Mechanisms and residues responsible for slow activation and Ba$^{2+}$ block of the cardiac muscarinic K$^+$ channel, Kir3.1/Kir3.4, were investigated using site-directed mutagenesis. Mutagenesis of negatively charged residues located throughout the pore of the channel (in H5, M2, and proximal C terminus) reduced or abolished slow activation. The strongest effects resulted from mutagenesis of residues in H5 close to the selectivity filter; mutagenesis of residues in M2 and proximal C terminus equivalent to those identified as important determinants of the activation kinetics of Kir2.1 was less effective. In giant patches, slow activation was present in cell-attached patches, lost on excision of the patch, and restored on perfusion with polyamine. Mutagenesis of residues in H5 and M2 close to the selectivity filter also decreased Ba$^{2+}$ block of the channel. A critical residue for Ba$^{2+}$ block was identified in Kir3.4. Mutagenesis of the equivalent residue in Kir3.1 failed to have as pronounced an effect on Ba$^{2+}$ block, suggesting an asymmetry of the channel pore. It is concluded that slow activation is principally the result of unbinding of polyamines from negatively charged residues close to the selectivity filter of the channel and not an intrinsic gating mechanism. Ba$^{2+}$ block involves an interaction with the same residues.

Rectification of the constitutively active classical inward-rectifying K$^+$ channels, such as Kir2.1, has been extensively studied and much is known about the underlying mechanisms. However, rectification of the cardiac muscarinic K$^+$ channel (Kir3.1/Kir3.4) is less understood. Rectification of Kir2.1 is due to intracellular block of the channel pore at potentials positive to the K$^+$ equilibrium potential, $E_K$, by intracellular Mg$^{2+}$ and positively charged aliphatic amines, such as spermine, spermidine, and putrescine (polyamines) (1–3). Upon hyperpolarization to potentials negative to $E_K$, current through Kir2.1 activates via instantaneous and time-dependent (time constant, <10 ms) phases. These components are thought to reflect rapid unblock of the channel by Mg$^{2+}$ and putrescine followed by a slower unblock by spermine and spermidine (2, 4). Kinetically, activation of Kir3.1/Kir3.4 is different. On hyperpolarization, again an instantaneous activation of current is observed followed by a time-dependent phase, but in this case the time-dependent phase is best described by a double exponential function with time constants of tens and hundreds of milliseconds (5). In addition, whereas the time constant of activation of Kir2.1 shows steep voltage dependence (6), that of Kir3.1/Kir3.4 is only weakly voltage-dependent (7). The slow component of activation is only observed in heteromultimeric Kir3 channels containing the subunit Kir3.1 (such as the cardiac muscarinic K$^+$ channel, which is a heterotetramer of two Kir3.1 and two Kir3.4 subunits (Refs. 8 and 9)); homomeric channels formed from Kir3.4 subunits alone show kinetics comparable to that of Kir2.1 (10). The difference in kinetics between Kir2.1 and Kir3.1/Kir3.4 has been suggested to be the result of an intrinsic gating mechanism in Kir3.1/Kir3.4 (11–13), possibly involving the C terminus of Kir3.1 in a manner analogous to N-type inactivation of voltage-gated K$^+$ channels (14, 15). In this case, slow activation would involve the slow unblock of the pore of the channel by the C terminus of Kir3.1. However, the time-dependent activation of Kir3.1/Kir3.4, even though it is approximately 2 orders of magnitude slower than that of Kir2.1, may also be the result of the unblock of the channel by polyamines. Upon depolarization, there is a time-dependent decay (or “relaxation”) of the cardiac muscarinic K$^+$ current. In inside-out patches from rabbit atrial cells, this relaxation has been shown to be the result of block by intracellular polyamines (16). If slow activation on hyperpolarization is the converse of the decay on depolarization (as has been suggested by Simmons and Hartzell (Ref. 5)), it follows that it is the result of dissociation of polyamines from the channel, rather than an additional intrinsic gating mechanism. One aim of this study was to test this. Block of the pore of Kir3.1/Kir3.4 by positively charged polyamines (or any other blocking particle, such as the C terminus of Kir3.1) could possibly be the result of an interaction with negatively charged residues within the pore of the channel. This is the case in Kir2.1 and mutation of two negatively charged residues in Kir2.1, Asp-172 and Glu-224, is known to reduce polyamine sensitivity and consequently reduce rectification (17, 18). Another aim was to seek equivalent residues in Kir3.1/Kir3.4. It is well known that inward-rectifying K$^+$ channels, such as Kir3.1/Kir3.4, are blocked by Ba$^{2+}$ and it is possible that this is also the result of interaction with negatively charged residues within the pore of the channel (19). In Kir2.1, at least, there is an overlap between polyamine and Ba$^{2+}$ interaction sites (20). The final aim of this study was to identify residues involved in Ba$^{2+}$ block of Kir3.1/Kir3.4.

**EXPERIMENTAL PROCEDURES**

Oocytes were injected with cRNA encoding wild-type or mutant Kir3.1 subunit, wild-type or mutant Kir3.4 subunit, and the hD2 receptor (the latter permits activation of the channel). When cRNA for a mutant subunit was injected, cRNA for the wild-type second subunit was injected (except in one case when cRNA for mutant forms of both
subunits was injected). Site-directed mutations were generated using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA) with oligonucleotides supplied by Sigma-Genosys (Cambridge, United Kingdom). Mutations were verified by fluorescent cycle sequencing using Taq DNA polymerase (PerkinElmer Life Sciences) on an Applied Biosystems 373A automated DNA sequencer (PerkinElmer Life Sciences). In vitro transcription of Kir 3.1, Kir 3.4, and hDcr cRNAs was performed using SP6 RNA polymerase (RiboMAX, Promega, Madison, WI). All cDNA templates were subcloned into a Xenopus high expression vector. Stock solutions of cRNA were diluted in nuclease-free water to give the appropriate concentrations prior to injection. Oocyte preparation, injection of cRNA, and two-microelectrode voltage clamp recordings were performed as described previously (21). The bath solution contained (in mM): KCl 90, HEPES 5, EDTA 5, pH 7.35 (KOH). To maintain channel activity in detached inside-out patches, 10 mM MgCl2 did not affect the results obtained. Microelectrodes were filled with 3 M KCl and were ~1 megohm in resistance. In the majority of recordings, oocytes were held at 0 mV and 750-ms pulses were applied to potentials ranging between −130 and +40 mV. In some experiments on Kir3.1/Kir3.4, pulses were 1.5 s in duration, and, in experiments on Kir3.1/Kir3.4-N179D, pulses were 3 s in duration. Control oocytes were injected with nuclease-free water; recordings from oocytes showing significant endogenous currents were rejected. Giant patch experiments were performed as described previously (21). The bath solution contained (in mM): KCl 90, NaCl 20, MgCl2 4.5, MgATP 2.5, EGTA 5, HEPES 10, pH 7.35 (KOH) (total free Mg2+ −5 mM). In some experiments the bath solution contained (in mM): KCl 90, NaCl 20, MgCl2 4.5, MgATP 2.5, EGTa 5, HEPES 10, pH 7.35 (KOH) (total free Mg2+ −5 mM). In some experiments the bath solution contained (in mM): KCl 90, HEPES 5, CaCl2 2, pH 7.4 (KOH). In some experiments the solution also contained 3 mM MgCl2; the absence or presence of MgCl2 did not affect the results obtained. Microelectrodes were filled with 3 M KCl and were ~1 megohm in resistance. In the majority of recordings, oocytes were held at 0 mV and 750-ms pulses were applied to potentials ranging between −130 and +40 mV. In some experiments on Kir3.1/Kir3.4, pulses were 1.5 s in duration, and, in experiments on Kir3.1/Kir3.4-N179D, pulses were 3 s in duration. Control oocytes were injected with nuclease-free water; recordings from oocytes showing significant endogenous currents were rejected. Giant patch experiments were carried out using the technique described by Hilgemann and Lai (22). The bath solution contained (in mM): KCl 90, NaCl 20, MgCl2 4.5, MgATP 2.5, EDTA 5, HEPES 10, pH 7.35 (KOH) (total free Mg2+ −5 mM). In some experiments the bath solution contained (in mM): KCl 90, HEPES 5, EDTA 5, pH 7.35 (KOH). To maintain channel activity in detached inside-out patches, 10 μM GTPγS1 was also included in the bath solution. Pipette solution contained (in mM): KCl 96, CaCl2 1.8, MgCl2 1, dopamine 10 μM, ascorbic acid 1, HEPES 10, pH 7.35 (KOH). Pipettes had resistances between 90 and 200 kilohms; this allowed recording of currents of the order of 0.5–10 nA/patch. Data are presented as mean ± S.E. (number of oocytes). Data comparisons were made by one-way analysis of variance, unless stated, followed by comparisons with control data using Dunnett’s test. Differences were considered to be statistically significant if p < 0.05.

RESULTS

Effect of H5 Mutations on Slow Activation—A typical recording of currents from an oocyte expressing wild-type Kir3.1 and Kir3.4 subunits is shown in Fig. 1A. The recording illustrates the strong rectification of the channel with little outward current being present at potentials positive to Emin (−0 mV). The currents in response to hyperpolarizing pulses demonstrate the rapid and slow components of activation of the current. As a measure of the slow activation of the current, we have expressed the current developed after 50 ms of a pulse to −100 mV as a percentage of the steady-state current (at the end of the pulse). For the wild-type channel, this parameter was 83 ± 1% (n = 6).

To identify the mechanism underlying the slow activation, we mutated residues within the pore region of the channel that could potentially act as a binding site for a positively charged gating particle (see Introduction). First, the H5 pore region was targeted. Previously, Kofuji et al. (13) identified the residue F137 in the H5 region of Kir3.1 as having an important effect on channel activation; the mutation Kir3.1-F137S abolished slow activation. However, Kofuji et al. (13) expressed Kir3.1-F137S alone. This means the recordings were either made from a high expression vector. The abbreviation used is: GTPγS, guanosine 5′-3-O-(thio) triphosphate.
Kir3.4-E147Q failed to alter slow activation. A summary of the activation properties of the wild-type and mutant channels is shown in Fig. 2; current at 50 ms during a pulse to −100 mV as a percentage of steady-state current is shown.

Effect of Other Mutations on Slow Activation—In Kir2.1 two negatively charged residues have been implicated in polyamine binding, time-dependent activation on hyperpolarization and rectification: an aspartate residue in the M2 domain (Asp-172) and a glutamate residue in the C terminus (Glu-224) (17, 18, 24, 25). Because Kir2.1 forms homomeric channels, there are four Asp-172 and four Glu-224 residues per channel. In Kir3.1/Kir3.4 there are only two negatively charged residues in the M2 region equivalent to Kir2.1-D172, because whereas Kir3.1 has an aspartate residue at the equivalent position (Kir3.1-D173), Kir3.4 has a neutral asparagine residue (Kir3.4-N179). In Kir3.1/Kir3.4 there are only two negatively charged residues in the C terminus equivalent to Kir2.1-E224, because whereas Kir3.4 has a glutamate residue at the equivalent position (Kir3.4-E231), Kir3.1 has a serine residue (Kir3.1-S225). These negatively charged residues in Kir3.1/Kir3.4 have been mutated to neutral residues.

The effect of mutating Asp-173 of Kir3.1 to a neutral glutamine residue (Gln) is shown in the first column of Fig. 3A (upper traces show currents from wild-type channel for comparison). The mutation Kir3.1-D173Q abolished slow activation (current at 50 ms, 99 ± 0.3%, n = 6, of steady state; see also Fig. 2), as did mutation to a neutral alanine residue (Kir3.1-D173A; Fig. 2). It was not possible to obtain recordings after mutation of Kir3.1-D173 to a neutral asparagine residue (Asn), because of the absence of measurable currents. Interestingly, the mutation Kir3.1-D173S failed to produce a significant effect on gating kinetics (Fig. 2). This may be due to the polar nature of the serine (Ser) side chain (the side chain could potentially form an interaction site for a positively charged blocking particle). The mutation Kir3.4-E231Q produced channels with kinetic properties illustrated in the second column of Fig. 3A; slow activation was reduced (current at 50 ms, 95 ± 1%, n = 6, of steady state; see also Fig. 2), but not as markedly as with many of the other mutations discussed above.

With Kir2.1, mutation of Asp-172 and Glu-224 together reduces rectification, such that significant outward currents may be observed at potentials positive to $E_K$ (18). With Kir3.1/Kir3.4, the combination of the two analogous mutations (Kir3.1-D173Q/Kir3.4-E231Q) reduced slow activation as illustrated in the second column of Fig. 3A (see also Fig. 2). However, the effects of the two mutations on slow activation were not additive, and the effect of the double mutation was no greater than that of Kir3.1-D173Q alone (Fig. 2; t test, p > 0.05). Furthermore, the double mutation did not reduce rectification (outward currents were still small; Fig. 3A).

Direct sequence comparison may not identify equivalently positioned residues. There may be structural differences between Kir2.1, Kir3.1, and Kir3.4 such that other residues in Kir3.1 (or Kir3.4) may have a more prominent role than Kir3.4-E231. In Kir3.1, the nearest negatively charged residues by

![Fig. 2. Summary of the effect of all mutations studied on activation kinetics.](image-url)
sequence alignment are Glu-198 and Asp-206. The mutation Kir3.1-E198Q produced a modest reduction in slow activation (Fig. 2); the effect was less marked than that of other mutations studied. Kir3.1-D206N failed to produce a significant alteration in gating kinetics (Fig. 2).

Thus far, we have considered the effects on activation of removing negatively charged residues. As noted above, Kir3.1 has a ring of four negatively charged residues within the channel pore formed by Asp-172, whereas Kir3.1/Kir3.4 has only two negatively charged residues (Kir3.1-D173) at the equivalent position. By mutating the neutral asparagine residue, Asn-179, of Kir3.4 to an aspartate residue, the heteromultimer formed upon assembly with Kir3.1 will also have such a ring of four negatively charged residues at the equivalent position. Currents recorded from an oocyte expressing Kir3.4-E145Q, which was obtained in the presence of a higher concentration (shown). Means ± S.E. are shown (prop = 5, 6, 12 and 5 for WT, Kir3.1-F137S/Kir3.4, Kir3.1-E139Q/Kir3.4, Kir3.1/Kir3.4-E145Q, and Kir3.1/Kir3.4-N179D, respectively). Membrane current has been normalized to the steady-state current at −130 mV in the absence of Ba2+. The concentrations of Ba2+ used were 0, 3, 10, 30, and 100 μM in all cases apart from Kir3.1/Kir3.4-E145Q for which the concentrations used were 0, 1, 3, 10, and 30 mM.

Wild-type Kir3.1/Kir3.4 had a half-maximal activation potential, V0.5, of −39 ± 3 mV and a slope factor, k, of 13 ± 1 mV. The activation curves for Kir3.1-F137S/Kir3.4 and Kir3.1-E139Q/Kir3.4 are significantly less steep (k of 25 ± 1 and 22 ± 1 mV, respectively; p < 0.01, t test), although V0.5 (−42 ± 4 and −38 ± 3 mV, respectively) is comparable to that of the wild-type channel. Kofuji et al. (13) failed to observe this change in the activation curve with the mutation Kir3.1-F137S; however, in their experiments Kir3.1/Kir3.4 was expressed alone, whereas in the present study it was expressed with Kir3.4 (see above).

**Ba2+ Block**—Block of wild-type and mutant Kir3.1/Kir3.4 is shown in Fig. 4. Currents shown in Fig. 4A were obtained under control conditions, whereas those in Fig. 4B were recorded in the presence of 30 μM Ba2+ (with the exception of Kir3.4-E145Q, which was obtained in the presence of a higher concentration). The dotted lines show the steady-state wild-type current (far left) recorded upon a pulse to −130 mV either in the absence or presence of Ba2+. Currents from mutant channels have been scaled so that the steady-state current at −130 mV in the absence of Ba2+ is the same as that for the wild-type channel. This scaling procedure allows the Ba2+ sensitivity of the wild-type and mutant channels to be compared. Fig. 4C shows current-voltage relationships for the steady-state current plotted for different concentrations of Ba2+. From the data in Fig. 4C, dose-response curves were generated for each potential. Fig. 5A shows the dose-response curves at −130 mV for the wild-type and mutant channels (dose-response curve for wild-type channel shown in every case). These have been fitted by the Hill equation (assuming a Hill coefficient of 1, i.e. one Ba2+ interacts with one channel to cause block). The dissociation constant, Kd, at −130 mV for Ba2+ block of the wild-type channel is 14 ± 1 μM (p = 5) (Fig. 5A). The mutation Kir3.1-F137S produced a reduction of Ba2+ block as
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**Fig. 5.** Dose-response curves and voltage dependence of Ba\(^{2+}\) block. A. dose-response curves for steady-state Ba\(^{2+}\) block at −130 mV. Current remaining in the presence of Ba\(^{2+}\) as a percentage of the control current is plotted against the Ba\(^{2+}\) concentration. Steady-state current at the end of the pulse (duration: Kir3.1/Kir3.4-N179D, 3 s; other channels, 750 ms) was measured. Means ± S.E. are shown for wild-type channel (Kir3.1/Kir3.4) (n = 8), Kir3.1-F137S/Kir3.4 (n = 5), Kir3.1-E139Q/Kir3.4 (n = 6), Kir3.1/Kir3.4-E145Q (n = 6), and Kir3.1/Kir3.4-N179D (n = 5). B. Dose-response curves for steady-state block of Kir3.1/Kir3.4-E145Q. Data are fitted with the Hill equation: $y = 100 - 100([Ba^{2+}]/(K_d + [Ba^{2+}]^n))$, where $y$ is the percentage current remaining, $[Ba^{2+}]$ is the Ba\(^{2+}\) concentration, and $n$ is the Hill co-efficient (assumed to be 1). The most effective mutation was Kir3.1-E139Q, which reduced Ba\(^{2+}\) block at −130 mV by 80% compared with wild-type channel (Fig. 5B). The effects of the mutations on Ba\(^{2+}\) sensitivity are comparable to the effects on slow activation (cf. Figs. 2 and 6A). Fig. 6B shows the $K_d$ at −130 mV plotted against the measure of slow activation used (current at 50 ms as a percentage of steady state) for each Kir3.1/Kir3.4 channel (wild-type and mutant). Kir3.3-E145Q has been excluded as it is perhaps a special case (see above). Fig. 6B shows that there is a significant correlation between the two sets of measurements ($r = 0.0004$). Therefore, in Kir3.1/Kir3.4 residues involved in slow activation also influence Ba\(^{2+}\) block.

From dose-response curves at different potentials, $K_d$ at −130 mV for Ba\(^{2+}\) block is shown in Fig. 6A. The $K_d$ for the wild-type channel is highlighted by the dotted line. Mutations in the H5 region had the biggest effect, mutations in the M2 region had smaller effects, and mutations in the C terminus had no effect. The effect of Kir3.4-E145Q was 100 fold larger than that of any other mutation and in this respect is in a special category (see “Discussion”). The effects of the mutations on Ba\(^{2+}\) sensitivity are comparable to the effects on slow activation (cf. Figs. 2 and 6A). Fig. 6B shows the $K_d$ at −130 mV plotted against the measure of slow activation used (current at 50 ms as a percentage of steady state) for each Kir3.1/Kir3.4 channel (wild-type and mutant). Kir3.3-E145Q has been excluded as it is perhaps a special case (see above). Fig. 6B shows that there is a significant correlation between the two sets of measurements ($r = 0.0004$). Therefore, in Kir3.1/Kir3.4 residues involved in slow activation also influence Ba\(^{2+}\) block.

A summary of the effect of all mutations investigated on the $K_d$ at −130 mV for Ba\(^{2+}\) block is shown in Fig. 6A. The $K_d$ for the wild-type channel is highlighted by the dotted line. Mutations in the H5 region had the biggest effect, mutations in the M2 region had smaller effects, and mutations in the C terminus had no effect. The effect of Kir3.4-E145Q was 100 fold larger than that of any other mutation and in this respect is in a special category (see “Discussion”). The effects of the mutations on Ba\(^{2+}\) sensitivity are comparable to the effects on slow activation (cf. Figs. 2 and 6A). Fig. 6B shows the $K_d$ at −130 mV plotted against the measure of slow activation used (current at 50 ms as a percentage of steady state) for each Kir3.1/Kir3.4 channel (wild-type and mutant). Kir3.3-E145Q has been excluded as it is perhaps a special case (see above). Fig. 6B shows that there is a significant correlation between the two sets of measurements ($r = 0.0004$). Therefore, in Kir3.1/Kir3.4 residues involved in slow activation also influence Ba\(^{2+}\) block.

From dose-response curves at different potentials, $K_d$ was calculated for each potential, and the $K_d$ has been plotted against the membrane potential in Fig. 5B for the wild-type and mutant channels (data for wild-type channel shown in every case). The plots of $K_d$ versus membrane potential in Fig.
Summary of the effect of all mutations studied on Ba$^{2+}$ block. A, mean ± S.E. $K_d$ at −130 mV for Ba$^{2+}$ block of the wild-type and mutant channels. For the mutated channels, on the abscissa the name of the mutated subunit is given (if only one subunit is named, the second subunit will be wild-type). *, significantly different from wild-type channel (n = 5 except: Kir3.1-E139Q/Kir3.4, Kir3.1-E141Q/Kir3.4, Kir3.1-D173Q/Kir3.4, Kir3.4-N179D/Kir3.4, Kir3.1-D206N/Kir3.4, n = 9; Kir3.1/Kir3.4-E145Q, n = 12). B, mean ± S.E. $K_d$ for Ba$^{2+}$ block for the wild-type and mutant channels (from A) plotted against mean ± S.E. current at 50 ms during a pulse to −100 mV as a percentage of steady-state current at the end of the pulse (from Fig. 2). Data for Kir3.1/Kir3.4-E145Q not included. The solid line shows the results of a regression to the data (p < 0.0004).

5B have been fitted by an exponential function of voltage according to the Woodhull model of channel block (26).

$$K_d = K_{d(0)} \exp(-\delta \times VF/RT)$$

$K_d$ is the dissociation constant at the membrane potential (V), $K_{d(0)}$ is the dissociation constant at 0 mV, $\delta$ is the fraction of the electric field experienced by the ion at its binding site, z is the ion valence, and $F$, $R$, and $T$ have their usual meanings. The gradient of the line represents $\delta$. Fig. 5B shows that Ba$^{2+}$ block of the wild-type channel was voltage-dependent and Ba$^{2+}$ sensitivity was greater at more negative potentials, as shown previously (21). For the wild-type channel, $\delta$ was 0.31 ± 0.01 (n = 10). Both Kir3.1-F137S and Kir3.1-E139Q generated an upward shift in the plot (Fig. 5B) highlighting the decreased Ba$^{2+}$ sensitivity of the channel. The mutation Kir3.1-F137S, failed to alter the gradient of the plot significantly (δ, 0.30 ± 0.02; n = 5) suggesting this mutation does not alter the position of the Ba$^{2+}$ binding site in the pore. However, the mutation Kir3.1-E139Q significantly altered the gradient (δ, 0.22 ± 0.01; n = 6), suggesting a shift in the Ba$^{2+}$ binding site to a shallower position in the channel. With Kir3.4-E145Q the $K_d$ appeared to show little voltage dependence and δ was significantly changed (0.0041 ± 0.0096; n = 12), suggesting the normal site for Ba$^{2+}$ block was removed (Fig. 5B). With Kir3.4-E145Q, the residual block by Ba$^{2+}$ possibly reflects an interaction with a weak external blocking site perhaps analogous to the site of external Ca$^{2+}$ and Mg$^{2+}$ block of Kir3.1/Kir3.4 (21).

The mutation Kir3.4-N179D shifted the plot of $K_d$ against membrane potential downwards and significantly increased the steepness of the plot (δ, 0.62 ± 0.03; n = 5) (Fig. 5B). The increase in δ suggests that this mutation altered the site of Ba$^{2+}$ block in the channel.

Fig. 6B shows the time-dependent onset of Ba$^{2+}$ block during hyperpolarizing pulses. Some of the mutations investigated altered the rate of onset of Ba$^{2+}$ block. Fig. 7A shows the time course of block by 30 μM Ba$^{2+}$ of wild-type and mutant channels during a pulse to −60 mV (wild-type data shown in every panel). Averaged (n = 8) normalized Ba$^{2+}$-sensitive current is plotted against time from the beginning of the pulse. Block of current developed in a manner best described by a double exponential function. The time constant of the slower exponential component ($\tau_{slow}$) is plotted against membrane potential in Fig. 7B for wild-type and mutant channels (wild-type data shown in every panel). $\tau_{slow}$ showed little voltage dependence in the wild-type channel (Fig. 7B). The mutations Kir3.1-F137S and Kir3.1-E139Q slowed the onset of block (Fig. 7A) and increased the voltage dependence of $\tau_{slow}$, block becoming substantially slower at more positive potentials. The mutation Kir3.4-N179D produced the greatest change in the kinetics of Ba$^{2+}$ block. Fig. 7A shows that it substantially slowed the onset of block (note the change in time scale for the traces). With the
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Currents were initially recorded in the cell-attached mode and showed slow activation comparable to that for currents from the whole oocyte (n = 8; Fig. 8B). Upon formation of an inside-out patch and perfusion of the intracellular surface of the patch, slow activation was lost (n = 12) and replaced with rapid activation (Fig. 8B). This was observed when Mg\textsuperscript{2+} was present in the perfusate (free Mg\textsuperscript{2+} ~5 mM) (n = 8) or when it was absent (n = 4) (indicating Mg\textsuperscript{2+} is not a major determinant of slow activation). In five cases, it was possible to reapply 1 mM spermine to the intracellular side of the patch; this led to the re-establishment of slow activation (Fig. 8C). This suggests that slow activation during a hyperpolarizing pulse does involve an interaction between polyamines and the channel.

**DISCUSSION**

The present study has shown alteration (sometimes profound) of both slow activation on hyperpolarization and Ba\textsuperscript{2+} sensitivity by mutation of various pore residues, K\textsuperscript{+} dependence of the slow activation, and loss of slow activation on excision of inside-out patches and restoration on exogenous application of polyamine. It is concluded that slow activation on hyperpolarization is the result of unbinding of polyamines from the pore of the channel and polyamines interact with a variety of sites in the H5, M2, and C-terminal regions. Ba\textsuperscript{2+} blocks the channel by interacting with sites in the H5 and M2 regions.

**Nature of Slow Activation**—The only argument against the involvement of polyamine unbinding in the time-dependent activation of Kir3.1/Kir3.4 current during hyperpolarizing pulses has been the slowness of the activation (in Kir3.1/Kir3.4, the time constant is ~10-fold greater than that for Kir2.1). However, the present study has obtained various types of evidence to indicate that the slow activation of Kir3.1/Kir3.4 is the result of a slow unbinding of polyamines from the channel: (i) mutations (Kir3.1-D173Q, Kir3.4-E231Q) equivalent to two mutations of Kir2.1 (Kir2.1-D172N, Kir2.1-E224Q) known to greatly reduce polyamine binding and time-dependent activation in Kir2.1 (17, 18) also reduce time-dependent activation of Kir3.1/Kir3.4, (ii) raising external K\textsuperscript{+} accelerates the time-dependent activation of Kir2.1 and raising the external K\textsuperscript{+} concentration has the same effect in Kir3.1/Kir3.4, and (iii) excision of inside-out patches results in loss of slow activation and exogenous application of polyamines results in its restoration. If the slow activation of Kir3.1/Kir3.4 is produced by polyamines unbinding from the channel pore, it cannot be the result of an intrinsic gating mechanism as proposed elsewhere (11–13). However, the present study does not preclude an explanation comparable to that suggested for Kir2.1 by Lee et al. (29), in which a polyamine molecule interacts with a part of the channel to form a blocking ball (however, we have failed to identify a site analogous to E224 in Kir2.1 at which such a complex is proposed to form).

**Role of Mg\textsuperscript{2+}**—The time-dependent activation of Kir3.1/Kir3.4 was lost both in the absence and presence of Mg\textsuperscript{2+} at the intracellular face of the channel. Thus, despite the ability of intracellular Mg\textsuperscript{2+} to produce strong rectification during depolarizing pulses, it apparently unbinds from the channel instantaneously during hyperpolarizing pulses (30) and so does not contribute to time-dependent activation.

**Comparison of Time-dependent Activation in Kir2.1 and Kir3.1/Kir3.4**—It is being suggested that time-dependent activation of Kir3.1/Kir3.4 current during hyperpolarizing pulses, like that of Kir2.1 current, is the result of polyamine unbinding despite the difference in time course of activation of the two channels. What could account for the difference in kinetics? There are two possible explanations. (i) In Kir3.1/Kir3.4, there is Kir3.1-F137 in the H5 region, whereas in Kir2.1 there is no equivalent phenylalanine residue, and in Kir3.1/Kir3.4, E145 is replaced with a glutamine residue. (ii) Kir2.1 has an N-terminal mutation Kir3.4-E145Q, time-dependent block during hyperpolarizing pulses was not seen (Fig. 4B), this suggests that Ba\textsuperscript{2+} block of this mutant is instantaneous.

**Evidence of Polyamine Involvement in Slow Activation**—The experiments to this point have identified residues involved in interactions with a putative gating particle, but have thus far failed to identify the gating particle. Time-dependent activation of Kir2.1 is dependent on external K\textsuperscript{+} concentration and is reduced as the external K\textsuperscript{+} concentration has the same effect in Kir3.1/Kir3.4, and (iii) excision of inside-out patches results in loss of slow activation and exogenous application of polyamines results in its restoration. If the slow activation of Kir3.1/Kir3.4 is produced by polyamines unbinding from the channel pore, it cannot be the result of an intrinsic gating mechanism as proposed elsewhere (11–13). However, the present study does not preclude an explanation comparable to that suggested for Kir2.1 by Lee et al. (29), in which a polyamine molecule interacts with a part of the channel to form a blocking ball (however, we have failed to identify a site analogous to E224 in Kir2.1 at which such a complex is proposed to form).

![Fig. 8. Mechanism of slow activation. A, top, currents recorded from an oocyte expressing the wild-type channel (Kir3.1/Kir3.4) in 40 (left) and 300 (right) mM external K\textsuperscript{+}. A, bottom, mean ± S.E. (n = 6) current at 50 ms during a pulse to −100 mV as a percentage of steady-state current plotted against the external K\textsuperscript{+} concentration. Data fitted with a curve to guide the eye. B, currents from cell-attached patch (top) and after excision of the patch into a polyamine free solution (bottom) during pulses to between −130 and +50 mV from a holding potential of 0 mV. C, currents from a cell-attached patch, after excision of the patch into a polyamine-free solution, and after application of 1 mM spermine to the inside of the excised patch during a pulse to −100 mV from a holding potential of 0 mV. The currents have been normalized to the current at the end of the pulse (1.2, 0.6, and 0.5 nA, in the cell-attached patch, excised patch in polyamine-free solution, and excised patch with 1 mM spermine, respectively).](image-url)
Kir3.4 when this residue is substituted kinetics become Kir2.1-like. (ii) In Kir3.1/Kir3.4, there are two closely spaced rings of negatively charged aspartate residues in the H5 region formed by Kir3.1-E139, Kir3.1-E141, Kir3.4-E145, and Kir3.4-E147, resulting in a total of eight negatively charged residues at this point (although results show that only Kir3.1-E139 and Kir3.4-E145 are “active”). In contrast, Kir2.1 has only four negatively charged residues at the equivalent point (although the one glutamate residue that is present, Kir2.1-E138, is equivalent to the active glutamate residue in Kir3.1 and Kir3.4). In Kir3.1 and Kir3.4, when residues Kir3.1-E139 and Kir3.4-E145 are substituted, kinetics become Kir2.1-like. In Kir3.1/Kir3.4, there are just two negatively charged residues in the M2 region (Kir3.1-D173), whereas in Kir2.1 there are four at the equivalent position (Kir2.1-D172). However, this difference was not expected to account for the difference in kinetics between Kir3.1/Kir3.4 and Kir2.1 (in Kir3.1/Kir3.4, whenever active negatively charged residues have been removed, slow activation has been reduced). This supposition was confirmed because, when an additional negatively charged residue was added at this point in Kir3.1/Kir3.4 by the mutation Kir3.4-N179D (so that the number of negatively charged residues at this point was the same as in Kir2.1), slow activation was enhanced, not lost. In conclusion, Kir3.1-F137, Kir3.1-E139, and Kir3.4-E145 could possibly be the reasons for the difference in kinetics, although the reason why Kir2.1-E138 does not fulfill the same role as Kir3.1-E139 and Kir3.4-E145 is not clear (but see below).

In Kir2.1, the two sites shown to be involved in time-dependent activation on hyperpolarization and polyamine interaction identified so far are in the M2 region (Kir2.1-D172) and the proximal C terminus (Kir2.1-E224) (18). The M2 region and the proximal part of the C terminus are thought to form part of the channel pore. Thus far, sites in the H5 region of Kir2.1 have not been shown to be involved. The involvement of Kir2.1-D172 in the M2 region and Kir2.1-E224 in the proximal C terminus is highly critical, because after mutation of these residues rectification is altered such that significant outward currents are observed during depolarizing pulses (18). Polyamine interaction with Kir3.1/E139 could be different, because in the present study (i) mutations of the H5 region had major effects on slow activation and the effects of mutations on slow activation roughly depended on the location of the mutations according to the sequence H5 > M2 > C terminus, and (ii) the combination of mutations, Kir3.1-D173Q and Kir3.4-E231Q, did not significantly affect rectification (outward current during depolarizing pulses was still small as compared with inward currents during hyperpolarizing pulses). The major effect of H5 pore mutations on slow activation in Kir3.1/Kir3.4 suggests that polyamines bind higher (i.e., further toward the extracellular face of the channel) in Kir3.1/Kir3.4 than in Kir2.1. Polyamines may bind higher in Kir3.1/Kir3.4 because of greater access to this part of the channel; it is possible the intracellular opening of the channel is wider in Kir3.1/Kir3.4. If this is correct, this perhaps explains why Kir2.1-E138 does not play the same role as Kir3.1-E139 and Kir3.4-E145.

Nature of Slow Inactivation of Kir3.1/Kir3.4—Loss of slow activation of Kir3.1/Kir3.4 by the mutations Kir3.1-F137S, Kir3.1-D139Q, and Kir3.4-D145Q (Fig. 1) as well as excision of inside-out patches (Fig. 8) revealed a slow time-dependent “inactivation” during hyperpolarizing pulses. The cause of the inactivation is not known (see Owen et al. (21) for a discussion in relation to Kir2.1), but it is interesting that the same inactivation is observed normally in Kir2.1.

Ba$^{2+}$ Block—The present study has shown that mutations Kir3.1-F137S, Kir3.1-E139Q, and Kir3.1-D173Q produced substantial decreases in Ba$^{2+}$ sensitivity. Residues Kir3.1-F137, Kir3.1-E139, and Kir3.1-D173 must, therefore, be in a position to influence Ba$^{2+}$ binding (the conclusion regarding the latter residue is supported by the finding that Ba$^{2+}$ sensitivity was increased when another negatively charged residue was added at this point by the mutation Kir3.4-N179D). All of these residues are close to the selectivity filter. However, the effects of the mutations above on Ba$^{2+}$ sensitivity were small compared with the effect of Kir3.4-E145Q. This mutation appeared to abolish normal Ba$^{2+}$ block (the $K_B$ at $-130$ mV was decreased $~500$-fold, and the voltage dependence of Ba$^{2+}$ block was lost). The mutation Kir3.4-E145Q also resulted in the loss of selectivity,2 and it is likely that the loss of Ba$^{2+}$ selectivity is the result of the loss of selectivity, i.e., Ba$^{2+}$ passes through the channel rather than blocking it. These findings are consistent with Ba$^{2+}$ blocking the channel by binding near or in the selectivity filter. This agrees with recent findings published by Jiang and MacKinnon (31) showing the site of Ba$^{2+}$ block in the KCSA channel. In Kir2.1 the site of Ba$^{2+}$ block may be slightly different to that in Kir3.1/Kir3.4 and it may lie deeper within the channel, because $\delta$ is $0.54$ (20) for Kir2.1 and 0.31 for Kir3.1/Kir3.4. The mutation Kir3.4-N179D makes Kir3.1/Kir3.4 more closely resemble Kir2.1 by adding an extra negatively charged residue at this position (see above); this mutation also increased $\delta$ to 0.62 and thus made Ba$^{2+}$ block of Kir3.1/Kir3.4 more like that of Kir2.1.

In previous studies, mutagenesis has provided evidence concerning block of inward-rectifying K$^+$ channels: Kir1.1 is less sensitive to Ba$^{2+}$ than Kir2.1. This can be accounted for by a single difference in amino acid residue in the H5 region: a valine residue in Kir1.1 (V121) and a threonine residue in Kir2.1 (T142). In Kir1.1, the mutation V121T abolished this difference (32). In Kir1.1/Kir3.4, the equivalent residues are Kir3.1-T143 and Kir3.4-T149. In Kir1.1/Kir3.4, Kir3.1-D173 appears not to be involved in Ca$^+$ block (33), whereas in Kir2.1 the equivalent residue (Asp-172) is involved in Sr$^{2+}$ block (34).

Further structural information may be gleaned from the findings that the equivalent mutations, Kir3.1-E139Q and Kir3.4-E145Q, had substantial effects on slow activation (and also Ba$^{2+}$ block), whereas the equivalent mutations, Kir3.1-E141Q and Kir3.4-E147Q, did not (and neither did they affect Ba$^{2+}$ block). This suggests that these two sets of residues are performing in a similar manner in Kir3.1 and Kir3.4. This in turn suggests symmetry in the channel. It also suggests that Kir3.1-E141 and Kir3.4-E147 are not in a position to interact with positively charged species in the channel pore (whereas Kir3.1-E139 and Kir3.4-E145 are). However, the effects of the equivalent mutations, Kir3.1-E139Q and Kir3.4-E145Q, on Ba$^{2+}$ sensitivity are unequal, and this suggests that residues Kir3.1-E139 and Kir3.4-E145 are not structural equivalents. This finding suggests that there may be asymmetry in the structure of Kir3.1/Kir3.4. In Kir2.1, Yang et al. (35) suggested that Glu-138 (equivalent to Kir3.1-E139 and Kir3.4-E145) forms a salt bridge with Arg-148 (also present in Kir3.1 and Kir3.4), because mutation of Glu-138 resulted in a non-functional channel and the double mutation E138R/R148E restored function (although it also resulted in loss of selectivity). In the present study, mutation of either Kir3.1-E139 or Kir3.4-E145 did not result in loss of function, but, because Kir3.1/Kir3.4 is a heteromultimer, either mutation resulted in loss of only two of the four glutamate residues and in Kir2.1 expression of channels comprising wild-type Kir2.1 and Kir2.1-E138Q subunits also did not result in loss of function (35).
The overall picture of Kir3.1/Kir3.4 coming from these data is that of a channel with significant asymmetry within the pore. The intracellular side is widely flared permitting the access of polyamine molecules that penetrate to the narrowest part of the channel, the selectivity filter, where they form strong interactions with various negatively charged residues. Ba\(^{2+}\) appears to block the channel by binding at a similar site.

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