution at pH 7.4 (Fig. 7*B*; representative of 4 patches); Excised patches exposed to Mg^{2+}-free solution at pH 9.1 (Fig. 7*C*; representative of 10 patches). Polyamines are basic compounds and their potency is related to the number of charges they carry (Lopatin, Makhina & Nichols, 1995) which would be neutralized at high pH. Indeed, rectification of cloned channels has been shown to be markedly reduced under alkaline conditions (Lopatin, Makhina & Nichols, 1994). Therefore, it was surprising that we did not find loss of rectification of Kir channels under this condition. We do not suggest, however, that these data dispute the accepted explanation for the mechanism of rectification identified for cloned channels, rather it is more likely that they illustrate the difficulty in removing polyamines in our recordings (*see* Discussion).

Gating Kinetics

Patches containing single Kir channels were seen infrequently (8%). Figure 8*A* illustrates channel activity at −90 mV from one such patch. In fact, this is the only patch on which kinetic analysis could be performed (long enough recording period and no contaminating channels). Open- and closed-time histograms are illustrated in Fig. 8*B*. The line drawn for the open time histogram was fitted by a single exponential function, indicating a single open state of mean duration 71 msec. Closed times were fitted by a double exponential function of time constants, 1.4 and 79 msec. It was noted that channel activity would often disappear for periods of minutes before reopening, implying that the channel also entered an additional, more stable closed state.

Discussion

We find that the surface membrane of *Xenopus* embryonic skeletal myocytes contain large numbers of inward rectifier K^+ channels 1–2 days after Stage 15. Matsuda & Stanfield (1989) estimated Kir channel density to be 0.09–0.36/μm^2 in mammalian myotubes. Since we observed channels in 6 times as many patches (using similar size electrodes) in some of which they were too numerous to count, the above range is an underestimate for *Xenopus* myocytes and may be much closer to the density calculated from fluctuation analysis of 4/μm^2 in frog skeletal muscle (Schwarz, Neumcke & Palade, 1981). This comparison, however, does not take into account the location of the channels. In adult muscle the majority of Kir channels are assumed to reside in the transverse tubular system (Standen & Stanfield, 1979; Ashcroft, Heiny & Vergara, 1985). Nevertheless, at early developmental times, when the myocytes are morphologically undifferentiated, they contain inward rectifier K^+ current (Moody-Corbett & Gilbert, 1990; Spruce & Moody, 1992). In fact, this study demonstrates that, even at later

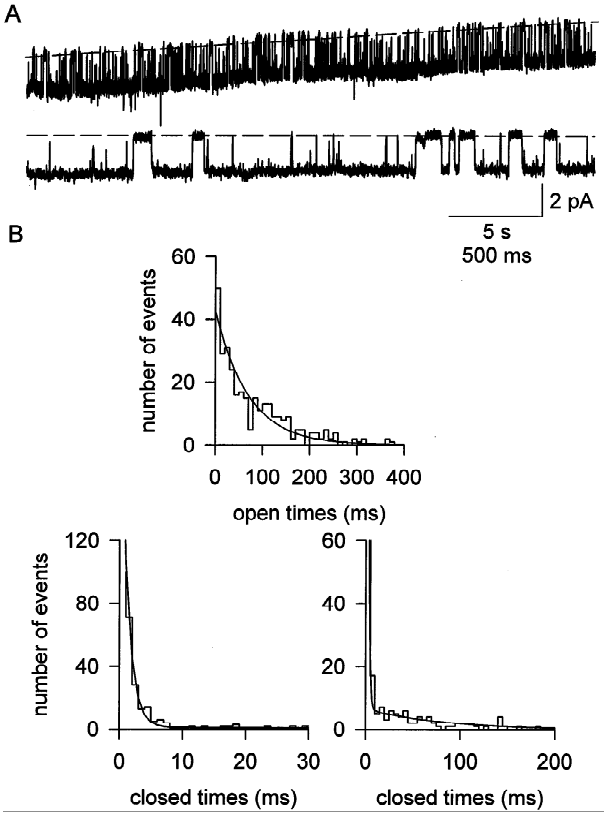

Fig. 8. Kinetic analysis of Kir channel activity. (*A*) The top trace plots the full recording at −90 mV of a patch containing a single Kir channel. An extract of this activity is shown below (note different label for time scale bar). Filter 1 kHz; Sample rate 2 kHz. (*B*) Histograms of open and closed times for the channel activity shown in (*A*). The open time histogram was formed using 10 msec bins. The line is a fit to the equation, $A.\exp(-t/\tau_o)$, where $A = 43$ and $\tau_o = 71$ msec. The closed time histograms are plotted using bin widths of 1 and 5 msec (left and right, respectively). The lines are a fit to the equation, $A.\exp(-t/\tau_{c1}) + B.\exp(-t/\tau_{c2})$, where $A = 169$ or 843 (left and right histograms), $B = 1$ or 7, $\tau_{c1} = 1.4$ msec and $\tau_{c2} = 79$ msec.

times in development, when the *Xenopus* myocytes contain a functional T-system (they are striated and can be stimulated to contract by K^+-depolarization), large numbers of channels reside in the surface membrane. Whole-cell recordings at similar developmental times, however, suggest that the Kir channels are now also present in the T-system (Moody-Corbett & Gilbert, 1990). Together, these results would imply that during embryonic development Kir channels are inserted in the surface membrane first and located to the T-system later. The functional significance of the surface membrane location of Kir channels may be to more accurately stabilize the resting membrane potential, particularly in the region of the developing neuromuscular junction, at times when it would otherwise fluctuate markedly due to changing levels of expression of other ion channel types and electrogenic pumps.

The characteristics of the Kir channels with the largest conductance in *Xenopus* myocytes show many features in common with those found in mammalian myotubes (Matsuda & Stanfield, 1989): (i) openings of long duration at voltages negative to E_K and the absence of outward current at positive voltages; (ii) similar conductances (*Xenopus* myocytes; 20 pS in 120 mM $[K^+]_o$: mammalian myotubes; 21 pS in 155 mM $[K^+]_o$; (iii) lack of voltage dependence of steady-state channel opening probability. Furthermore, skeletal muscle Kir channels and those strongly rectifying K^+ channels from other tissues are also alike: e.g., astrocytes (Ranson & Sontheimer, 1995) and a leukaemia cell line (Wischmeyer et al., 1995). Significantly, the conductance and long open times of the cloned inward rectifier channel, Kir2.1, closely resemble the present results also (Kubo et al., 1993*a*). Nevertheless, there is an important difference between our results and those of other studies.

In preliminary experiments, we have not found evidence for modulation of Kir channel function. Thus, the open state probability is not different comparing cell-attached and excised, inside-out patches. Further, exposure of the cytoplasmic face of an excised patch to a solution containing either ATP and Mg^{2+} or GTP-γS has no significant effect on P_{open}. In contrast, in a basophilic leukaemia cell line, the activity of Kir channels which rundown after patch excision can be restored by ATP and, in addition, loading of cells with GTP-γS causes loss of channel activity in cell-attached patches (Wischmeyer et al., 1995). Similarly, GTP-γS produces inhibition of whole-cell inward rectifier K^+ currents in a differentiating mammalian myoblast cell line (Wieland & Gong, 1995). The activity of cloned channels can also be modulated. Thus, excision of a patch containing heterologously expressed Kir2.1 channels leads to rundown of activity, restored by an ATPase-dependent process as well as by kinase-dependent phosphorylation (Fakler et al., 1994). The explanation for the difference between our results and these other studies may be that retaining the association of Kir channels with proteins, including enzymes and other biochemical modulators either in macropatches or in intact cells is necessary to observe modulation. Support for this hypothesis is provided by experiments which showed lack of biochemical modulation of single Kir2.1 channel activity in excised oocyte membranes (Kubo et al., 1993*a*). Future experiments will investigate the modulation of whole-cell current in the myocytes. It is also possible that the activity of the smaller conductance Kir channels may be modulated. We have not been able to quantify this because of difficulty in measuring P_{open} for these channels.

Inward rectifier K^+ current in skeletal muscle is blocked with high potency by Ba^{2+} (Standen & Stanfield, 1978). We have characterized this block of the single channels revealing that it acts on a slow time scale, binds

with 1:1 stoichiometry and with a dissociation constant of 8 μM at −90 mV, agreeing well with the results of Standen and Stanfield (1978). Whole-cell recordings were used to establish the voltage dependency of Ba^{2+} block of inward rectifier K^+ current (I_{IR}) in the embryonic myocytes. The degree of block was increased substantially by hyperpolarization, changing e-fold every 21 mV, similar to results in adult frog muscle (Standen & Stanfield, 1978). For the cloned Kir2.1 channels expressed in oocytes, voltage dependent block of macroscopic currents by Ba^{2+} is also evident (Kubo et al., 1993*a*).

Recordings from the *Xenopus* myocytes can be interpreted to suggest the presence of three additional, lower conductance, Kir channels with otherwise similar features to the Kir channel characterized most fully here. There is good evidence from other sources to favor this. Thus, in addition to Kir2.1, other members of the Kir2.0 channel gene family have been cloned. Interestingly, mRNA for Kir2.3 channels whose single channel conductance is about half that of Kir2.1 has been identified in skeletal muscle (HIR; Périer, Radeke & Vandenberg, 1994). In addition, skeletal muscle also contains mRNA for Kir2.2, although the conductance of the expressed channels is greater than that of Kir2.1 (Takhashi et al., 1994). The possibility of forming heteromultimeric channels with intermediate conductances using any of the expressed genes clearly exists. Furthermore, native Kir channels with different conductances have been described in mammalian myotubes (Matsuda & Stanfield, 1989) and, notably, *Xenopus* myocytes (Hancock et al., 1996), although a total of only two separate Kir channels were found in each study. An alternative explanation of these data is that each current level actually represents a highly stable subconductance state of a single Kir channel protein. In fact, both the number and relative amplitude of these openings compared to the ''full amplitude'' Kir channel are the same as for the subconductance states of the Kir channel present in mammalian myotubes (Matsuda & Stanfield, 1989). Cardiac inward rectifier K^+ channels have been shown to exist in four substates (Mazzanti et al., 1996), a finding which the authors suggest is compatible with the proposed tetrameric structure of inward rectifier K^+ channels (Yang, Jan & Jan, 1995). Finally, cloned Kir2.1 channels also exhibit substates, though only three have been identified (Davies et al., 1996).

There is now abundant evidence to show that rectification in cloned Kir2.1 (and other) channels is entirely explained by block by internal Mg^{2+} (e.g., Stanfield et al., 1994; Taglialatela et al., 1994) and polyamines (e.g., Ficker et al., 1994; Lopatin et al., 1994). It is important to assess whether this is also true for native strong inward rectifiers. Studies of heterologously expressed cloned channels may suggest that rectification can be relatively easily removed by perfusion of the excised face of a membrane patch with Mg^{2+}-free solution. In our experiments on native Kir channels, the results did not conform to this expectation. For all channels (Kir and smaller channels), not even perfusion of the patch with alkaline pH solution (to neutralize the charges on the polyamines) removes rectification. Although Kir2.1 channels have recently been proposed to contain an intrinsic gating mechanism in addition to that caused by polyamine and Mg^{2+} block (Aleksandrov et al., 1996), we would still have expected to see outward currents in the absence of blocking compounds. The simplest explanation is that it is very difficult to remove the ''sticky'' polyamines under our conditions. When outward currents through single cloned Kir channels have been described previously, the range of resistances of the patch electrodes in these experiments, where noted (Wible et al., 1994), is of the order of five times lower than those used in our recordings. Therefore, for compounds which ''stick'' easily to membranes, the small electrodes and the amount by which the membrane has been drawn inside (seals generally took a number of seconds to form) will be crucial factors in restricting their removal. Nevertheless, we do not expect that more mobile compounds (such as GTP-γS) would be prevented from accessing the cytoplasmic surface. Channels within membrane patches excised from muscle using similar sized electrodes can be easily modulated by chemicals applied via a perfusing solution (Spruce, Standen & Stanfield, 1987).

The support of the Wellcome Trust is gratefully acknowledged.

References

Aleksandrov, A., Velimirovic, B., Clapham, D.E. 1996. Inward rectification of the IRK1 K^+ channel reconstituted in lipid bilayers. *Biophys. J.* **70:**2680–2687

Ashcroft, F.M., Heiny, J.A., Vergara, J. 1985. Inward rectification in the transverse tubular system of frog skeletal muscle studied with potentiometric dyes. *J. Physiol.* **259:**269–291

Davies, N.W., Shelton, P.A., Conley, E.C., Stanfield, P.R. 1996. Subconductance states of the inward rectifier Kir2.1 expressed in murine erythroleukaemia (MEL) cells. *J. Physiol.* **495.P:**89P

Ernsberger, U., Spitzer, N.C. 1995. Convertible modes of inactivation of potassium channels in *Xenopus* myocytes differentiating in vitro. *J. Physiol.* **484:**313–329

Fakler, B., Brändle, U., Glowatzki, E., Zenner, H.-P., Ruppersberg, J.P. 1994. $K_{ir}2.1$ inward rectifier K^+ channels are regulated independently by protein kinases and ATP hydrolysis. *Neuron* **13:**1413–1420

Ficker, E., Taglialatela, M., Wible, B. A., Henley, C. M., Brown, A.M. 1994. Spermine and spermidine as gating molecules for inward rectifier K^+ channels. *Science* **266:**1068–1072

Gurdon, J.B. 1977. Methods for nuclear transplantation in amphibia. *Methods Cell Biol.* **16:**125–139

Hagiwara, S. 1983. Membrane potential-dependent ion channels in cell membrane. Phylogenetic and developmental approaches. pp [4, 5, 14, 20] Raven Press, New York

Hagiwara, S., Jaffe, L.A. 1979. Electrical properties of egg cell membranes. *Annu. Rev. Biophys. Bioeng.* **8:**385–416

Hagiwara, S., Miyazaki, S., Moody, W., Patlak, J. 1978. Blocking effects of barium and hydrogen ions on the potassium current during anomalous rectification in the starfish egg. *J. Physiol.* **279:**167–185

Hancock, S., Moody-Corbett, F.L., Virgo, N.S. 1996. Potassium inward rectifier and acetylcholine receptor channels in embryonic *Xenopus* muscle cells in culture. *J. Neurobiol.* **29:**354–366

Ho, K., Nichols, C.G., Lederer, W.J., Lytton, J., Vassilev, P.M., Kanazirska, M.V., Hebert, S.C. 1993. Cloning and expression of an inwardly rectifying ATP-regulated potassium channel. *Nature* **362:**31–38

Honoré, E., Lazdunski, M. 1995. Biophysical, pharmacological and developmental properties of ATP-sensitive K^+ channels in cultured myotomal muscle cells from *Xenopus* embryos. *Pfluegers Arch.* **429:**607–616

Katz, B. 1949. Les consantes électriques de la membrnae du muscle. *Arch. Sci. Physiol.* **3:**285–299

Keller, R.E., Danilchik, M., Gimlich, R., Shih, J. 1985. The function and mechanism of convergent extension during gastrulation of *Xenopus laevis. J. Embryol. Exp. Morphol.* **89**(Suppl.):185–209

Kubo, Y., Baldwin, T.J., Jan, Y.N., Jan, L.Y. 1993*a*. Primary structure and functional expression of a mouse inward rectifier potassium channel. *Nature* **362:**127–133

Kubo, Y., Reuveny, E., Slesinger, P.A., Jan, Y.N., Jan, L.Y. 1993*b*. Primary structure and functional expression of a rat G-protein-coupled muscarinic potassium channel. *Nature* **364:**802–806

Lopatin, A., Makhina, E.N., Nichols, C.G. 1994. Potassium channel block by cytoplasmic polyamines as the mechanism of intrinsic rectification. *Nature* **372:**366–372

Lopatin, A., Makhina, E.N., Nichols, C.G. 1995. The mechanism of inward rectification of potassium channels: ''Long-pore plugging'' by cytoplasmic polyamines. *J. Gen. Physiol.* **106:**923–955

Matsuda, H., Stanfield, P.R. 1989. Single inwardly rectifying potassium channels in cultured muscle cells from rat and mouse. *J. Physiol.* **414:**111–124

Mazzanti, M., Assandri, R., Ferroni, A., DiFrancesco, D. 1996. Cytoskeletal control of rectification and expression of four substates in cardiac inward rectifier K^+ channels. *FASEB J.* **10:**357–361

Moody-Corbett, F., Gilbert, R. 1990. Inward rectifier potassium current on embryonic *Xenopus* muscle cells at different times in culture. *Dev. Br. Res.* **55:**139–142

Nieuwkoop, P.D., Faber, J. 1967. Normal table of *Xenopus laevis* (Daudin). Amsterdam, North-Holland

Ohmori, H., Yoshida, S., Hagiwara, S. 1981. Single K^+ channel currents of anomalous rectification in cultured rat myotubes. *Proc. Natl. Acad. Sci. USA* **78:**4960–4964

Périer, F., Radeke, C.M., Vandenberg, C.A. 1994. Primary structure and characterization of a small-conductance inwardly rectifying potassium channel from human hippocampus. *Proc. Natl. Acad. Sci. USA* **91:**6240–6244

Ranson, C., Sontheimer, H. 1995. Biophysical and pharmacological characterisation of inwardly rectifying K^+ currents in rat spinal cord astrocytes. *J Neurophysiol.* **73:**333–346

Schwarz, W., Neumcke, B., Palade, P.T. 1981. K-current fluctuations in inward-rectifying channels of frog skeletal muscle. *J. Membrane Biol.* **63:**85–92

Spruce, A.E., Moody, W.J. 1992. Developmental sequence of expression of voltage-dependent currents in embryonic *Xenopus laevis* myocytes. *Dev. Biol.* **154:**11–22

Spruce, A.E., Standen, N.B., Stanfield, P.R. 1987. Studies of the unitary properties of adenosine-5′-triphosphate-regulated potassium channels of frog skeletal muscle. *J. Physiol.* **382:**213–236

Standen, N.B., Stanfield, P.R. 1978. A potential and time-dependent blockade of inward rectification in frog skeletal muscle fibres by barium and strontium ions. *J. Physiol.* **280:**169–191

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

Stanfield, P.R., Davies, N.W., Shelton, P.A., Khan, I.A., Brammar, W.J., Standen, N.B., Conley, E.C. 1994. The intrinsic gating of inward rectifier K^+ channels expressed from the murine IRK1 gene depends on voltage, K^+ and Mg^{2+}. *J. Physiol.* **475:**1–7

Taglialatela, M., Ficker, E., Wible, B.A., Brown, A.M. 1994. C-terminus determinants for Mg^{2+} and polyamine block of the inward rectifier K^+ channel IRK1. *EMBO J.* **14:**5532–5541

Takahashi, N., Morishige, K., Jahangir, A., Yamada, M., Findlay, I., Kurachi, Y. 1994. Molecular cloning and functional expression of cDNA encoding a second class of inward rectifier potassium channels in the mouse brain. *J. Biol. Chem.* **269:**23274–23279

Wible, B.A., Taglialatela, M., Ficker, E., Brown, A.M. 1994. Gating of inwardly rectifying K^+ channels localized to a single negatively charged residue. *Nature* **371:**246–249

Wieland, S.J., Gong, Q.-H. 1995. Modulation of a potassium conductance in developing skeletal muscle. *Am. J. Physiol. Cell Physiol.* **268:**C490–C495

Wischmeyer, E., Lentes, K.-U., Karschin, A. 1995. Physiological and molecular characterisation of an IRK-type inward rectifier K^+ channel in a tumour mast cell line. *Pfluegers Arch.* **429:**809–819

Yang, J., Jan, Y.N., Jan, L.Y. 1995. Determination of the subunit stoichiometry of an inwardly rectifying potassium channel. *Neuron* **15:**1441–1447