The Selectivity Filter May Act as the Agonist-activated Gate in the G Protein-activated Kir3.1/Kir3.4 K⁺ Channel*

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The Kir3.1/Kir3.4 channel is activated by Gβγ subunits released on binding of acetylcholine to the M₂ muscarinic receptor. A mechanism of channel opening, similar to that for the KcsA and Shaker K⁺ channels, has been suggested that involves translocation of pore lining transmembrane helices and the opening of an intracellular gate at the “bundle crossing” region. However, in the present study, we show that an extracellular gate at the selectivity filter is critical for agonist activation of the Kir3.1/Kir3.4 channel. Increasing the flexibility of the selectivity filter, by disrupting a salt bridge that lies directly behind the filter, abolished both selectivity for K⁺ and agonist activation of the channel. Other mutations within the filter that altered selectivity also altered agonist activation. In contrast, mutations within the filter that did not affect selectivity had little if any effect on agonist activation. Interestingly, mutation of bulky side chain phenylalanine residues at the bundle crossing also altered both agonist activation and selectivity. These results demonstrate a significant correlation between agonist activation and selectivity, which is determined by the selectivity filter, and suggests, therefore, that the selectivity filter may act as the agonist-activated gate in the Kir3.1/Kir3.4 channel.

K⁺ channels open and close in response to a number of stimuli including voltage or intracellular ligands such as G proteins. The position of the gate or gates that control channel opening and closing remains a matter of interest. Studies on the proton-gated KcsA K⁺ channel (1, 2), the voltage-gated Shaker K⁺ channel (3–5) and the G protein-activated Kir3.2 channel (6, 7) or ATP-sensitive Kir6.x (8, 9) inward rectifier K⁺ channels suggest that an intracellular gate is formed by the bundle crossing region of the channel. These studies are consistent with spin labeling measurements made in the KcsA channel that suggest that during proton activation the second transmembrane (TM2) domains rotate and tilt away from the axis of the pore about a pivot point in a scissoring-type motion (1, 2). This work is supported by crystallization of the MthK bacterial K⁺ channel in the open state (10), which shows a pivot or hinge point at a highly conserved glycine residue at position 83 (equivalent to position 99 in the KcsA channel).

However, other parts of the channels may be associated with channel opening, because although access of large MTS reagents (such as MTSET; radius —2.9 Å) to the inner vestibule of a Ca²⁺-activated K⁺ channel (SK) is state-dependent, smaller MTS reagents (such as MTSEA; radius —1.8 Å, compared with K⁺ radius of 1.33 Å) can access as far as the selectivity filter equally well in open and closed channel states (11). Similarly, access of large MTS reagents to a cyclic nucleotide-gated channel (CNG1) is gated, while Ag⁺ (radius 1.27 Å) has state-independent access (12). This suggests that, in some channels at least, the bundle crossing may not be sufficient to impede the passage of permeant ions through the pore. On channel activation, other than the large movements of the TM2 domains that are described above, small movements at the inner portion of the selectivity filter of the KcsA channel have been observed in spin labeling experiments (1). It is therefore possible that this region of the channel may also act as a gate.

These results demonstrate a significant correlation between agonist activation and selectivity, which is determined by the selectivity filter, and suggests, therefore, that the selectivity filter may act as the agonist-activated gate in the Kir3.1/Kir3.4 channel.

EXPERIMENTAL PROCEDURES

Molecular Biology—Mutations in the Kir3.1 or Kir3.4 channel subunits were made using site-directed PCR mutagenesis and confirmed by sequencing. Plasmids (pTLNII or pOES) containing wild-type or mutant Kir3.1 or Kir3.4 channel subunits or the hD₂ (human dopamine) or M₂ (muscarinic acetylcholine (ACh)₃ receptor (required for agonist activation of the channel) were linearized using MluI or NotI (New England Biolabs, Beverly, MA) and transcribed in vitro using SP6 or T7 RNA polymerase (Riboprobes®; Promega, Madison, WI).

Electrophysiology—Xenopus oocytes were prepared as described previously (23). Oocytes were injected with 50 nl of cRNA encoding wild-type or mutant Kir3.1 (30 ng/µl) and Kir3.4 (30 ng/µl) as well as hD₂ (3.8 ng/µl). In a few experiments, M₂ (3.8 ng/µl) was injected instead of hD₂.

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* The abbreviations used are: TM2, transmembrane 2; ANOVA, analysis of variance.
there was no difference in the behavior of the wild-type Kir3.1/Kir3.4 channel with the h6b and M2 receptors (and relevant agonist). In the case of the mutations, Kir3.4[E145Q], Kir3.4[R155E], Kir3.4[E145R,R155E], Kir3.4 [F181A], and Kir3.4[F187A], cRNA was injected at 10 times the concentration to obtain adequate currents. Oocytes were incubated for 36–96 h at 19 °C in Barth's medium (in mM): 88 NaCl, 1 KCl, 2.4 NaHCO3, 0.82 MgSO4, 0.33 Ca(NO3)2, 0.41 CaCl2, 1.25 sodium pyruvate, 0.1 mg/ml neomycin (Sigma, Poole, UK), 100 units/0.1 mg/ml penicillin/streptomycin mix (Sigma), pH 7.4 (NaOH).

Oocytes were generated using pClamp software (Axon Instruments, Union City, CA) filtering at 500 Hz and sampling at 2 kHz. Voltage protocols were created using Clampfit software (Axon Instruments) and SigmaPlot (SPSS Science, Chicago, IL) software. Data are given as means ± S.E. (n = number of oocytes). Statistical analysis was performed using a Student's t test or ANOVA as appropriate. p values of <0.05 were considered to signify a significant difference.

**RESULTS**

**Effect of Mutation of the Bulky Hydrophobic Residues at the Bundle Crossing**—The comparative model of the Kir3.1/Kir3.4 channel (Fig. 1) shows a narrowing of the permeation pathway at the bundle crossing formed by the large side chains of the phenylalanine residues at position 181 in Kir3.1 and 187 in Kir3.4 (Fig. 1, A and C). The recently crystallized KirBac1.1 bacterial inward rectifier K+ channel also possesses phenylalanine residues at the equivalent position (P146 (22)). Because their large hydrophobic side chains are ideally positioned to prevent movement of water through the narrow bundle crossing, the four phenylalanine residues in KirBac.1 were proposed to be involved in channel activation (22). We have investigated the role of these phenylalanine residues in agonist activation of the Kir3.1/Kir3.4 channel.

**Modeling**—A comparative model of the Kir3.1/Kir3.4 tetrameric channel was constructed based on the crystal structure of KcsA as described previously (24, 25).

**Fig. 1.** Comparative model of the Kir3.1/Kir3.4 channel tetramer. A, model of the M1-P-M2 domains of the Kir3.1/Kir3.4 tetramer based on the KcsA crystal structure. The backbones and side chains of residues of interest are shown. B, an enlarged view of the P-loop. Charged residues associated with the selectivity filter (Kir3.1[E139]/Kir3.4[E145] and Kir3.1[R149]/Kir3.3[R155]) are shown as well as Kir3.1[A142] and Kir3.4[T148]. C, an enlarged view of the phenylalanine residues (Kir3.1[F181] and Kir3.4[F187]) at the bundle crossing of the channel. D, an alignment of a part of the selectivity filter (left) and the lower part of the second transmembrane domain (right) in Kir2.1, Kir3.1, and Kir3.4. Residues of interest are highlighted in red. Residues known from previous studies (6, 7) to abolish agonist activation are highlighted in blue.
fect on the agonist dependence of the channel. This can be seen in Fig. 2, which shows current traces and mean current-voltage relationships for the mutant channel, and also in Fig. 3A (bar 4), which shows the agonist-activated current (at –130 mV) of various mutant channels normalized to that of the wild-type channel recorded from the same batch of oocytes. Although mutation to a methionine residue in all four subunits had no effect, mutation in either Kir3.1 or Kir3.4 alone did have a modest effect on agonist dependence (Fig. 3A, bars 2 and 3).

The mutation, Kir3.1[F181M], reduced agonist activation by 30.9 ± 2.3% (n = 15; ANOVA, p < 0.05) compared with that of the wild-type channel, while the mutation, Kir3.4[F187M], increased agonist activation by 42.1 ± 3.9% (n = 5; ANOVA, p < 0.05) compared with that of the wild-type channel (Fig. 3A, bars 2 and 3). The effect of replacement of the phenylalanine residues with small alanine residues (Kir3.1[F181A]/Kir3.4[F187A]) was much greater (Figs. 2 and 3A, bar 5). The double mutation, Kir3.1[F181A]/Kir3.4[F187A], abolished agonist activation; the agonist-activated current was reduced by 114 ± 4% (n = 6; ANOVA, p < 0.05) compared with that of the wild-type channel.

In proline-scanning experiments of the TM2 domain (7), introduction of proline residues affected agonist activation with an α-helical periodicity (residues highlighted in blue in Fig. 1D). The double mutation, Kir3.1[S170P]/Kir3.4[S176P], had the greatest effect on agonist activation and produced this by introducing a kink into the helix of the TM2 domains (7). In the present study, the double mutation, Kir3.1[S170P]/Kir3.4[S176P], reduced agonist activation by 80.5 ± 4.9% (Fig. 2; n = 5, ANOVA, p < 0.05) compared with that of the wild-type channel (Fig 3A, bar 6).

As a control, we investigated the effect of the mutation of an aspartate residue within the TM2 domain (D173 in Kir3.1; equivalent to D172 in Kir2.1) that is thought to be involved with polyamine block of inward rectifier K+ channels. The mutation, Kir3.1[D173Q], had no effect on agonist activation; agonist-activated current through the Kir3.1[D173Q]/Kir3.4 mutant channel was 93.1 ± 1.0% that of the wild-type channel (n = 2; ANOVA, not significant).

**Mutations that Disrupt the Selectivity Filter Abolish Agonist Activation**—Fig. 1B shows an enlarged view of the part of the P-loop that forms the selectivity filter of the Kir3.1/Kir3.4 channel. As in the Kir2.1 channel (27), a salt bridge is thought to exist behind the selectivity filter between the negatively charged glutamate residue at position 139 (Glu-139) and the positively charged arginine residue at position 149 (Arg-149) in the Kir3.1 subunit and between Glu-145 and Arg-155 in the Kir3.4 subunit (see also the alignment in Fig. 1D).

Fig. 4 shows the effect of agonist application on mutant Kir3.1/Kir3.4 channels in which the salt bridge behind the selectivity filter had been broken by neutralizing or reversing the charges at Glu-139/Glu-145 (in Kir3.1 and Kir3.4, respectively) and/or Arg-149 in the Kir3.1 subunit and between Glu-145 and Arg-155 in the Kir3.4 subunit.
salt bridge in the Kir3.4 subunit (but not the Kir3.1 subunit, see below), by the Kir3.4[E145Q], Kir3.4[R155E] and Kir3.4[E145R,R155E] mutations, had dramatic effects on agonist activation. Current through these mutant channels was not activated by agonist, and the channels were constitutively active (Fig. 4). Again, this can also be seen in Fig. 3A (bars 8–10).

The agonist-activated current was reduced by 89.5 ± 1.9, 109.4 ± 11.2 and 120.4 ± 13.3% in the Kir3.1/Kir3.4[E145Q], Kir3.1/Kir3.4[R155E] and Kir3.1/ Kir3.4[E145R,R155E] mutant channels, respectively (n = 5–6; ANOVA, p < 0.05) when compared with that of the wild-type channel. Interestingly, only breaking the salt bridge in the Kir3.4 subunit made the channel constitutively active. Breaking the salt bridge in Kir3.1, by the mutation Kir3.1[E139Q], did not affect agonist activation (Fig. 3A, bar 7).

To investigate the effect of disrupting the salt bridge on channel activation further, we measured the sensitivity of the wild-type channel and the Kir3.1/Kir3.4[E145Q] mutant channel (taken as an example of a mutant channel that lacks agonist activation) to Gβγ activation. Fig. 5A shows current through the wild-type channel and the Kir3.1/Kir3.4[E145Q] mutant channel recorded with and without injection of 3 ng/oocyte of Gβγ. Injection of 3 ng/oocyte of Gβγ activated the wild-type channel, but had no effect on the Kir3.1/Kir3.4[E145Q] mutant channel. Panels B and C of Fig. 5 show mean current-voltage relationships and dose-response curves for a range of Gβγ concentrations. At each concentration tested, Gβγ activated the wild-type channel, but failed to activate the Kir3.1/Kir3.4[E145Q] mutant channel (Fig. 5, B and C). These data show that the Kir3.1/Kir3.4[E145Q] mutant channel, in which the salt bridge has been broken, is insensitive to Gβγ activation as well as to agonist activation.

Thus far, we have described mutant channels that do not respond to agonist, such as Kir3.1/Kir3.4[E145Q], as being constitutively active. However, it is possible that these channels are instead highly sensitive to Gβγ. Mutant channels may be maximally activated by endogenous Gβγ and so do not respond to agonist. To distinguish between these two possibilities, we co-expressed Gaq, which sequesters endogenous Gβγ and abolishes basal current through the wild-type channel (26). Fig. 6A shows continuous current recordings at −80 mV from oocytes injected with wild-type or Kir3.1/Kir3.4[E145Q] mutant channels in the absence or presence of 3 ng/oocyte of Gaq. These experiments were performed with the M₃ muscarinic ACh receptor. In the absence of Gaq, substantial basal current was recorded through the wild-type channel and on application of agonist (10 μM ACh), current was increased (Fig. 6A, left as observed previously, e.g., Fig. 2). Co-expression of Gaq abolished basal current, but not the agonist-activated current, through the wild-type channel (Fig. 6A, left). In contrast, basal current through the Kir3.1/Kir3.4[E145Q] mutant channel was not affected by Gaq (Fig. 6A, right). Furthermore, in the absence and presence of Gaq, agonist application still failed to activate the channel (Fig. 6A, right). These results are confirmed by the mean data in Fig. 6B, which shows mean current amplitudes in the absence and presence of ACh for the wild-type and mutant channels with and without Gaq. These data suggest that the Kir3.1/Kir3.4[E145Q] mutant channel is indeed constitutively active and not highly sensitive to Gβγ.

Also highlighted in Fig. 1B are the alanine residue at position 142 in Kir3.1 and the threonine residue that is in the equivalent position in Kir3.4 (Thr-148). In Kir3.1, this site has been shown to be important in high affinity block of the channel by Cs⁺, Rb⁺, and Ba²⁺ (28, 29). Fig. 7 shows the effect of mutation at this site on agonist activation. Replacement of the alanine residue in Kir3.1 with a threonine residue (Kir3.1[A142T]; Fig. 7 left), or the threonine residue in Kir3.4 with an alanine residue (Kir3.4[T148A]; Fig. 7 middle) significantly reduced the extent of channel activation on application of agonist. This effect was modest in the case of the Kir3.3[A142T]/Kir3.4 mutant channel and large in the case of the Kir3.1/Kir3.4[T148A] mutant channel. Fig. 3A (bars 11 and 12) shows that the agonist-activated
current at −130 mV was reduced by 32.6 ± 3.8% and 61.5 ± 1.3%, in the case of the Kir3.1[Δ142T]/Kir3.4 and Kir3.1/Kir3.4[Δ142T]/Kir3.4[Δ148A] mutant channels, respectively, (n = 5; ANOVA, p < 0.05) when compared with that of the wild-type channel. Curiously, the double mutation, Kir3.1[Δ142T]/Kir3.4[Δ148A], had no effect on agonist activation (Fig. 3A, bar 13; Fig. 7; n = 5; not significant).

**Correlation between Agonist Activation and Selectivity**—The salt bridge behind the selectivity filter is likely to be important for the correct conformation of the selectivity filter (30). The dramatic effect of disruption of the salt bridge in Kir3.4 could, therefore, be the result of the disruption of the selectivity filter, rather than the salt bridge per se. It is possible that the effects of the mutation of Ala-142 and Thr-148 in Kir3.1 and Kir3.4 may again be the result of disruption of the selectivity filter. To test this, the effect of the various mutations on channel selectivity as well as agonist activation was investigated. Fig. 3A shows a measure of channel selectivity. In these experiments, the 90 mM K⁺ in the extracellular solution was replaced with 90 mM Rb⁺. Rb⁺ permeated the wild-type channel to a certain extent; at −130 mV, Rb⁺ current was 0.35 ± 0.01 (n = 5) the size of the K⁺ current. However, in the case of the mutant channels in which the salt bridge in Kir3.4 had been broken and in the case of the Kir3.1/Kir3.4[Δ148A] mutant channel, Rb⁺ permeation was dramatically increased; Rb⁺ current was 0.88 ± 0.06, 1.33 ± 0.07, 0.92 ± 0.02, and 0.58 ± 0.03 the size of the K⁺ current in Kir3.1/Kir3.4[Δ145Q], Kir3.1/Kir3.4[Δ155E], Kir3.1/Kir3.4[Δ145Q, Δ155E], and Kir3.1/Kir3.4[Δ145Q, Δ155E] mutant channels, respectively (n = 5–6; ANOVA, p < 0.05), suggesting that, in these channels, selectivity was reduced or abolished (see also Ref. 31). Interestingly, selectivity was also altered by the double mutation, Kir3.1[Δ142T]/Kir3.4[Δ148A]; Rb⁺ current was 0.64 ± 0.02 the size of the K⁺ current (n = 5; ANOVA, p < 0.05). However, selectivity was not affected in the Kir3.1[S170P]/Kir3.4[S176P] or Kir3.1[D173Q] mutant channels; Rb⁺ currents were 0.29 ± 0.02 and 0.32 ± 0.01 the size of the K⁺ current, respectively (n = 2–5; ANOVA, not significant). Fig. 3C shows that agonist dependence and channel selectivity are significantly correlated (r² = 0.47, p < 0.02). These data suggest that the selectivity filter of the Kir3.1/Kir3.4 channel may be the agonist-activated gate.

**Rate of Agonist Activation**—In Fig. 3C, the Kir3.1[Δ139Q]/Kir3.4 mutant channel (point 7) is an outlier: whereas disrupting the salt bridge in the Kir3.1 subunit by the mutation, Kir3.1[Δ139Q], had no effect on the size of the agonist-activated current, it did reduce selectivity; Rb⁺ current was 0.81 ± 0.06 the size of the K⁺ current (n = 5; ANOVA, p < 0.05). However, although the size of the agonist-activated current was not altered, the rate of agonist activation of the Kir3.1[Δ139Q]/Kir3.4 mutant channel was. Fig. 8 shows the rate of agonist activation in the wild-type channel and those mutant channels (including the Kir3.1[Δ139Q]/Kir3.4 mutant channel) in which agonist
dependence was not completely abolished. Typical continuous current traces recorded at -80 mV during the rapid application of the agonist, dopamine, are shown in Fig. 8A. Cells were perfused initially with solution containing no agonist. This was then rapidly exchanged with solution containing dopamine. Fig. 8B shows the mean 10–90% rise time of agonist-activated current in the wild-type and mutant channels. Wild-type current activated in 7.7 ± 0.6 s (n = 13) on application of agonist. Activation was significantly slowed by the mutation Kir3.1[E139Q]; current activated in 11.1 ± 1.2 s (n = 6; ANOVA, p < 0.05). The rate of agonist activation was also slowed by a number of other mutations. Channels in which one of the pairs of phenylalanine residues was replaced (the Kir3.1[F181M]/Kir3.4 and Kir3.1/Kir3.4[F187M] mutant channels) activated more slowly on application of agonist; current activated in 10.8 ± 1.1 s and 12.0 ± 1.0 s, respectively (n = 4–5; ANOVA, p < 0.05). Also the mutations Kir3.4[T148A] and Kir3.1[A142T]/Kir3.4[T148A] slowed channel activation; current activated within 13.6 ± 0.6 s and 12.0 ± 0.3 s, respectively (n = 5; ANOVA, p < 0.05). In contrast, mutation of both of the pairs of phenylalanine residues to methionine residues (Kir3.1[F181M]/Kir3.4[F187M]) or of the alanine residue in Kir3.1 to a threonine residue (Kir3.1[A142T]) had no effect on the rate of agonist activation (n = 6–8; not significant).

Spermine Can Permeate the Channel in the Absence of Agonist—We have previously shown that polyamines, such as spermine, can permeate the Kir3.1/3.4 channel (32–34). Fig. 9 shows that agonist is not required for spermine to permeate the wild-type channel. Fig. 9A (left) shows typical wild-type currents recorded in the presence of 90 mM K+ and in the absence and presence of agonist. Agonist-activated current and mean current-voltage relationships are also shown. The data are similar to those in Fig. 2. Fig. 9A (right) shows currents recorded from the same cell when the 90 mM K+ was replaced with 90 mM spermine. With spermine as the charge carrier, substantial current was recorded showing that spermine can permeate the wild-type channel (32–34). A substantial spermine current was present in the absence of agonist and this was not increased by agonist application (Fig. 9A, right). The spermine current through the wild-type channel was small, but a large spermine current was recorded through channels consisting of Kir3.1 subunits alone (Fig. 9B). In these experiments, the mutation, Kir3.1[F137S], was made to restore K+ current (35). In the Kir3.1[F137S] channel, as in the wild-type channel, whereas K+ current was agonist-dependent, the spermine current did not depend on agonist (Fig. 9B).

**DISCUSSION**

**The Bundle Crossing May Not be the Agonist-Activated Gate**—Our comparative model of the Kir3.1/Kir3.4 channel predicts a narrowing of the permeation pathway at the bundle crossing created by bulky side-chain phenylalanine residues (Fig. 1C) just as in the KirBac1.1 crystal structure (22). It is proposed by Kuo et al. (22) that the presence of large hydrophobic residues at this position may act as a barrier to ion permeation. Consistent with this idea, replacement of the phenylalanine residues in Kir3.1/Kir3.4 with small side chain alanine residues, but not with bulky side chain methionine residues, produced channels that were constitutively active (Figs.
However, these residues may not form the gate that opens and closes in response to agonist activation, because replacement with methionine residues, as in Kir2.1, had only minimal effects on agonist activation (Figs. 2 and 3A) and did not render the channel constitutively active, like the Kir2.1 channel. Furthermore, replacement with alanine residues reduced ion selectivity at the same time as agonist activation (Fig. 3B), suggesting that the effect on agonist activation may be due to disruption of a gate at the selectivity filter, not the bundle crossing. The state-independent access of small MTS reagents (11) and Ag⁺/H₁₁₀₀₁ (12) to the inner vestibule of some K⁺/H₁₁₀₀₁ channels also suggests that the bundle crossing does not form a significant barrier to ion permeation.

**The Selectivity Filter May Act as the Agonist-Activated Gate**—Neutralization of a glutamate residue in the P-loop (Kir3.4[E145Q]) of the Kir3.1/Kir3.4 channel abolishes K⁺ selectivity (31) and reduces Ba²⁺ block of the channel (23). The residue E145 in Kir3.4 is thought to form a salt bridge behind the selectivity filter (27). It may provide the selectivity filter with its tension and hence hold it in its correct conformation (30). We refer to this salt bridge as a bowstring that gives the bow (the selectivity filter) its tension. In the present study, we found that the Kir3.4[E145Q] mutation, and others that disrupt the salt bridge in Kir3.4 such as Kir3.4[R155E] and Kir3.4[E145R,R155E], abolished agonist-activation of the channel rendering them constitutively active (Figs. 3A and 4). Kir3.1/Kir3.4 is a G protein-activated channel and is activated by binding of Gβγ subunits to the cytoplasmic domain of the channel (see Ref. 36 for review). The Kir3.4[E145Q] mutation also abolished activation on binding of Gβγ (Fig. 5). The inability of agonist and exogenous Gβγ to activate the Kir3.1/Kir3.4[E145Q] mutant channel could be the result of a high sensitivity to Gβγ and, therefore, the channel is already maximally activated by endogenous Gβγ. However, current was recorded through the Kir3.1/Kir3.4[E145Q] mutant channel even when endogenous Gβγ had been lowered by co-expression of Gαq (Ref. 26; Fig. 6). Furthermore, under these conditions, agonist still failed to activate the channel. This suggests, there-
fore, that the mutant channels are constitutively active rather than hypersensitive to Gβy. Each of the mutations in Kir3.4 that abolished selectivity for K⁺ also abolished agonist activation (Fig. 3), suggesting that disruption of the normal conformation of the selectivity filter to such an extent that it cannot discriminate between monovalent ions renders the channel constitutively active and, therefore, that the selectivity filter itself acts as the agonist-activated gate.

Interestingly, disruption of the salt bridge in Kir3.1 had comparatively little effect on channel properties: the mutation Kir3.1[E139Q] had no effect on the amplitude of the agonist-activated current (Fig. 4), although it did slow the rate of agonist activation (Fig. 8). It also increased Rb⁺ current to some degree (Fig. 3B). However, using a wider spectrum of monovalent ions, we have previously shown that the effect of the Kir3.1[E139Q] mutation on selectivity is considerably less than that of the equivalent mutation, Kir3.4[E145Q] (31). This suggests that disruption of the salt bridge in Kir3.1 has a more subtle effect on the structure of the selectivity filter. This may be explained by asymmetry of the Kir3.1/Kir3.4 channel pore: the contribution of the two Kir3.1 and two Kir3.4 subunits to the structure of the selectivity filter may not be equal. There is already evidence for this: for example, the tyrosine residue of the Kir3.4 GYG motif, but not of the Kir3.1 GYG motif, is required for selectivity (37). Also, the mutation Kir3.4[D145Q] alters Ba²⁺ block and inward rectification whereas the equivalent mutation, Kir3.1[E139Q], does not (31).

Replacement of the threonine residue at position 148 in Kir3.4 also significantly reduced the response of the channel to agonist (Figs. 3A, 7, and 8). This mutation also altered selectivity for K⁺ (Fig. 3B) suggesting that it probably also alters the conformation of the selectivity filter. Thr-148 probably lines the selectivity filter, because in the Kir2.1 channel mutation at this site (T141A) reduces both Cs⁺ (28) and Ba²⁺ (29) block. Interestingly, this effect in the Kir3.1/Kir3.4 channel was also asymmetrical: the reverse mutation in Kir3.1 (Kir3.1[A142T]) had considerably less effect on the amplitude of the agonist-activated current (Figs. 3A and 7) and no effect on selectivity (Fig. 3B).

The experiments shown in Fig. 9, where spermine was used as a charge carrier, suggest that spermine acts as a foot-in-the-door at the selectivity filter. We have previously shown that spermine can permeate the Kir3.1/Kir3.4 channel (34) suggesting that it can access the selectivity filter. The results in Fig. 9 show that spermine can access the selectivity filter and permeate the channel not only in the presence, but also in the absence, of agonist. This suggests that spermine binding to the selectivity filter acts as a foot-in-the-door: it opens the selectivity filter and this allows permeation.

Proton-activation of the KcsA channel is associated with relatively large movement of the TM2 helices that open the aperture of the bundle crossing (1). Subsequent work on this and other K⁺ channels suggests that the TM2 helices tilt away from the central pore axis about a pivot point so that an intracellular gate at the bundle crossing opens (see Introduction). However, small movement was also observed at the KcsA
selectivity filter on proton-activation (1). It is possible that these relatively small movements alter the conformation of the selectivity filter and effectively gate the access of K⁺. The idea of a flexible selectivity filter is supported by observations that the filter adopts one conformation in the presence of low extracellular K⁺ and another in the presence of high extracellular K⁺ (38), and also it was recently shown that different distortions of the filter could produce channels with differing conductance (39). The idea that the selectivity filter might move during channel opening has been suggested previously. The affinity of the Kir3.1/Kir3.4 channel to block by Ba²⁺ and Cs⁺ is reduced on binding of Gβγ (40) and, furthermore, mutations within the Kir3.1/Kir3.4 pore helix (Kir3.1[E141Q]/Kir3.4[E147Q]) stabilize the single channel open state (41). Several other studies suggest that selectivity may be coupled to gating. Replacement of the second glycine in the GYG motif of the Kir3.2 G protein-activated channel with a serine residue (as occurs in the Weaver mutation) alters both selectivity and agonist activation (20, 21). Also, the selectivity of cyclic nucleotide-gated channels for Ca²⁺ over Na⁺ increases with open probability (42) and the selectivity spectrum of a mutant Shaker channel changes as it passes through subconductance states on its way to the fully open state (43).

**Working Hypothesis of Agonist Activation**—How does the selectivity filter of the Kir3.1/Kir3.4 channel act as a gate when the activating signal, the binding of Gβγ, occurs within the cytoplasmic domain of the channel? Given the wealth of evidence supporting the idea of an intracellular gate and the evidence reported in this study, it is possible that two gates are present in the Kir3.1/Kir3.4 channel: one at the bundle crossing and one at the selectivity filter. A similar suggestion was recently proposed for the Kir1.1 channel in which the intracellular gate is regulated by internal pH and an extracellular gate at the selectivity filter is regulated by external K⁺ (44). Whether the two gates of the Kir3.1/Kir3.4 channel are coupled to one another or act independently would require further investigation. Another possibility is that a lateral force exerted by Gβγ binding at the cytoplasmic domain could be transduced not only to the TM2 domains, causing them to rotate and translate (7, 22), but also across the glycine hinge to induce subtle conformational changes within the selectivity filter. Many of the observations reported in this study support this latter hypothesis; both agonist activation and selectivity were altered by the replacement of phenylalanine residues at the bundle crossing, suggesting that changes at the bundle crossing can indeed alter the conformation of the selectivity filter. Also, disruption of the salt bridge that maintains the tension of the selectivity filter abolished agonist activation and selectivity, suggesting that relaxation of the filter renders the signal transduced from the cytoplasmic domain on binding of Gβγ unnecessary. Introduction of a proline kink within the TM2 domain (by the double mutation Kir3.1[S170P]/Kir3.4[S176P]) abolished agonist activation, but did not affect selectivity; this double mutation perhaps had a more subtle effect on the selectivity filter. However, of course, Gβγ binding presumably also opens the channel without causing a major change in selectivity. These data sug-
gest, therefore, that transduction of the activating stimulus to the selectivity filter may explain why mutations at both intracellular and extracellular sides of the channel affect agonist activation and, also, the correlation between agonist activation and selectivity for $K^+$. 

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