Coupling of Voltage Sensing to Channel Opening Reflects Intrasubunit Interactions in Kv Channels

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Abstract
Voltage-gated K⁺ channels play a central role in the modulation of excitability. In these channels, the voltage-dependent movement of the voltage sensor (primarily S4) is coupled to the (S6) gate that opens the permeation pathway. Because of the tetrameric structure, such coupling could occur within each subunit or between adjacent subunits. To discriminate between these possibilities, we analyzed various combinations of an S4 mutation (R401N) and an S6 mutation (P511G) in hKv1.5, incorporated into tandem constructs to constrain subunit stoichiometry. R401N shifted the voltage dependence of activation to negative potentials while P511G did the opposite. When both mutations were introduced in the same α-subunit of the tandem, the positive shift of P511G was compensated by the negative shift of R401N. With each mutation in a separate subunit of a tandem, this compensation did not occur. This suggests that for Kv channels, the coupling between voltage sensing and gating reflects primarily an intrasubunit interaction.

Key words: hKv1.5 • gating • S4 segment • S6 gate • dimers

Introduction
Voltage-gated K⁺ channels (Kv channels) respond to changes in the electric field across the cell membrane by opening or closing the ion-conducting pore. They are composed of four individual α-subunits each containing six membrane-spanning helices (S1–S6) and a pore loop between S5 and S6. The S4 segment makes up the main part of the channel’s voltage sensor that moves and/or reorients in response to a change in the membrane potential (for review see Bezanilla, 2000). This repositioning triggers the opening (or closing) of the cytoplasmic activation gate, which is considered to be located in the lower part of S6 (del Camino and Yellen, 2001). The crystal structures of the bacterial K⁺ channels KcsA, MthK, KirBac, and KvAP suggest that the pore forming regions S5–P–S6 of each subunit are assembled in a tetrameric configuration with intra- and intersubunit interactions (Doyle et al., 1998; Jiang et al., 2002, 2003; Kuo et al., 2003). Based on these structures, a gating model has been proposed in which the movement of the voltage sensor (regions S2–S4), proposed to be directly attached to the S5 helix, might open the pore by pulling S5 away from the central pore axis and causing the S6 helix to follow. In this model, S5 and S6 move together as one unit, suggesting an intrasubunit interaction between voltage sensing and gate opening (Jiang et al., 2003). However, this mechanism could be different in eukaryotic Kv channels because the structure of the voltage sensor may differ from the KvAP’s voltage sensor “paddle” (Gandhi et al., 2003; Laine et al., 2003; Lee et al., 2003; Ahern and Horn, 2004; Starace and Bezanilla, 2004) and because of the presence of a functionally important PX̂P motif in the S6 domain that is absent in KvAP (Labro et al., 2003). Furthermore, other channel segments might be involved in gating since several studies have provided evidence for interactions between the S4–S5 linker and residues in the COOH-terminal part of S6 in HERG, HCN, and Shaker (Lu et al., 2002; Tristani-Firouzi et al., 2002; Ding and Horn, 2003; Decher et al., 2004). Since subunit cooperativity is a recurrent feature in the various kinetic models of channel gating (Tytgat and Hess, 1992; Zagotta et al., 1994; Schoppa and Sigworth, 1998), the question arises whether the mechanism that couples voltage sensing to gate opening reflects an interaction between neighboring subunits or whether it occurs within the same subunit. This is an important question because the answer will determine whether this coupling mechanism acts largely independently within each subunit or whether it reflects a highly cooperative interaction between neighboring subunits. To discriminate between both hypotheses, we analyzed an S4 mutation in combination with an S6 mutation in human Kv1.5 tandem constructs to control the subunit stoichiometry (Liman et al., 1992).

Materials and Methods
Molecular Biology

hKv1.5 was expressed using a pBK-CMV expression vector and mutations were introduced as described previously (Labro et al., 2003).
2003). Tandem constructs were obtained by creating a first and a second hKv1.5/pBK-CMV vector, each representing the first and the second subunit of the tandem. In the first vector, the stop codon was removed (replaced by a serine codon) together with the introduction of an Xba1 restriction site. In the second vector, an Xba1 site was inserted three nucleotides upstream of the start ATG. Tandem constructs were subsequently created by cutting the hKv1.5 sequence out of the first vector with Hind3/Xba1 and ligate it in the second vector that had been digested with Hind3/Xba1. This yielded a tandem with two complete hKv1.5 sequences linked together with a three amino acid–long linker. The muta-
tions R401N or P511G or double mutant R401N/P511G were
introduced separately in the first or second vector before creating
the tandems. Note that the hKv1.5 numbering used here refers
to the original numbering of Tamkun et al. (1991) and used in
our subsequent papers; in the revised numbering (accession no.
P22460), R401 is R403 and P511 is P513.

Electrophysiology

Ltk− cells were cultured and transfected (1 μg cDNA) as de-
scribed previously (Labro et al., 2003). Current recordings were
made with an Axopatch-200B amplifier (Axon Instruments) in
the whole cell configuration at room temperature; current re-
cordings were low pass filtered and sampled with a Digidata-
1200A data acquisition system (Axon Instruments). Command
voltages and data storage were controlled with pClamp8 software
(Axon Instruments). Patch pipettes were pulled from 1.2-mm
quick-fill borosilicate glass capillaries (World Precision Instru-
ments) with a P-2000 puller (Sutter Instruments) and heat pol-
ished. The cells were perfused continuously with a bath solution
containing (in mM) NaCl 130, KCl 4, CaCl2 1.8, MgCl2 1, HEPES
10, glucose 10, adjusted to pH 7.35 with NaOH. The pipettes were
filled with intracellular solution containing (in mM) KCl 110,
K4BAPTA 5, K3ATP 5, MgCl2 1, HEPES 10, and adjusted to pH 7.2
using KOH. Experiments were excluded from analysis if the volt-
age errors originating from the series resistance exceeded 5 mV.

The holding potential was −80 mV in most experiments, but
set to −90 mV for mutants containing the R401N mutation. The
interpulse interval was at least 15 s, but was increased up to 45 s
as needed for some mutants to prevent accumulation of slow in-
activation. Voltage protocols (voltage range and step duration)
were adjusted based upon the different biophysical properties of
mutant channels. Activation and deactivation time constants
were determined with a single or double exponential function;
the goodness of the fit was judged by inspection of the residuals.

Figure 1. Biophysical properties of channels containing WT-hKv1.5, R401N, P511G, or R401N+P511G monomers. (A) Raw current
traces elicited by the voltage protocol shown on top. From left to right, the current recordings of WT-hKv1.5, the mutant R401N, the
double mutant R401N+P511G, and the mutant P511G. The horizontal bar on the left indicates the zero current level. Notice the
difference in the threshold of activation. (B) Voltage dependence of activation. R401N (circles) shifted the activation curve toward
negative values while P511G (open circles) shifted it toward positive potentials, compared with WT-hKv1.5 (dotted line). Note that the
double mutant R401N+P511G (inverted triangles) had an activation curve that was shifted toward WT values compared with P511G. (C)
Activation and deactivation kinetics. R401N had time constants that were slightly slower than WT (open triangles). In contrast, P511G
displayed marked slowing of activation and deactivation. The double mutant R401N+P511G had activation time constants similar to those
of P511G but deactivated extremely slow (clearly slower than R401N or P511G).
and substitutions (indicated in bold). The residue numbering on top is for hKv1.5, with the top section, partial sequences of the S4 and S6 segments of WT-hKv1.5. The S4, the S6, and the double mutations are named according to their jgp.200409194/DC1.

The residue numbering in parentheses. Main section, voltage dependence of activation of WT and mutant channels formed by monomeric or tandem subunits, as indicated. The configuration of WT and mutant subunits within a tandem construct is represented as 1st 2nd subunit (starting from the NH2 terminus). The midpoint of activation ($V_{1/2}$), slope factor (k), and $\Delta G_0$ for the voltage dependence of activation were derived as described in MATERIALS AND METHODS. The results are expressed as mean ± SEM and n the number of cells. For raw current traces of dimers and tandem constructs see Figs. S2 and S3 (http://www.jgp.org/cgi/content/full/jgp.200409194/DC1).

### TABLE I

**Biophysical Parameters for Voltage Dependence of Activation**

| Monomeric     | $V_{1/2}$ | k    | $\Delta G_0$ | n  |
|---------------|-----------|------|--------------|----|
| WT-hKv1.5     | -14.3 ± 1.0 | 5.8 ± 0.2 | -1.35 ± 0.11 | 9  |
| R401N         | -47.9 ± 1.7 | 9.3 ± 0.9 | -2.81 ± 0.29 | 5  |
| P511G         | 44.2 ± 1.2  | 8.6 ± 0.2 | 2.79 ± 0.09  | 10 |
| R401N+P511G   | 21.6 ± 1.2  | 10.0 ± 0.4 | 1.18 ± 0.08  | 5  |

| Tandem constructs | $V_{1/2}$ | k    | $\Delta G_0$ | n  |
|-------------------|-----------|------|--------------|----|
| 1st               | 2nd       |      |              |    |
| WT                | WT        | -6.5 ± 1.2 | 6.3 ± 0.2 | -0.56 ± 0.11 | 12 |
| R401N             | R401N     | -46.2 ± 0.6 | 9.9 ± 0.4 | -2.55 ± 0.11 | 4  |
| R401N             | WT        | -17.9 ± 1.6 | 7.7 ± 0.2 | -1.27 ± 0.12 | 9  |
| WT                | R401N     | -15.6 ± 1.5 | 8.4 ± 0.3 | -1.01 ± 0.10 | 7  |
| P511G             | P511G     | 32.0 ± 1.0  | 9.2 ± 0.4 | 1.90 ± 0.11  | 7  |
| P511G             | WT        | 26.2 ± 0.9  | 13.6 ± 0.3 | 1.05 ± 0.08 | 8  |
| WT                | P511G     | 35.8 ± 0.4  | 11.9 ± 0.4 | 1.64 ± 0.06 | 6  |
| R401N+P511G      | R401N+P511G | 12.7 ± 1.6 | 9.0 ± 0.4 | 0.77 ± 0.10 | 8  |
| R401N+P511G      | WT        | 10.0 ± 1.3  | 9.2 ± 0.3 | 0.59 ± 0.08 | 12 |
| WT                | R401N+P511G | 13.0 ± 2.0 | 10.8 ± 0.6 | 0.66 ± 0.07 | 7  |
| P511G             | R401N     | 24.1 ± 0.8  | 13.1 ± 0.5 | 1.01 ± 0.07 | 6  |
| R401N             | P511G     | 31.8 ± 3.0  | 11.5 ± 0.5 | 1.51 ± 0.16 | 7  |

Activation curves were fitted with a Boltzmann equation: $y = 1/(1 + \exp[-(E - V_{1/2})/k])$, with E the applied voltage, $V_{1/2}$ the midpoint potential, and k the slope factor. 1/k corresponds to $kT/\epsilon$ with $\epsilon$ the equivalent charge, and F, R, and T have their usual meaning. The Gibbs free energy of activation at 0 mV ($\Delta G_0$) was calculated by $\Delta G_0 = 0.2389zFV_{1/2}$, with the factor 0.2389 to express the values in cal/mol (Li-Smerin et al., 2000). Results are expressed as mean ± SEM.

### Online Supplemental Material

The online supplemental material (Figs. S1, S2, and S3) is available at http://www.jgp.org/cgi/content/full/jgp.200409194/DC1. Fig. S1 shows current traces and properties of channels originating from either a cotransfection of WT and P511G subunits or from the tandem construct 1:WT_2:P511G. It demonstrates that the biophysical properties of channels originating from a cotransfection are clearly different from those of channels resulting from the tandem construct. In addition, it further highlights that both subunits of the tandem construct are integrated in the tetramer. Figs. S2 and S3 show the raw current recordings of all dimers and tandem constructs used.

### RESULTS

Channel activation is thought to occur in a succession of conformational states that include the movement or reorientation of the voltage sensor and the opening of the channel gate. It has been suggested that the lower part of S6 forms the cytoplasmic activation gate while S4 has been considered to be the main part of the voltage-sensing domain. Therefore we selected in hKv1.5, a Shaker-type K+ channel, one mutation in S4 and another in S6 that both altered the energetics of activa-
Intrasubunit S4–S6 Gating Linkage. These mutations were chosen on the basis of their location as well as their functional effects.

In the S6 segment, we used a glycine substitution of the second proline of the highly conserved PXP motif (Table I). This mutation (P511G) shifted the voltage dependence of activation by 60 mV toward positive potentials (Fig. 1, A and B; Table I), and slowed the kinetics of activation and deactivation ~104-fold compared with WT-hKv1.5 (Fig. 1 C) (Labro et al., 2003). The mutation in S4 (R401N) neutralized the second positive charge of the S4 segment and caused a hyperpolarizing shift of ~30 mV in the voltage dependence of activation (Fig. 1, A and B; Table I), similar to the effects of the corresponding R365N mutation in Shaker (Miller and Aldrich, 1996). The activation and deactivation time constants were slightly slower compared with WT-hKv1.5 (Fig. 1 C).

When both mutations were combined in the same subunit (R401N/P511G), the positive shift (P511G) was partially compensated by the negative shift (R401N), and the midpoint was shifted back toward WT values (Fig. 1, A and B; Table I). However, the compensation did not occur for the time constants. To express these shifts in midpoints as differences in the free energy between the closed and the open state, we calculated ΔG0 (at 0 mV) for each mutant as described in experimental procedures. Consistent with the shifts of the activation curves, both mutations altered the ΔG0 compared with WT (Table I) and the combination of both mutations (double mutant R401N+P511G) had a compensatory effect on the ΔG0. According to the KvAP crystal structure (Jiang et al., 2003) and the latest Shaker channel models (Durell et al., 2004; Shrivastava et al., 2004), both residues R401 and P511 are most likely separated in space and their compensatory effect should thus not be due to close packing effects.

To discriminate between an inter- or intrasubunit interaction, it was necessary to control the stoichiometry of subunits in the tetrameric channel. Therefore, tandem subunits were constructed with predetermined locations of the S4 and the S6 mutations. The WT-hKv1.5 dimer had a voltage dependence of activation that was only slightly shifted (~8 mV) toward positive potentials, compared with channels derived from WT-hKv1.5 monomers (Fig. 2; Table I). The biophysical properties of channels containing the R401N dimer were not significantly different from those containing the corresponding monomer (Fig. 3; Table I). For the P511G mutation the voltage dependence of activation of the dimer was shifted 12 mV toward negative potentials compared with the monomer (Fig. 3; Table I). The activation and deactivation time constants of both were comparable (Fig. 2 C). The biophysical properties of channels containing the R401N dimer were not significantly different from those containing the corresponding monomer (Fig. 3; Table I). For the P511G mutation the voltage dependence of activation of the dimer was shifted 12 mV toward negative potentials compared with the monomer (Fig. 3; Table I). The activation and deactivation time constants of both were comparable (Fig. 2 C). The biophysical properties of channels containing the R401N P511G dimer compared well to those of the monomer (Fig. 1 C; Fig. 3 B), with slowed deactivation kinetics.
These results showed that the functional properties of channels composed of WT, R401N, P511G, or R401N+P511G monomers or dimers were similar. Therefore, the use of dimer constructs had minimal effects on gating. It has been suggested that only one of the subunits in a tandem construct may be incorporated in the functional tetrameric channel protein (McCormack et al., 1992). To exclude this possibility, we created tandem constructs that contained one mutant (R401N or P511G) subunit and one WT subunit in either the configuration mutant–WT or WT–mutant. If only a single subunit of the tandem would be included in the tetramer, we would expect clear functional differences between these two opposite configurations (mutant–WT and WT–mutant). The tandem with an R401N mutation in the first subunit and WT as second subunit (i.e., a 1:R401N_2:WT configuration) had properties that were indistinguishable from those of the opposite configuration (1:WT_2:R401N), but both were clearly different from the R401N dimer (Fig. 4 A; Table I). Both tandems had a midpoint of activation that was only 10 mV more negative than that of a WT-hKv1.5 dimer (Fig. 4 A; Table I). Furthermore, the time constants of activation and deactivation of these tandems compared more to those of the R401N dimer than those of the WT dimer, taking into consideration the shifts in voltage dependence of activation (Fig. 4 B). The tandems with a 1:WT_2:P511G and a 1:P511G_2:WT configuration (Fig. 4 C, top) had a positive midpoint potential similar to the P511G dimer and markedly different from WT (Fig. 4 C). In contrast to the R401N/WT tandems, the kinetics of the channels expressed from either the 1:WT_2:P511G or the 1:P511G_2:WT tandem were best fitted with a double exponential function in which the slow component compared well to the P511G gating, as expected if the slowly gating P511G subunit forms part of the tetramer. To confirm further the integration of both subunits of a dimer in a channel, the properties of the 1:WT_2:P511G tandem were compared with those of a cotransfection of WT and P511G subunits (Fig. S1, available at http://www.jgp.org/cgi/content/full/jgp.200409194/DC1). In the case of the cotransfection, a mixed population of channels with binomially distributed ratios of subunits is expected. The biophysical properties derived from the currents of this mixed population were clearly different from the ones obtained with the tandem construct, strengthening the idea that both subunits of the dimer were integrated in a functional channel. All together, these results with oriented tandems and cotransfections strongly indicate that both subunits of the tandem constructs were assembled in a tetrameric channel. The raw current traces for all constructs in Fig. 4 are shown in Fig. S2 (available at http://www.jgp.org/cgi/content/full/jgp.200409194/DC1).

The results above indicated that the R401N mutation could compensate for the positive shift of the P511G mutation in a monomeric (Fig. 1) and tandem configuration (Fig. 3). Furthermore, in tandems with one WT and one mutant subunit, channel opening was found to be determined largely by the gating machinery with the most positive voltage dependence of activation, which was WT for the WT/R401N cases (Fig. 4 A), and P511G for the WT/P511G combinations (Fig. 4 C). Thus, combining a single S4 and S6 mutation in all possible positional combinations in tandem subunits should allow us to determine if the gating machinery that couples voltage sensitivity to gate opening is an inter- or intrasubunit interaction. To investigate the hypothesis of an intrasubunit interaction, tandem constructs with one WT and one double mutant (R401N+P511G) subunit were created (Fig. 5 A, top). The reference construct for these experiments was the...
R401N+P511G dimer (both subunits containing both mutations), the condition in which the S4 mutation always compensates the S6 mutation independent of an inter- or intrasubunit interaction. The tandems with either the 1:WT_2:R401N+P511G or the 1:R401N+P511G_2:WT configuration resulted in functional channels with a voltage dependence of activation nearly indistinguishable from the reference R401N+P511G dimer (Fig. 5 A; Table I). The activation and deactivation time constants of both R401N/WT tandem configurations were similar to those of the R401N dimer. The voltage dependence of activation of the tandem with the opposite configuration 1:P511G_2:WT (red circles) was slightly shifted toward negative potentials compared with the P511G dimer; but compared with WT (dotted line), the midpoint was still at positive potentials. (D) Time constants of both P511G/WT tandem configurations were similar to those of the P511G dimer. For raw current traces of dimers and tandem constructs see Fig. S2 (available at http://www.jgp.org/cgi/content/full/jgp.200409194/DC1).

To exclude an intersubunit interaction, tandem constructs with both the S4 and the S6 mutation in a separate subunit (Fig. 5 C, top) were used. The channels containing the 1:R401N_2:P511G tandem had a midpoint potential similar to the P511G dimer (Fig. 5 C; Table I), indicating that no compensation of the voltage dependence of activation had occurred. The tandem with the opposite configuration 1:P511G_2:R401N resulted in a channel with an activation curve that was slightly shifted (∼8 mV) toward negative potentials compared with the P511G dimer. However, compared with the reference R401N+P511G dimer, it was still
quite positive (Fig. 5 C). The activation and deactivation time constants of both 1:R401N_2:P511G and 1:P511G_2:R401N were also best approximated with a double exponential function. Despite this, both configurations deactivated faster than the reference R401N+P511G dimer, unrelated to the shifted voltage dependence of activation (Fig. 5 D).

**DISCUSSION**

A voltage-gated K⁺ channel operates by sensing the transmembrane voltage (S4 being the main component of the voltage sensor) and subsequently opening a gate (in Shaker-type channels, the lower part of S6 contributes to the intracellular gate). Since these channel proteins are tetramers of tightly associated α-subunits, this coupling might occur within the same subunit or between neighboring α-subunits. To address this question, we selected mutations in S4 and S6 that shifted the voltage dependence of activation in the opposite direction. We hypothesized that we could take advantage of the compensation of these shifts when both mutations “communicated” with each other through a coupling mechanism within or between subunits. To exploit this strategy, at least two requirements had to be fulfilled: (1) both mutations should affect gating independently and their shifted voltage dependencies should compensate each other, and (2) the stoichiometry of subunits within a channel should be controlled.

The S4 (R401N) and S6 (P511G) mutation shifted the voltage dependence of activation, compared with WT-hKv1.5, toward negative (Miller and Aldrich, 1996)
and positive potentials, respectively (Fig. 1 and 3; see also Labro et al., 2003). Combining both mutations shifted the activation curve back toward WT (Fig. 1 B). Since both mutations are located in different regions of the channel protein, their compensatory effect on channel gating would therefore not be due to close packing of both residues, and we hypothesize that they communicated through a coupling mechanism. To control the stoichiometry of the mutations within a functional channel, tandem constructs were used (Tytgat and Hess, 1992). Tandems with one mutant (R401N or P511G) and one WT subunit indicated that both subunits of the tandem participated in a functional channel (Fig. 4) (McCormack et al., 1992). Furthermore, a tandem with two “different” subunits displayed, in contrast with homodimeric tandems, two components in the kinetics of current activation. Finally, the comparison of a cotransfection with a tandem construct supported the idea that both subunits of the tandem were integrated in a functional channel.

The use of these mutations in a controlled stoichiometry allowed us to address whether the coupling mechanism originates within or between subunits. The intrasubunit nature of the interaction was supported by the results of the tandems 1:R401N+P511G_2:WT and 1:WT_2:R401N+P511G because (1) the midpoint potential was similar to the reference R401N+P511G dimer and (2) both tandems deactivated extremely slow, a feature typical for the double mutant R401N+P511G and not observed with the tandems 1:R401N_2:P511G and 1:P511G_2:R401N (Fig. 5, B and D). Furthermore, the results for the tandem configurations 1:R401N_2:P511G and 1:P511G_2:R401N do not support an intersubunit interaction since the midpoint was similar to that of the P511G dimer and that of the tandem constructs with a WT subunit instead of the mutant R401N (tandems 1:WT_2:P511G and 1:P511G_2:WT, respectively; Fig. 4 B).

In the gating model based on the 3D crystal structure of the bacterial voltage-gated K⁺ channel KvAP, the movement of the voltage sensor “paddle” exerts force on S5, through the S4–S5 linker, pulling S5 away from the central pore axis (Jiang et al., 2003). It was suggested that S5 and S6 move together as one unit, resulting in pore widening. This model is consistent with an intrasubunit interaction, but admittedly, the deduction of a dynamic gating mechanism from a static crystal structure remains inconclusive. Moreover, recent results suggest that the voltage sensor of Shaker-type channels differs from that of KvAP (Gandhi et al., 2003; Laine et al., 2003; Lee et al., 2003; Ahern and Horn, 2004; Durell et al., 2004; Shrivastava et al., 2004; Starace and Bezanilla, 2004). An alternative gating hypothesis for eukaryotic K⁺ channels is based on the direct coupling of the S4–S5 linker to the COOH-termi-
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