Independence and Cooperativity in Rearrangements of a Potassium Channel Voltage Sensor Revealed by Single Subunit Fluorescence

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abstract Voltage-gated potassium channels are composed of four subunits. Voltage-dependent activation of these channels consists of a depolarization-triggered series of charge-carrying steps that occur in each subunit. These major charge-carrying steps are followed by cooperative step(s) that lead to channel opening. Unlike the late cooperative steps, the major charge-carrying steps have been proposed to occur independently in each of the channel subunits. In this paper, we examine this further. We showed earlier that the two major charge-carrying steps are associated with two sequential outward transmembrane movements of the charged S4 segment. We now use voltage clamp fluorometry to monitor these S4 movements in individual subunits of heterotetrameric channels. In this way, we estimate the influence of one subunit's S4 movement on another's when the energetics of their transmembrane movements differ. Our results show that the first S4 movement occurs independently in each subunit, while the second occurs cooperatively. At least part of the cooperativity appears to be intrinsic to the second S4 charge-carrying rearrangement. Such cooperativity in gating of voltage-dependent channels has great physiological relevance since it can affect both action potential threshold and rate of propagation.

key words: potassium channel • cooperativity • fluorescence • voltage-sensing • gating

INTRODUCTION

The model of Hodgkin and Huxley (1952) for the activation of voltage-dependent potassium channels postulates that voltage controls the conductance of membranes by changing the equilibrium between two states (resting and activated) of four identical and independent charged membrane particles. This hypothesis was supported by the finding that potassium channels are composed of four subunits (MacKinnon, 1991), each containing an S4 segment that consists of a sequence of basic residues conserved within the primary structure of voltage-gated ion channels, and therefore hypothesized to confer voltage sensitivity (Noda et al., 1984; Greenblatt et al., 1985; Guy and Seetharamulu, 1986). The idea that S4 is the Hodgkin and Huxley voltage sensing gating particle has been supported by recent evidence based on S4 accessibility to internal and external solutions (Yang and Horn, 1995; Larsson et al., 1996; Yang et al., 1996; Yusaf et al., 1996; Starace et al., 1997; Baker et al., 1998), on total gating charge measurements after neutralization of its basic residues (Aggarwal and MacKinnon, 1996; Seoh et al., 1996), and on real-time fluorescence measurement of its motion (Mannuzzu et al., 1996; Cha and Bezanilla, 1997, 1998; Baker et al., 1998). These studies showed that S4 traverses the membrane, moves across the membrane electric field in the direction expected for the voltage sensor, and can account for the majority of the gating current that is generated during channel activation (acidic residues in S2 and S3 may also contribute to the gating charge; Seoh et al., 1996; Cha and Bezanilla, 1997).

While the description of Hodgkin and Huxley captures some of the essential features of voltage-dependent gating (four identical gating particles, a sequence of transitions that yields sigmoidicity to channel opening), it has required two major modifications to account for the details of potassium channel gating. First, voltage sensor movement occurs not in one but in several steps (Stühmer et al., 1991; Schoppa et al., 1992; Bezanilla et al., 1994; McCormack et al., 1994; Schoppa and Sigworth, 1998a,b,c). At least two of these steps are generated by sequential movements of S4 (Baker et al., 1998). Second, several lines of evidence indicate that late transitions that follow the major gating charge carrying steps are cooperative (Hurst et al., 1992; Tytgat and Hess, 1992; Bezanilla et al., 1994; Lin et al., 1994; Sigg et al., 1994; Zagotta et al., 1994a,b; Zheng and Sigworth, 1997, 1998; Schoppa and Sigworth, 1998a,b,c; Smith-Maxwell et al., 1998a,b; Ledwell and Aldrich, 1999). The most successful models for gating of the *Shaker* potassium channel describe it as a series of independent charge carrying transitions followed by at least one concerted or highly cooperative transition.

Cooperativity in channel activation represents an issue of fundamental importance for understanding the mechanics of channel function. Subunit interaction in activation is expected to have important physiological consequences by influencing voltage sensitivity in the

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same way that cooperative interaction among binding sites affects the sensitivity of an allosteric protein to ligand concentration. Such cooperativity could account for the discrepancies previously described between voltage sensitivity and number of charges in S4 (Stühmer et al., 1989; Papazian et al., 1991; Sigworth, 1994; Tang and Papazian, 1997; Smith-Maxwell et al., 1998a,b).

Recent studies from the Aldrich laboratory have demonstrated a role for S4 in cooperativity in channel activation (Smith-Maxwell et al., 1998a,b; Ledwell and Aldrich, 1999). Smith-Maxwell et al. (1998a,b) found that a triple mutation in the *Shaker* S4, inserting the sequence of the Shaw S4, makes the voltage dependence of activation shallow and shifts it to positive voltages, while making the ionic current activate slowly, with a single exponential time course that lacks the sigmoidicity of *Shaker.* These effects were accounted for by slowing a final cooperative transition that follows the two independent charge-carrying steps per subunit according to the model of Zagotta et al. (1994b). As a consequence, this step, which itself appears to carry a small amount of gating charge (Smith-Maxwell et al., 1998b; Ledwell and Aldrich, 1999), becomes rate limiting for channel opening. In other words, mutations in S4 can alter cooperative interactions between subunits in late steps of channel activation.

In this study, we used a novel approach to examine subunit interaction during activation. We did this by taking advantage of the ability of voltage-clamp fluorometry (Mannuzzu et al., 1996) to monitor particular gating rearrangements in site-specifically labeled protein segments of individual subunits in heterotetrameric *Shaker* channels. Our test consisted in determining whether or not S4 movement of a labeled subunit is influenced by coassembly with other subunits having different S4 gating properties. Absence of influence was taken to indicate independence and presence of influence to indicate cooperativity. This approach allowed us to evaluate whether cooperative interactions occur during the major S4 charge carrying movements that have been previously thought to be independent. We find that, of S4's two major charge-carrying steps, the first takes place independently in each subunit, whereas the second is influenced by cooperative interaction between S4 segments. Our results suggest that at least some of this cooperativity is intrinsic to the second major S4 movement. We interpret our results in light of the earlier kinetic models.

MATERIALS AND METHODS

Molecular Biology

Experiments were performed on nonconducting, ball-deleted ShH4 (W434F/Δ6-46) *Shaker* channels (Kamb et al., 1987; Hoshi et al., 1990; Perozo et al., 1993) after removal of two native cysteines (C245V and C462A) (Mannuzzu et al., 1996) to ensure that membrane-impermeant fluorescent thiol reagents would attach exclusively to a known position of cysteine addition (S352C) near the outer end of S4. Site-directed mutagenesis, dimer and tetramer fusion gene construction, cRNA synthesis, and cRNA injection into *Xenopus* oocytes were as described previously (Isacoff et al., 1990) except that T7 Ambion kits MEGAscript or mMESSAGE mMACHINE (Ambion Inc.) were used for the transcriptions.

For the coinjection experiments, a S352C:L382V or S352C/L382V:wild-type ratio of 1.5:8.5 was used to maximize the fraction of channels containing only one fluorophore binding subunit. For free association between subunits, these ratios are predicted to yield 52, 37, 10, 1, and ~0% channels with zero, one, two, three, or four labeled subunits, respectively, so that ~62% of the fluorescence will come from channels with one labeled subunit.

We ensured that linked heterotetrameric constructs with one labeled subunit produced channels with the subunit stoichiometry defined by their tandem linkage in two ways: (a) by comparing linked tetramers, such as A-B-B-B, to coinjections of cRNAs A + B at a ratio that favored B by 5.7-fold (this comparison produced identical results for studies with the L382V mutation); (b) by attempting to reduce the opportunity for intermolecular assembly of tetramers and favoring intramolecular assembly. This was done by injecting progressively more diluted cRNA encoding the linked tetramers to express channel protein at low enough levels so that the highest chance of interaction in endoplasmic reticulum would be between subunits of the same nascent protein. For the linked tetramer C*/S-W-W-W (S352C-TMRM/R365S linked to three wild-type subunits; Fig. 1) at high levels of expression there was a positive f1, with no voltage shift, and a negative f2, with a very minor leftward voltage shift, consistent with channels containing multiple C*/S subunits due to intermolecular assembly—precisely the situation we need to avoid. At fourfold lower expression levels of C*/S-W-W-W, both f1 and f2 were positive, f1 was not shifted, and f2 was shifted far to the left. Further lowering of expression did not change the behavior. The results are consistent with intertetramer assembly at high expression levels, but not over the range of lower expression levels on which we based our analysis.

Voltage Clamp Fluorometry

Voltage clamp fluorometry, oocyte preparation and incubation, and tetraglycine maleimide (TGM)[1] blocking were performed as described earlier (Mannuzzu et al., 1996). In brief, defolliculated, cRNA-injected oocytes were incubated for 4–6 d at 12°C, either in MBSH [88 mM NaCl, 1 mM KCl, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM HEPES, pH 7.5] or ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6). At this temperature, channel protein is made, but little is expressed at the plasma membrane. Cysteines of native oocyte membrane proteins were then blocked by a 30-min incubation at room temperature in 0.1–0.5 mM TGM. The oocytes were subsequently washed, and then incubated for ~14 h at room temperature to incorporate the channels into the plasma membrane. Fluorescent labeling was done for 30 min on ice with 5–10 μM tetramethylrhodamine-5-maleimide (TMRM) (Molecular Probes).

TGM was synthesized by incubating 25 mM tetraglycine (TG; Aldrich Chemical Co.), dissolved in 0.1 M NaCl, 0.1 M Naphosphate, pH 7.25, with 10 mM succinimidyl trans-4-(*N*-maleimidylmethyl)cicloexane-1-carboxylate (SMCC; Molecular Probes) for 1 h at 37°C. TGM was purified from the unreacted components by

[1]*Abbreviations used in this paper:* f-v, fluorescence–voltage; TGM, tetraglycine maleimide; TMRM, tetramethylrhodamine-5-maleimide; q-v, charge–voltage.

HPLC, using a semipreparative C18 reversed phase column, and an acetonitrile gradient (+0.1% trifluoroacetic acid). TGM concentration was measured according to Sedlak and Lindsay (1968).

The two-electrode voltage clamping was performed with a CA-1 amplifier (Dagan Corp.). External solution was NaMES (110 mM NaMES, 2 mM $Ca(MES)_2$, 10 mM HEPES, pH 7.5). Capacitance compensation was performed from a holding potential of +60 mV (or +100 mV for channels carrying the L382V mutation). A photomultiplier tube (HC120-05; Hamamatsu Phototonics) was used for fluorescence measurements.

Since fluorophore attachment could alter gating, and interaction between fluorophores bound to neighboring subunits could alter the fluorescence report of gating movements, two means were used to ensure that differences in fluorescence–voltage relations (f-v's) between channels did not arise from differences in the number of labeled subunits per channel. First, f-v's were compared, and found to be similar, in channels composed of subunits with similar voltage dependence, but with a different number of labeled subunits (e.g., see Fig. 7 B). Second, photodestruction of TMRM molecules bound to the 352C homotetramer (labeled to saturation) produced only a small (<5 mV) leftward shift in the f-v (Fig. 2). This indicates that TMRM–TMRM interaction between subunits had no significant effect on our comparisons, and that, if anything, it led to a slight underestimate of the degree of the cooperative influence. Nevertheless, to avoid this complication, comparisons were also made between channels made of distinct subunits, but in which only one subunit was labeled (Fig. 7 B).

All comparisons were made within the same batch of oocytes in the same experiment under the same labeling conditions. Values are reported as mean ± SEM.

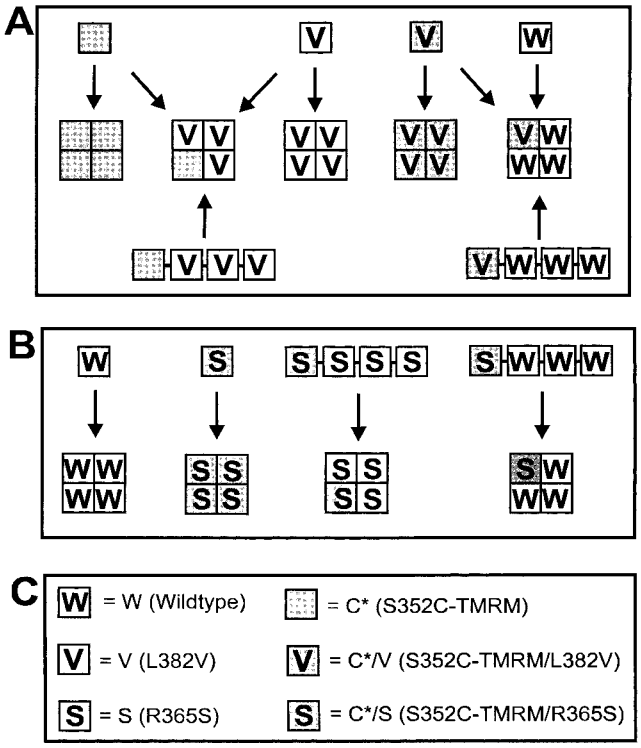

Figure 1. Subunit stoichiometry in heterotetrameric channels. Cartoon shows expected subunit arrangement of labeled (shaded) and unlabeled (unshaded) subunits that have either wild-type (blank) or voltage-shifted (V and S) S4 gating. The identity of each subunit shown in A and B, and the corresponding abbreviations used in this paper, are shown in C. (A) Homo- and heterotetrameric channels (middle) expressed in the experiments involving the L382V mutant (V). Heterotetrameric channels with one labeled subunit were favored in coinjection of pairs of cRNAs encoding labelable and unlabelable subunits (e.g., C* + V) (top), or defined by injection of a single cRNA encoding four linked subunits (bottom). (B) Subunit stoichiometry in the experiments involving expression of the R365S mutant (S). For expression of heterotetrameric channels, subunits were covalently linked in all experiments to constrain their stoichiometry.

Data Analysis

Charge–voltage (q-v) and fluorescence–voltage (f-v) relations were obtained using Clampfit 6 (Axon Instruments). q-v's were constructed from the integrated "off" gating currents, evoked by repolarizations to −80 mV, after long enough depolarizations (25–500 ms) for the "on" gating current to decay to completion. f-v's were constructed from the amplitudes of the on fluorescence changes. Although detailed models of *Shaker* activation may require functions more complicated than Boltzmann to describe *Shaker*'s q-v, it has been shown that Boltzmann functions fit very well the q-v curves of wild-type and mutant *Shaker* channels (Stefani et al., 1994; Bezanilla et al., 1994; Baker et al., 1998). Therefore, we estimated the voltage parameters of both q-v and f-v curves by fitting the data points to Boltzmann functions using Origin 3.0 (Microcal).

RESULTS

We designed a method to exclusively monitor a specific set of *Shaker* protein rearrangements in single subunits

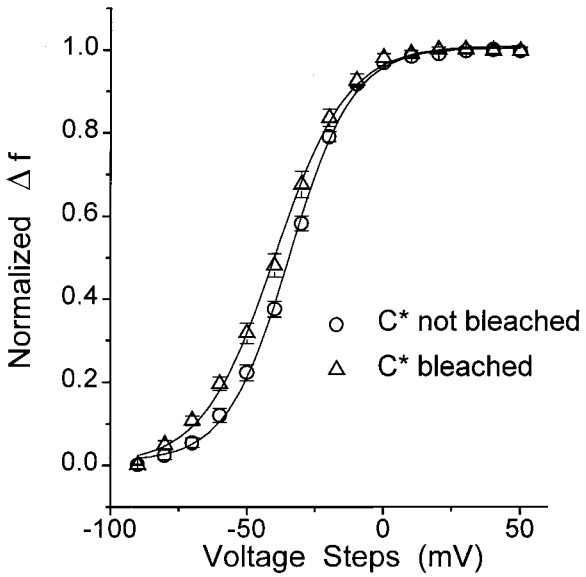

Figure 2. Dependence of fluorescence on the number of labeled subunits. Possible interactions between fluorophore bound to neighboring subunits does not distort the fluorescence of 352C-TMRM (C*), since the f-v of 352C channels labeled to saturation is very little affected by bleaching to a degree that decreases Δf by 80%. Solid lines represent fit of the data points to a Boltzmann function: $y = B/\{1 + \exp[-z\,(x - v)/25]\}$. C* not bleached: $V = -34.7 \pm 0.9$, $z = 2.17 \pm 0.09$. C* bleached: $v = -39.6 \pm 1.5$, $z = 1.95 \pm 0.05$. $n = 10$ for both.

during channel gating transitions. The method was based on three elements.

First, a cysteine substitution at S352 near the outer end of S4 allowed for the site-specific attachment of a sulfhydryl-reactive fluorescent probe. As shown earlier (Baker et al., 1998), and further documented below, fluorescence of this labeled site tightly correlates with S4's voltage-sensing transmembrane rearrangements. Second, coinjection was used to express channels made of a combination of subunits with and without the cysteine mutation (Fig. 1). Coinjections were weighted to favor the unlabeled subunit, yielding a large fraction of channels with only one labeled subunit that provided the bulk of the fluorescent signal (see materials and methods). These constraints on stoichiometry were further assured by tandem linkage of subunits (Isacoff et al., 1990; Fig. 1). Third, mutations that alter specific activation transitions were employed to make heteromeric channels in which the labeled and unlabeled subunits differ in known gating steps (Fig. 1). The expectation was, if certain activation transitions are cooperative, then gating movements in the labeled subunit would be altered by the presence of unlabeled subunits with distinct energetics of specific S4 gating steps.

To monitor the activation movements of S4, position S352C in S3–S4, near the NH_2 terminus of S4, was labeled with TMRM (see materials and methods). Homotetramers made of S352C labeled with TMRM (C*) showed large fluorescence changes in response to voltage steps (Fig. 3 A), with a steady state fluorescence–voltage relation that was statistically indistinguishable from the voltage dependence of gating charge movement (q-v) (Fig. 3 B). This indicates that C* fluorescence can serve as a reporter of S4's charge-carrying conformational changes in a labeled subunit.

Evidence for Cooperativity in Channel Activation

The first voltage-shifting mutation chosen was L382V (Lopez et al., 1991) (referred to as V in this paper), which decreases the steepness of the voltage dependence of a late component of the gating charge, and shifts its $v_{1/2}$ to positive values, without altering the total gating charge (Schoppa et al., 1992). This effect has been accounted for by a reduction in the forward bias of a concerted step that follows the main charge carrying steps in each subunit (Schoppa et al., 1992; Sigworth, 1994; Schoppa and Sigworth, 1998b,c). As previously shown for comparisons of wild-type and L382V homotetramers (Schoppa et al., 1992), the voltage dependencies of gating of C* and V homotetramers differed considerably in the positive range (Fig. 4 A). In addition, the foot of the f-v (and q-v) of C* homotetramers was shifted to the right compared with the q-v of V homotetramers (Fig. 4 A). Such a difference at the foot of the q-v is not seen between V and wild-type homotetramers (Schoppa et al., 1992), and it was due to a right shift of the C* q-v compared with both the V and wild-type q-v curves. The different behavior of the V and C* homotetramers in the whole voltage range of gating charge movement made it possible to look for interactions that affect both early and late transitions.

To examine how interactions between wild-type and V subunits influence S4 activation rearrangements, subunits that were wild type except for the cysteine labeling site substitution were coexpressed with unlabelable V subunits, or labelable V subunits were coexpressed with unlabelable wild-type subunits (W; Fig. 1 A).

Complementary RNAs (cRNAs) encoding the S352C subunit and the V subunit were coinjected into oocytes in a ratio chosen to optimize the number of channels containing three V subunits and one fluorophore-binding S352C subunit (Fig. 1 A, and see materials and methods), and then labeled with TMRM. The f-v of the S352C-TMRM (C*) subunit in these heterotetramers (C* + V in Fig. 4 A) was shallower and shifted to the right with respect to the C* homotetramers, approaching the shallow depolarized component of the V homotetramers (Fig. 4 A). The same result was obtained when subunit stoichiometry was constrained by using

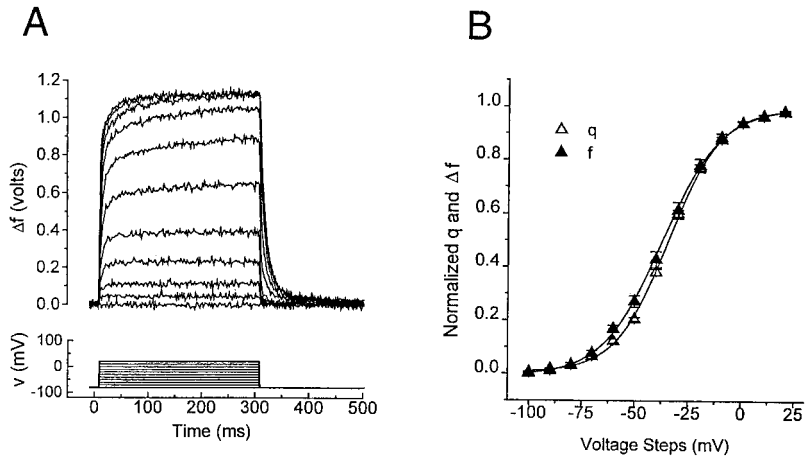

Figure 3. Voltage-dependent gating charge movement (Q) and fluorescence change (F) of TMRM-labeled S352C (C*). (A) Fluorescence recording (top) during voltage steps (bottom) from a holding potential of −80 to +20 mV in 10-mV increments. Each fluorescence trace is the average of five sweeps. (B) Normalized q-v (▲) and f-v (△) relations show a tight correlation, indicating that the f-v is a good indicator of the activation state of a C* subunit's S4. Solid lines represent the fit of the data points to a Boltzmann function (q:v = −34.6 ± 1.8 mV, z = 2.26 ± 0.05; f:v = −31.3 ± 2.26 mV, z = 2.21 ± 0.12, n = 12). Note that only fluorescence increase is seen across the measured voltage range.

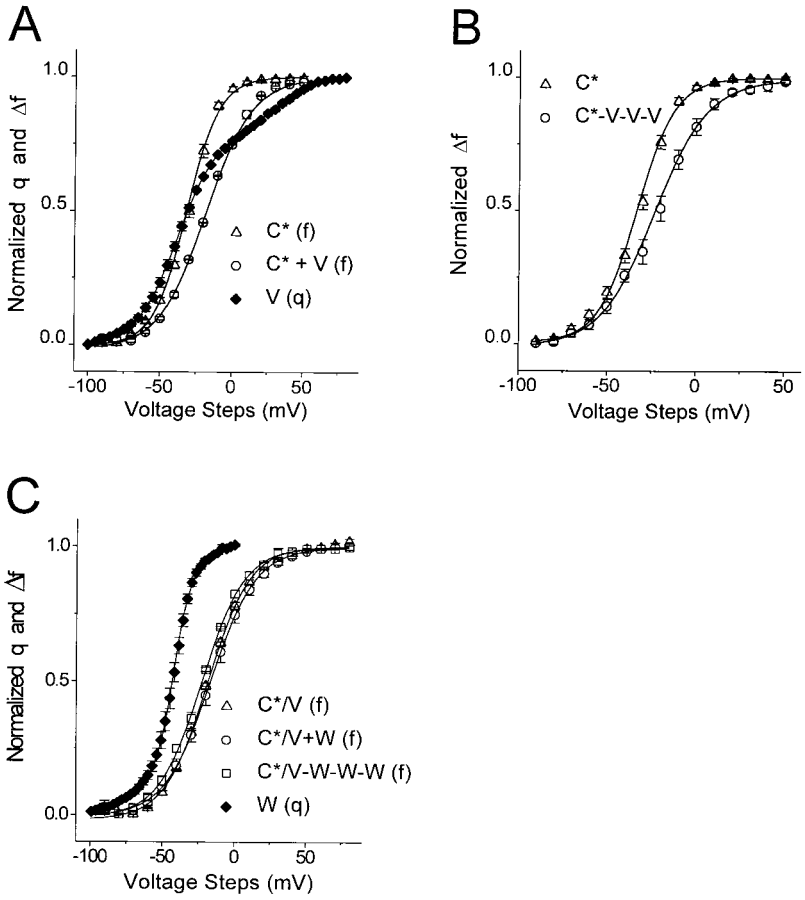

Figure 4. Cooperativity in wild-type S4 movement. (A) Normalized f-v from channels expressed in oocytes injected with cRNA encoding S352C (C*, △), or coinjected with a 1.5:8.5 ratio of S352C:L382V cRNA (C*+V, ○), and the normalized q-v of L382V channels (V, ◆). The presence of a majority of V subunits produces a rightward shift in the C* subunit f-v toward the even further rightward-shifted depolarized component of the V subunit q-v. Note the absence of a leftward shift toward the V subunit q-v at the foot of the C* f-v. Solid lines represent the fit of the data points to a single Boltzmann (C*+V: V = −18.8 ± 0.9 mV, z = 1.54 ± 0.05, n = 6) or to the sum of two Boltzmann functions (V: v_1 = −42.6 ± 1.9, z_1 = 1.84 ± 0.04, v_2 = +33.0 ± 2.0, z_2 = 1.38 ± 0.23, n = 5). (B) Normalized f-v of S352C (C*, △) and of the linked tetramer S352C-L382V-L382V-L382V (C*-V-V-V, ○: V = −20.6 ± 2.4 mV, z = 1.74 ± 0.11, n = 11). The linked heterotetramer with only one labelable subunit closely resembles the coinjection (A) that favors channels with zero or one labelable subunit, of which only the latter contribute to the fluorescence signal. (C) S4 gating of the labeled double-mutant S352C-TMRM/L382V (C*/V, △) is not changed by coassembly with three wild-type subunits (W, ◆), as shown by the comparison of the normalized f-v of the homotetramer C*/V to that of the heterotetramer with a 1:3 ratio of C*/V to W subunits (obtained by coinjection of C*/V and W cRNA in a 1:8.5 ratio (C*/V + W in the figure, ○) or by injection of the C*/V-W-W-W–linked cRNA, □). (C*/V: v = −18.8 ± 0.8, z = 1.70 ± 0.07; C*/V + W: v = −16.7 ± 4.2, z = 1.62 ± 0.07; C*/V-W-W-W: v = −22.9 ± 1.0, z = 1.68 ± 0.16, n = 5). The normalized q-v of the unlabeled wild type is also shown (W; see Table I for the results of the Boltzmann relation fit).

the linked tetramer C*-V-V-V (Fig. 4 B). The effect on the single C* subunit of the three V subunits was strong enough to make the f-v indistinguishable from that of the homotetramer of the double mutant 352C*/382V (C*/V) (Fig. 4, A and C).

In contrast to the pronounced influence of the V subunits on the C* subunit's rearrangements at positive voltages, the foot of the f-v of the C* subunit was not shifted toward the q-v of the V homotetramer (Fig. 4 A). This suggests that interactions between subunits affect late transitions, but not early transitions.

The voltage-shifting influence of the three V subunits on a single C* subunit could result from a strong cooperative influence of the V subunits, or, alternatively, the L382V mutation may eliminate a cooperative interaction that normally makes the q-v comparatively steep. To distinguish between these possibilities, we determined whether a V subunit remains sensitive to the cooperative influence exerted by wild-type subunits. This was done in channels composed of a single labeled C*/V subunit and three W subunits. In both coinjection of C*/V cRNA with an excess of W cRNA, and expression of the linked tetramer C*/V-W-W-W, the f-v's generated by the single C*/V subunit combined with three W subunits were virtually the same as the f-v of the C*/V homotetramers (Fig. 4 C). The gating currents of the C*/V-W-W-W channels, three quarters of which should be generated by W subunits, were intermediate in kinetics between those of the C*/V homotetramer and the W homotetramer (Fig. 5). These results demonstrate that the L382V mutation renders the subunit insensitive to cooperative influence on its S4 movement by wild-type subunits (Fig. 6), a remarkable effect that leaves the L382V subunit operating independently.

A Charge Neutralizing Mutation Better Resolves Charge Carrying Movements of S4

The above results suggest that early steps in activation are independent and that late steps are cooperative. However, the early and late charge carrying transitions could not be resolved in the f-v's of C*-V-V-V and C*/V-W-W-W (Fig. 4), preventing us from precisely identifying the cooperative step(s). This loss of resolution is

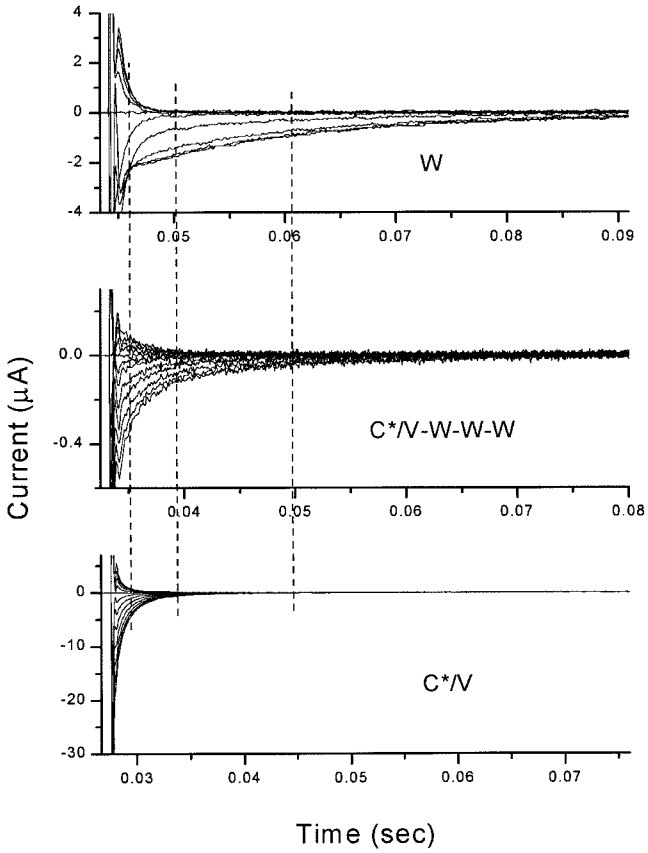

Figure 5. Off-gating currents recorded from oocytes expressing either wild type (W, top), labeled double mutant 352C/382V (C*/V, bottom), or linked heterotetramer C*/V-W-W-W (middle) channels. The gating currents were evoked by steps from −80 mV to voltages ranging from −100 to 0 mV in 10- mV increments, followed by repolarization to −60 mV. Vertical dashed lines correspond to the time constant of the slower component from a two-exponential fit of the off-gating current following the step to −20 mV (left dashed line = C*/V's time constant = 1.7 ms; middle dashed line = C*/V-W-W-W time constant = 6.0 ms; right dashed line = W time constant = 16.0 ms).

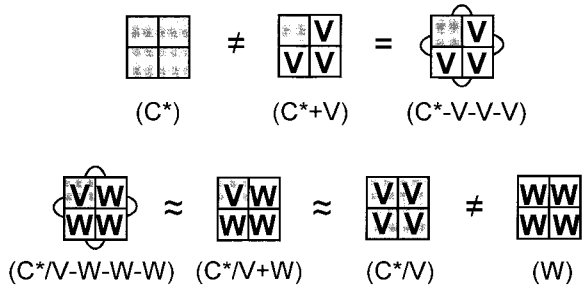

Figure 6. Cooperativity in late activation transitions. Diagram illustrates the main results of the L382V experiments. (Top) S4 gating of the labeled reporter subunit (C*) in heterotetramers containing three L382V subunits (V), obtained either by coinjection (middle) or by expression of the linked tetramer (right), is different from that of the same subunit in the homotetramer C* (left). (Bottom) The labeled L382V subunit (C*/V) behaves independently of the subunits with which it is assembled, since gating of C*/V coassembled with three W subunits, both in monomer coinjection (C*/V+W) and in linked tetramer (C*/V-W-W-W) experiments, is not different from gating of the C*/V homotetramer. The apparent uncoupling of a subunit from the cooperative influence of its neighbors by the L382V mutation suggests that a wild-type–like labeled subunit coassembled with three L382V subunits is voltage shifted because of the loss of wild-type cooperative interactions.

due to the combined influence of the cysteine mutation at S352 and to its conjugation with TMRM, as found earlier for the double mutant A359C-TMRM/L382V (Mannuzzu et al., 1996).

To determine more clearly which activation movement of S4 is cooperative, we employed another voltage-shifting mutation, R365S. Like other neutralizations of this second arginine in S4 (Perozo et al., 1994; Aggarwal and MacKinnon, 1996; Seoh et al., 1996), R365S shifts apart the voltage dependencies of the two major charge-carrying steps (q1 and q2) of channel activation (Bezanilla et al., 1994). The q1 and q2 steps correspond to two sequential outward movements of S4 between three distinct transmembrane topologies corresponding to the deactivated, intermediate, and activated states of the channel (Baker et al., 1998). Neutralization of R365 provided the possibility of directly relating cooperative interactions to transitions between these known conformations of S4. In addition, unlike L382V, R365S appears to maintain its cooperativity, since, as for wild-type channels, despite the similarity in the magnitude of charge carried by q1 and q2, the steepness of q2 is greater than that of q1 (Perozo et al., 1994; Fig. 7 A). This could be explained by positive cooperativity in q2, or, alternatively, by negative cooperativity in q1.

As we have previously shown (Baker et al., 1998), TMRM conjugated at S352C in the double mutant S352C/R365S (C*/S) experienced a two-step fluorescence change (f1 and f2) that well correlates in their voltage dependence with the two steps of charge movement (q1 and q2). This correspondence is clearly demonstrated in Fig. 7 A, which shows that the voltage parameters from the double Boltzmann fit to the q-v equally fits the f-v (solid lines in Figure 7A). This correlation was also confirmed by examining the kinetics of the C*/S fluorescence change. Fig. 8 (left) shows fluorescence traces from the linked tetramer C*/S-S-S-S, which had a kinetic behavior (data not shown) and steady state behavior (Fig. 7 B) indistinguishable from the C*/S homotetramer. From very negative voltages up until −30 mV, only a single component that increases fluorescence with depolarization was present. Further depolarization changed the fluorescence during the step (on fluorescence) to one in which a fast fluorescence increase was followed by a slower fluorescence decrease to a steady state level. As the step potential was made more positive, both the magnitude and rate of activation of the downward fluorescence compo-

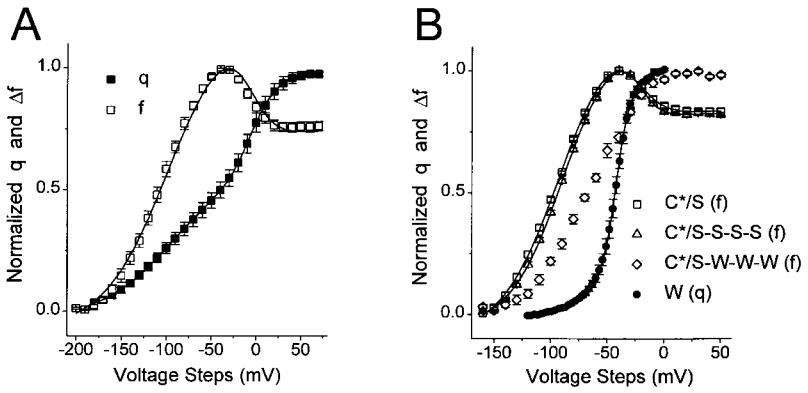

Figure 7. Subunit interaction in the C*/S-W-W-W heterotetramer. (A) Both the q-v (■) and f-v (□) of S352C/R365S (C*/S) have two components. The estimates from the fit of the q-v to the sum of two Boltzmann functions (v_1 = −94.0 ± 7.3 mV, z_1 = 0.98 ± 0.05, v_2 = −7.4 ± 2.5 mV, z_2 = 2.46 ± 0.15, mean ± SEM, n = 6) well fits the f-v (solid line) when $v_{1/2}$ and slope values are fixed, but the amplitudes and polarity of the two components are allowed to vary. This indicates that C* reports on both of S4's major outward activating movements. (B) Assembly of one C*/S subunit with three W subunits alters C*/S S4 movement. Graph includes f-v's of the linked tetramers C*/S-W-W-W (with C*/S = S352C-TMRM/R365S, and W = wild type; ◇) and C*/S-S-S-S (with S = R365S; △), and of C*/S homotetramers (□), as well as the q-v of W homotetramers (●). See Table I for the results of the Boltzmann relation fits.

nent increased, resulting in a progressively smaller and shorter lasting upward-going transient (Fig. 8 B, arrow). In the same voltage range (from about −20 to +50 mV), two components were also evident in the fluorescence upon repolarization (off fluorescence), a rapid fluorescence increase followed by a slower fluorescence decrease. This sequence of events in the on and off fluorescence change is consistent with the behavior, in wild-type channels, of q1 and q2 (Bezanilla et al., 1994), which we have shown to be better resolved, and preserved in character, in channels with an R365 neutralization (Baker et al., 1998).

The good correlation between f1 and q1, and between f2 and q2 (Fig. 7 A), indicates that TMRM at 352 reports on both steps of transmembrane S4 movement, making it possible to determine if the cooperative interactions occur during one or both of these charge carrying transitions.

Of the Two Sequential Transmembrane Charge Carrying Movements of S4, the First Appears to be Independent and the Second Cooperative

In the following experiments, the subunit carrying both the S352C and R365S mutations was labeled with TMRM (C*/S), and the S4 movements of this voltage-shifted subunit were examined in three contexts (Fig. 1 B): (a) in C*/S homotetramers, (b) in linked C*/S-S-S-S heterotetramers, made of one C*/S subunit and three unlabeled voltage-shifted R365S subunits (S), and (c) in linked C*/S-W-W-W heterotetramers, made of one C*/S

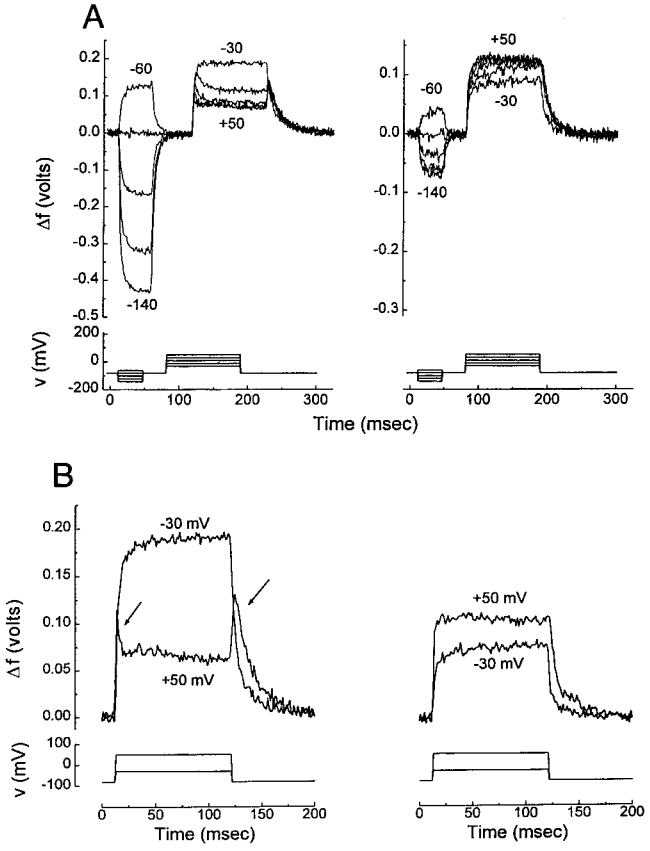

Figure 8. Fluorescence changes of the linked tetramers C*/S-S-S-S (left) and C*/S-W-W-W (right). (A) Fluorescence changes in a double-pulse experiment (five episodes, each composed of five epochs, sequentially in mV: −80/−140/−80/−30/−80, with 20-mV increments of the second and fourth epochs). Note that, for both channels, only a single component is present from very negative voltages up until −30 mV. With further depolarization, only C*/S-S-S-S (left) shows two kinetic components both in the "on" and in the off fluorescence: an upward transient, which rapidly decays to a steady state level. These components are the kinetic expressions of S4's three gating states (resting, intermediate, and activated), which are well separated in their voltage dependence by the mutation R365S. As a result of the cooperative interaction in q2, coassembly of one C*/S subunit with three W subunits restores the direction of the f2 fluorescence change to that of C* (compare right with Fig. 3 A). (B) Fluorescence changes in response to voltage steps from −80 to −30 and +50 mV. Arrows points to the on and off transients shown by C*/S-S-S-S, but absent in C*/S-W-W-W, as they are in wild type (Fig. 3 A).

subunit and three unlabeled wild-type subunits. The rationale behind these experiments was that comparison of the fluorescence of C*/S in these channels should make it possible to resolve the influence of wild-type subunits on the q1 and q2 steps of S4 movement.

The f-v of the C*/S homotetramers was virtually indistinguishable from that of the linked tetramer C*/S-S-S-S (Fig. 7 B and Table I). In contrast, activation gating of the single C*/S reporter subunit was clearly influenced by its coassembly with three W subunits (Fig. 7 B). This influence was seen not only in the voltage dependence of fluorescence, but also in the direction of the f2 fluorescence change, which became positive (Figs. 7 B and 8, right). A free parameter fit of the C*/S-W-W-W f-v to the sum of two Boltzmann functions gave an estimate for the $v_{1/2}$ of f2 that was significantly different ($P = 1.2 \times 10^{-6}$) from that of C*/S-S-S-S. The three W subunits shifted the $v_{1/2}$ of the C*/S f2 in the hyperpolarized direction, so that it approached the $v_{1/2}$ of the f2 of W homotetramers (Fig. 9 A, solid line; Table I). In other words, the W subunits exerted a cooperative influence in f2. Unlike the evidence for cooperativity in f2, the f1 estimated from the free fit of the C*/S-W-W-W f-v to the sum of two Boltzmann functions was not significantly different from that of C*/S-S-S-S (Table I), indicating that the q1 transition in the reporter subunit is not substantially influenced by the other subunits in the channel. This is consistent with the absence of a shift toward more depolarized voltages in the foot of the f-v of the C*/S-W-W-W tetramer.

The above conclusion that W subunits affected q2 but not q1 motion of the C*/S subunit S4 is supported by the fact that the f-v of C*/S-W-W-W was closely approximated by the sum of two fixed Boltzmann functions, one having the voltage parameters of the f1 of C*/S-S-S-S, and the other having the voltage parameters of the q2 of W homotetramers (Fig. 9 B, solid line). In contrast, a double Boltzmann composed of the opposite combination of parameter values (the f1 from the q1 of W homotetramers and f2 of C*/S-S-S-S), failed to fit the f-v of C*/S-W-W-W (Fig. 9 B, dotted line). Finally, a sum of two Boltzmann functions made of both f1 and f2 of C*/S-S-S-S, but with reversed f2 polarity, also did not fit the data (Fig. 9 B, dashed line).

In summary, the good agreement between the f1 of C*/S-W-W-W and that of C*/S-S-S-S points to an absence of cooperativity in q1. The large shift to the left of the C*/S subunit's f2 in C*/S-W-W-W channels, towards the q2 values of W homotetramers, is consistent with a strong cooperativity in the q2 movement of S4.

TABLE I

Boltzmann Function Fits for Experiments Involving the R365S Charge Neutralization

	v_1	z_1	v_2	z_2	n
	mV		*mV*		
W	−59.3 ± 2.3	1.58 ± 0.12	−42.9 ± 1.4	4.28 ± 0.28	6
C*/S	−92.9 ± 0.7	1.04 ± 0.06	−22.4 ± 1.0	2.24 ± 0.19	4
C*/S-S-S-S	−90.9 ± 1.4	1.06 ± 0.03	−21.6 ± 1.0	2.85 ± 0.18	9
C*/S-W-W-W	−87.7 ± 2.9	1.21 ± 0.11	−38.5 ± 3.1	2.71 ± 0.11	12

Values (mean ± SEM) of the midpoints (v_1 and v_2) and slopes (z_1 and z_2) of a fit to the sum of two Boltzmann functions for channels with defined subunit stoichiometries and arrangements.

DISCUSSION

In this paper, we used a new approach to study interactions between subunits in heteromeric membrane proteins. This method relies on the use of cysteine mutagenesis to introduce an attachment site for a thiol-reactive fluorophore into a specific functional domain of a subunit. These modified subunits are coexpressed with subunits that cannot be labeled because they do not contain accessible cysteines. In this manner, fluorescence measurements only monitor the structural rearrangements of the labeled domain in particular subunits without interference from signals in unlabeled subunits. This approach makes it possible to define how rearrangements in a specific domain of one subunit, such as those evoked by voltage change or ligand binding, are coupled to functional rearrangements in other subunits of a multimeric protein. The advantage of studying cooperativity in this manner is that it provides a clear indication of subunit interaction, circumventing the need to dissect apart gating or ionic currents, which are governed by the complex ensemble properties of all of the gating domains in each of the channel's subunits.

For this method to work, two criteria need to be met: (a) the fluorophore should report only on the conformational changes of the subunit to which it is bound, and (b) the fluorescence report of conformational rearrangements should not be distorted by interaction between fluorophores bound to multiple subunits of the same channel. In this context, we have shown that *Shaker* channels carrying the substitution S352C labeled with TMRM (C*) fulfill these two criteria. With respect to the first criterion, we showed that the fluorophore bound to this site is not sensitive to gating movements in neighboring subunits, since the f-v's of two distinct subunits (C*/V and C*/S) are different although they have the same three neighboring subunits (three Ws in the tetramers C*/V-W-W-W and C*/S-W-W-W; Figs. 4 and 7; Table I). Further, the f-v of C*/V was the same whether associated with the same subunits in the homotetramer C*/V or with different subunits in the heterotetramer C*/V-W-W-W (Fig. 4 C). The second criterion was met in that, despite the difference in the number of labeled subunits, the f-v's of C*/V and C*/S homotetramers, with four labeled subunits, were found to be equivalent to those of the linked heterotetramers

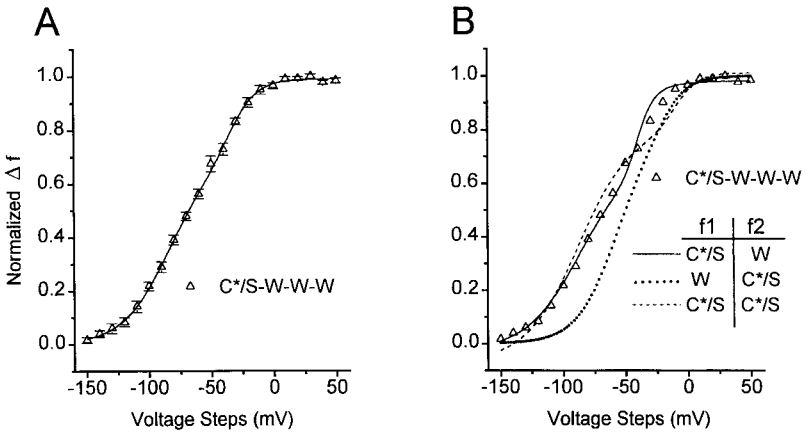

Figure 9. The single C*/S subunit S4 moves independently in q1, but is strongly influenced by the three W subunits in q2. (A) Free fit of the C*/S-W-W-W's f-v to the sum of two Boltzmann functions (solid line). See Table I for estimates of the voltage parameters. (B) The f-v of C*/S-W-W-W is closely approached by a constrained fit using the f1 voltage parameters of C*/S and the q2 voltage parameters of W (solid line), but not by constrained fits with the q1 of W and f2 of C*/S (dotted line), or the f1 and f2 of C*/S-S-S-S, with f2 polarity inverted (dashed line). Note that the foot of the heterotetramer's f-v does not show the shift toward more depolarized potentials expected for subunit interactions in q1 (dotted line).

C*/V-W-W-W and C*/S-S-S-S, with only one labeled subunit (Figs. 4 C and 7 B). This data is consistent with evidence that the S3–S4 loop of one subunit is far enough away from the S3–S4 loops of other subunits to allow for their simultaneous binding of multiple molecules of the peptide hanatoxin (Swartz and MacKinnon, 1997a,b). Additionally, since basic residues in S4 have been shown to interact with acidic residues in the S2 and S3 of the same subunit (Tiwari-Woodruff et al., 1997), the microenvironment around an S3–S4 fluorophore is also likely to be defined by the same subunit.

We used this approach to determine whether activation during gating involves cooperative interactions between subunits of the *Shaker* potassium channel. Our results, using two mutations that exert distinct effects on channel activation, indicate that a specific step of voltage sensing is cooperative, while another is independent. This cooperativity influences both the energetics of specific S4 activation states and the conformation of S4 or of the protein that immediately surrounds it.

Independent and Cooperative Steps in Activation

Our results with both the mutation L382V and R365S indicate that cooperative interactions occur in late activation transitions, but not in early ones. The results were particularly informative with the mutation R365S, which neutralizes the second basic residue in S4. This arginine is one of the key positive charges in S4. During activation it traverses practically the entire membrane-spanning protein and membrane electric field (Aggarwal and MacKinnon, 1996; Larsson et al., 1996; Seoh et al., 1996; Starace et al., 1997; Baker et al., 1998). In the resting and intermediately activated S4 membrane positions, it is buried deeply enough (Baker et al., 1998) to probably require electrostatic interactions with acidic residues in S2 and S3 for structural stability (Papazian et al., 1995; Tiwari-Woodruff et al., 1997). Based on the kinetics of q1 and q2 charge movements and on the relationship between magnitude of the charge and slope, we have concluded that the mutation R365C closely resembles wild type, except that the energetics of the three transmembrane topologies of S4 (associated with the deactivated, intermediate, and activated states) are altered, resulting in large voltage shifts (Baker et al., 1998).

We took advantage of the easily resolved q1 and q2 gating charge movements in S352C-TMRM/R365S subunits to correlate cooperative interactions with specific S4 activation movements using fluorescence. We found that the q1 gating rearrangement is insensitive to the identity of the other subunits in the tetramer. In contrast, the q2 rearrangement is significantly influenced by the other subunits. Thus, in the context of the q1–q2 model of gating charge movement, the results are consistent with cooperative interactions occurring only in q2.

Cooperative Interactions Affect Subunit Conformation

As shown in Fig. 3, upon depolarization, the S352C-TMRM homotetramers produce a fluorescence increase throughout the voltage range. The same is true for homotetramers of S352C-TMRM/L382V, despite the shift by the L382V mutation of the late charge carrying step to more depolarized voltages (Fig. 4 C). Although it is difficult to resolve distinct charge carrying components in these two homotetramers, it appears that all the major steps of S4 movement produce increases in fluorescence with activation for this labeling position. In contrast, although the f1 component of S352C-TMRM/R365S homotetramers also increases fluorescence with depolarization, f2 decreases fluorescence with depolarization (Fig. 7 A). Since fluorescence depends on the local fluorophore environment, this reversal of the direction of f2 indicates that the mutation R365S not only alters the relative energetics of S4 gating states, but that it also alters the conformation of the S3–S4 loop at position 352, and/or of its protein surround.

Interestingly, we found that the identity of the amino acid at position 365 is not the sole determinant of the ratio of brightness of the intermediate state versus the fully activated state for that subunit's S352C-TMRM.

Coassembly of one C*/S subunit with three W subunits restored the direction of the f2 fluorescence change to that of C*, with the fully activated state being brighter than the intermediate state (Figs. 7 B and 8). This indicates that subunit interaction during gating affects the protein conformation either of the S3–S4 or of the protein microenvironment around S3–S4. In summary, interaction between subunits influences: (a) the energetics of an S4's intermediate and fully extruded states, and (b) the conformation of the segment just external to S4 (or of its protein surround).

The cooperativity observed in q2 could be "intrinsic" to S4, in the sense that the transmembrane state of one S4 could directly or indirectly influence that of S4 in other subunits. Alternatively, the cooperativity could result from an "extrinsic" influence of other coupled transitions. The basis of this cooperativity is examined in the next two sections.

Possible Basis of Cooperativity in the Second Step of Outward S4 Movement

Measurements of ionic and gating currents by the Aldrich and Sigworth labs have led to models of *Shaker* channel activation that consist of two or three major charge carrying transitions that occur independently in each subunit. These are followed by a concerted step that: (a) either directly opens the channel, or immediately precedes channel opening, (b) carries between 1.4 and 1.8 e_0 per channel, and (c) is strongly forward biased (Hoshi et al., 1994; Zagotta et al., 1994a,b; Schoppa and Sigworth, 1998a,b,c; Smith-Maxwell et al., 1998a,b; Ledwell and Aldrich, 1999). Mutations that are modeled to reverse this forward bias separate the main voltage sensing movements from channel opening (Schoppa and Sigworth, 1998b,c; Ledwell and Aldrich, 1999), whereas a mutation modeled to increase the forward bias can actually cause the channel to open before all the voltage sensors have moved, leading to subconductances (Zheng and Sigworth, 1997, 1998; and see Chapman et al., 1997).

Our experiments are consistent with the q2 phase of S4 charge movement being cooperative. According to the models described above, q2 movements are intrinsically independent, but could be made functionally cooperative by being tightly coupled to an ensuing forward biased concerted transition. In this context, the apparent cooperativity of S4 would depend on the ratio of the forward and backward rate constants of the concerted transition for each contributing subunit. A prediction of such a model is that heteromeric channels composed of mixtures of W subunits (having a strongly forward-biased concerted transition) and V subunits (in which the same transition is slightly backward biased) would be expected to have an intermediate behavior weighted by the number of each subunit type. If the q2 movement of S4 borrowed cooperativity from the concerted step, then the S4 of a single V subunit should still experience cooperative influence from the three W subunits. Therefore, channels made of one V and three W subunits should still have a strongly forward-biased concerted step. Our results do not support this prediction. Instead, they show that the S4 of a V subunit is not influenced at all by other subunits with which it coassembles. This implies that the L382V mutation affects more than just the rates of the concerted step. One possibility is that the mutation directly eliminates an intrinsic cooperativity of q2.

How Are Subunits Coupled to Each Other?

Our results suggest that the intermediate to fully extruded motion of S4 is intrinsically cooperative. But how does the transmembrane state of one S4 affect that of S4 in other subunits? We have two possible indications about this mechanism. The first is the dependence of the q2 cooperativity on L382. L382 is located at the internal end of S4, in a segment that is not buried in the protein even when S4 is fully extruded at positive voltages (Larsson et al., 1996). Although the NH_2-termini of S4s appear to be too far apart from each other (Swartz and MacKinnon, 1997a,b) to directly interact, nothing is known about the distance between the intracellular ends of S4. Thus, L382 could be part of a structure that either directly couples S4 segments to one another when these are in the intermediate and/or fully extruded state (see discussion on the putative leucine zipper motif; McCormack et al., 1991; Durell et al., 1998) or indirectly couples S4s via interaction with *Shaker*'s internal activation gate (see Liu et al., 1997). The second point to consider is that interactions between subunits also change the environment of the S3–S4 loop near the NH_2 terminus of S4 (Figs. 7 B and 8). This suggests that S4 movements may also be coupled via structural rearrangements of the part of the channel protein surrounding S4, including the pore-forming S5 and S6.

Conclusions

We have used single subunit fluorescence to obtain evidence for cooperativity in a charge-carrying voltage sensor movement of S4 during the activation of *Shaker* potassium channel. Of the two sequential outward steps that S4 takes in response to membrane depolarization, the first appears to occur independently in the four subunits, and the second step appears to be cooperative. This cooperative interaction between subunits not only affects the relative energetics of specific S4 gating states, but also changes the conformation of the S3–S4 loop with respect to its protein environment. With this method in hand, it should now be possible to identify the structures that couple gating movements of

the S4s of different subunits, and S4 movements to *Shaker* channel gates.

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