Long Distance Interactions within the Potassium Channel Pore Are Revealed by Molecular Diversity of Viral Proteins*

Received for publication, February 3, 2004, and in revised form, April 22, 2004
Published, JBC Papers in Press, April 22, 2004, DOI 10.1074/jbc.M401184200

Sabrina Gazzarrini§, Ming Kang§, James L. Van Etten§§, Sascha Tayefeh¶, Stefan M. Kast¶¶, Dario DiFrancesco**‡‡, Gerhard Thiel§§¶¶, and Anna Moroni‡‡¶¶

From the *Department of Biology and Consiglio Nazionale delle Ricerche Istituto di Biofisica-Mi and **Department of Biomolecular Sciences and Biotechnology, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy, §Department of Plant Pathology and ¶¶Nebraska Center for Virology, University of Nebraska, Lincoln, Nebraska 68583-0729, Institutes for Inorganic and Physical Chemistry and ||Istituto Nazionale per la Fisica della Materia, Unità di Milano-Università, Via Celoria 16, 20133 Milano, Italy

Kcv is a 94-amino acid protein encoded by chlorella virus PBCV-1 that corresponds to the pore module of K+ channels. Therefore, Kcv can be a model for studying the protein design of K+ channel pores. We analyzed the molecular diversity generated by ~1 billion years of evolution on kcv genes isolated from 40 additional chlorella viruses. Because the channel is apparently required for virus replication, the Kcv variants are all functional and contain multiple and dispersed substitutions that represent a repertoire of allowed sets of amino acid substitutions (from 4 to 12 amino acids). Correlations between amino acid substitutions and the new properties displayed by these channels guided site-directed mutations that revealed synergistic amino acid interactions within the protein as well as previously unknown interactions between distant channel domains. The effects of these multiple changes were not predictable from a priori structural knowledge of the channel pore.

Potassium (K+) channels are membrane proteins that mediate K+ flux between the outside and the inside of the cell (1). They are found in all organisms, from bacteria to humans, where they control essential physiological properties, such as osmotic regulation and the duration and shape of action potentials. Potassium channels have recently been found in the genomes of chlorella viruses (2, 3) where they probably play a role in viral replication.

Potassium channels are modular proteins (4) and consist of three main components: 1) the pore, formed by two transmembrane helices and a loop between them (2TM/P),1 that controls selectivity and gating and is a universal feature of K+ channels; 2) four transmembrane helices (S1–S4) that, in voltage-gated (Kv) channels precede the pore and endow the channel with the capability of responding to voltage changes, and 3) cytoplasmic N- and C-terminal domains that exert several regulatory functions.

The recent crystallization of the bacterial K+ channels KcsA (5), MthK (6), and KirBac1.1 (7) have enhanced our understanding of the structural features of the K+ channel pore. These studies identified the selectivity filter within the pore as the structure responsible for ion selection and suggested that the bundle crossing formed by the inner pore helices constitutes the intracellular channel gate. However, channels are dynamic structures undergoing global conformational changes in response to effectors (such as voltage and ligands) that open and close the pore. To understand the mechanics of these movements, the coupling of the pore with other regulatory components of the channel as well as the interaction between structural elements within the pore itself need to be described.

In the past, structural-functional studies of ion channels have been performed using a “rational” design approach, relying on structural analysis and subsequent site-directed mutagenesis. However, interactions between multiple and dispersed amino acids are not easily predicted with this approach; consequently, other experimental approaches are required to discover these interactions (8, 9). Particularly useful are “irrational” protein design approaches, such as random mutagenesis generated by error-prone PCR. This “gene shuffling” technology randomizes the sequence producing multiple mutations, whose effects are not easily predicted by “a priori” structural knowledge because of the interplay of widely dispersed mutations (10). Like rational design, however, random mutagenesis does not satisfy the statistical complexity of evolution because of its lack of combinatoriality. The complexity of combinatorial protein design experiments implies that the number of possible sequences that can generally be created and screened (106–1012) is infinitesimal compared with the total number possible (294 = 10133) for the 94-residue Kcv protein (11).

This manuscript describes an alternative strategy for identifying functionally important long-range amino acid interactions within the pore region of a K+ channel. For this purpose, we exploit the quasi-unlimited statistical complexity of naturally evolved proteins in which each spontaneous mutation has been selected through millions of years in the context of the surrounding sequence to generate a modified variant of the original sequence. We used as an experimental system the K+ channel protein Kcv, from the chlorella virus PBCV-1, and a natural collection of functional Kcv-variants isolated from 40 additional chlorella viruses. Kcv is a model system to study the pore because this 94 amino acid protein consists only of the pore module (2TM/P) of K+ channels (2). Kcv forms a functional

1 The abbreviations used are: TM, transmembrane; wt, wild type.
K⁺ channel in several heterologous systems (2, 12, 13) and is therefore suitable for validation of functional properties through biophysical analysis. Because a functional Kcv channel is apparently required for the virus to replicate (2, 3), it is not surprising that all the Kcv variants, generated by the long evolutionary history of these viruses (14), are functional. They constitute a pool of prescreened (and therefore) functionalized genetic diversity in which selection has removed non-advantageous diversity from the pool of functionality. We have previously described six natural Kcv variants containing 4–12 amino acid substitutions; these variants have different kinetics and permeability properties relative to the reference channel PBCV-1 Kcv (3). Comparing patterns of amino acid substitutions with channel properties identified three sets of amino acid substitutions associated with three specific channel properties: 1) K⁺ current inactivation, 2) high Rb⁺ permeability, and 3) block by Cs⁺. Mutational analysis was used to determine whether amino acid substitutions in each set contributed singly or in combination to the channel property. Our results indicate that none of the properties resulted from a single amino acid substitution. Indeed, mutations that do not alter the channel behavior singly nevertheless exert a strong influence reverting the effects of other mutations. Each property is controlled by the synergistic interaction between amino acids located in different pore domains with no obvious physical interactions. Properties such as permeation by Rb⁺ and block by Cs⁺, expected to localize to the selectivity filter (15), are the result of a synergistic interaction at two positions, amino acid 19 in the outer helix, TM1, and amino acid 54 in the pore helix. Homology modeling of Kcv suggests that these two positions are far from each other and do not seem to contact the selectivity filter. Thus, these natural Kcv variants reveal interactions between distant domains and, most importantly, not predicted from the current static understanding of channel pore structure.

EXPERIMENTAL PROCEDURES

**Mutagenesis and Expression of the kcv Genes in Oocytes**—Kcv cDNA was cloned into pSGEM vector (a modified version of pGEM-HE, courtesy of M. Hollmann, Max Planck Institute for Experimental Medicine, Göttingen, Germany). Point mutations were created by the QuickChange method (Stratagene) and confirmed by sequencing the DNA insert. We prepared cDNA by T7 polymerase transcription and injected it (20–40 ng/oocyte) into Xenopus laevis oocytes, prepared according to standard methods (2). Measurements were performed 2–4 days after injection.

**Electrophysiology**—A two-electrode voltage clamp (Gene clamp 500; Axon Instruments) was used to record K⁺ currents from oocytes. Electrodes were filled with 3 M KCl and had a resistance of 0.2–0.8 MO (in 50 mM KCl). Oocytes were perfused at room temperature with a standard bath solution containing 50 mM KCl (or RbCl, NaCl, LiCl, CsCl, as indicated in figure legends and text), 1.5 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH adjusted to 7.4 with KOH, at a rate of 2 ml/min. Mannitol was used to adjust the osmolarity of the solution to 215 mosM.

The standard clamp protocol consisted of steps from the holding voltage of -20 mV to voltages in the range +80 mV to -160 mV; tail currents were measured at -80 mV. Instantaneous and steady-state currents were sampled after 10 ms and at the end of the voltage step (usually 800 ms), respectively.

**Ion Permeability**—Permeability ratios (Pᵢ/Pᵢ⁺) were calculated using the equation

\[ \frac{P_i}{P_i^+} = \frac{E_{rev,i} - E_{rev,+}}{RTF_i \ln \frac{P_i}{P_i^+}A_i} \]

where

- \(E_{rev,i}\) is the value in millivolts of the current reversal potential measured in the presence of 50 mM monovalent cation (either \(A_i\) or \(B_i\)) in the external solution,
- \(R\) is the gas constant,
- \(T\) is the absolute temperature,
- \(z\) is the ion charge, and
- \(F\) is Faraday's constant (1). 

**Diversity of Kcv proteins.** Alignment of 7 Kcv proteins. Each protein is named after a virus (PBCV-1, CA-4B, AL-2A, AN69C, MA-1D, NY-2A, NY-2B) representative of each group (3). Amino acid substitutions, compared with PBCV-1 Kcv, are highlighted in black. The assignment of putative structural domains is based on the alignment between PBCV-1 Kcv and KirBac1.1 (Ref. 7; Fig. 2): slide helix (S helix) (previously described as the 12 amino acid cytoplasmic N terminus in PBCV-1 Kcv (2, 12)), outer transmembrane domain (TM1), pore helix (P helix), selectivity loop (filter), inner transmembrane domain (TM2).

**Results**

Kcv (K⁺ chlorella virus) is a 94-amino acid protein encoded by the genome of PBCV-1 (family Phycodnaviridae), the prototype of a family of viruses that infect unicellular, eukaryotic, chlorella-like green algae (14). We have previously described the properties of Kcv and six new Kcv-like channels cloned from 40 chlorella viruses (2, 3).

Expression of the six Kcv-like proteins in Xenopus laevis oocytes showed that they all form K⁺-selective channels and that the properties of the six new Kcv-like channels, or subgroups among them, differ from those of the reference channel PBCV-1 Kcv (3). These differences include: 1) inactivation of inward current in K⁺ solutions (all new channels), 2) absence of inactivation of inward current in Rb⁺ solutions (all new channels except NY-2B Kcv) and 3) full block of inward current by external Cs⁺ (AN69C, MA-1D, NY-2A, and NY-2B Kcv).

A comparison of the currents recorded from PBCV-1 Kcv and MA-1D Kcv, a channel that displays all three new properties, is shown in Fig. 3, A and B. The opposite behavior is evident when comparing the inward
currents of the two channels: in K\(^+\), they activate in PBCV-1 and inactivate in MA-1D; in Rb\(^+\), they decrease in PBCV-1 and increase in MA-1D; and in Cs\(^+\), they are fully blocked in MA-1D but not in PBCV-1. We operationally describe these properties by the ratio of currents measured at the reference voltage of \(-100\) mV. As reported in Table I, the ratio of the current values measured in Rb\(^+\) and K\(^+\) solutions was \(<1\) for PBCV-1 Kcv and \(>1\) for MA-1D Kcv (IRb\(^+\)/IK\(^+\) = 0.4 ± 0.09 and 2.0 ± 0.2, respectively). In addition, the ratio between the current measured in the presence and in the absence of 10 mM Cs\(^+\) was approximately \(1\) for PBCV-1 Kcv and near 0 for MA-1D-Kcv (ICs\(^+\)/IK\(^+\) = 0.8 ± 0.04 and 0.1 ± 0.02, respectively).

Analysis of Common Amino Acid Substitutions—Alignment of the seven channels (Fig. 1) highlights common amino acid substitutions that could correlate with the above properties. Compared with PBCV-1 Kcv, all the new channels have two amino acid substitutions, in positions 19 and 54 in TM1 and in the pore helix, respectively. These substitutions might be related to the inactivation of inward currents in K\(^+\), because this is a property shared by all the new channels. Channels that do not show inactivation of inward current in Rb\(^+\) (all new channels except for NY-2B) have several conserved residue combinations which are absent in PBCV-1 and NY-2B (Leu-54–Val-64; Leu-54–Phe-66; Leu-54–Leu-78; Leu-54–Ile-84). The combination Leu-54–Phe-66 in the pore helix and selectivity filter is especially intriguing, given the important role of an aromatic residue (Phe or Tyr) in position 66 in the control of ion permeability in K\(^+\) channels (19). Finally, channels that are completely blocked by Cs\(^+\) (AN69C, MA-1D, NY-2A, and NY-2B Kcv) have three common amino acid substitutions in TM1 (Val-20, Thr-26, and Arg-29) compared with PBCV-1 Kcv, which is only slightly blocked by Cs\(^+\). To test the functional relevance of the amino acid combinations mentioned above, site-directed amino acid mutations were made to change the reference channel PBCV-1 Kcv into one of the new channels and vice versa. MA-1D Kcv was chosen for these studies because it exhibits the largest functional distance from PBCV-1 Kcv (it differs in all three properties) with the fewest amino acid substitutions (five substitutions).

Interactions between TM1 and the Pore Helix Affect Channel Properties—We examined the effects of single and double amino acid substitutions in positions 19 and 54 in MA-1D Kcv. Substitution at position 19 (V19F) by the corresponding residue from PBCV-1 Kcv produces a channel whose properties are substantially modified and are very similar, although not identical, to those of PBCV-1 Kcv (Fig. 3C, Table I). These similar-
FIG. 3. Different electrophysiological properties displayed by PBCV-1 and MA-1D Kcv channels. Each row shows the currents recorded in 50 mM K+, 50 mM Rb+, and 50 mM K+ plus 10 mM Cs+ solutions from a representative single oocyte. The current/voltage relation on the right shows K+ (■), Rb+ (□), and Cs+ (▲) Kcv channels. A, the inward currents of PBCV-1 Kcv show activating kinetics in K+ solution, strong reduction in Rb+ solution and a small reduction in the presence of 10 mM Cs+. B, the inward currents of MA-1D show opposite behavior; 1) inactivation in K+ solution; 2) increase in Rb+; 3) full block by external Cs+. C, strong effect of the V19F mutation on the properties of MA-1D Kcv. The mutant MA-1D channel resembles the PBCV-1 channel in regard to 1) kinetics in K+, 2) inward currents increase in Rb+, and 3) small reduction of inward currents by Cs+. It is noteworthy that, in the presence of 10 mM Cs+, the kinetics of this channel is most similar to that of PBCV-1 Kcv. The mutation is given in one-letter code.

FIG. 4. Synergistic effect of residues 19 and 54 on channel properties. A, comparison of current kinetics recorded in 50 mM K+ solution from MA-1D wt and mutants as indicated. As shown in Fig. 3C, mutation V19F creates the typical kinetics of PBCV-1. Although mutation L54I does not have any effect by itself, it completely suppresses the effect of mutation V19F in the double mutant. B, comparison of current/voltage relations from MA-1D wt and mutants: ■, 50 mM K+; ○, 50 mM K+ plus 10 mM Cs+. The strong Cs+ block observed in the wt is removed by mutation V19F. Mutation L54I does not have any effect but nevertheless suppresses the effect of mutation 19 in the double mutant. Mutations are given in one-letter code.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Summary of effects of single and double mutations performed in PBCV-1 and MA-1D Kcv channels</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBCV-1</td>
<td>Steady-state currents measured at −100 mV in 50 mM Rb+ or 50 mM K+ plus 10 mM Cs+ solutions, expressed as ratios of current measured in 50 mM K+ only. A value of IRb/IK lower than 1 indicates inactivation in Rb+ and a value higher than 1 indicates removal of inactivation. ICs+IK expresses the degree of Cs+ block and can range from 0 (full block) to 1 (no block). Data are mean of n = number of measured oocytes ± S.E. Mutations are given in one-letter code.</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>PBCV-1 Kcv</td>
<td></td>
</tr>
<tr>
<td>wt</td>
<td>0.4 ± 0.09 (n = 4) 0.8 ± 0.04 (n = 5)</td>
</tr>
<tr>
<td>F19V</td>
<td>0.5 ± 0.06 (n = 8) 0.9 ± 0.05 (n = 2)</td>
</tr>
<tr>
<td>I20V</td>
<td>0.6 ± 0.11 (n = 5) 0.4 ± 0.01 (n = 4)</td>
</tr>
<tr>
<td>M26T</td>
<td>0.4 ± 0.06 (n = 6) 0.9 ± 0.04 (n = 5)</td>
</tr>
<tr>
<td>K29R</td>
<td>0.2 ± 0.06 (n = 3) 0.3 ± 0.02 (n = 3)</td>
</tr>
<tr>
<td>I54L</td>
<td>1.6 ± 0.09 (n = 11) 0.7 ± 0.1 (n = 7)</td>
</tr>
<tr>
<td>F19V I54L</td>
<td>0.7 ± 0.05 (n = 5) 0.9 ± 0.03 (n = 5)</td>
</tr>
<tr>
<td>MA-1D</td>
<td></td>
</tr>
<tr>
<td>wt</td>
<td>2.0 ± 0.2 (n = 6) 0.1 ± 0.02 (n = 6)</td>
</tr>
<tr>
<td>V19F</td>
<td>0.5 ± 0.09 (n = 4) 0.7 ± 0.03 (n = 4)</td>
</tr>
<tr>
<td>V20I</td>
<td>2.0 ± 0.08 (n = 7) 0.1 ± 0.03 (n = 7)</td>
</tr>
<tr>
<td>T26M</td>
<td>2.9 ± 0.17 (n = 6) 0.1 ± 0.02 (n = 5)</td>
</tr>
<tr>
<td>R29K</td>
<td>2.3 ± 0.18 (n = 3) 0.1 ± 0.01 (n = 5)</td>
</tr>
<tr>
<td>L54I</td>
<td>1.7 ± 0.07 (n = 7) 0.1 ± 0.01 (n = 5)</td>
</tr>
<tr>
<td>V19F L54I</td>
<td>1.4 ± 0.11 (n = 5) 0.1 ± 0.03 (n = 4)</td>
</tr>
</tbody>
</table>

Ities include: 1) kinetics in K+ solution more similar to those of PBCV-1 Kcv because of the disappearance of the inactivation kinetics typically present in MA-1D wt Kcv; 2) decrease of inward current when Rb+ replaces K+ (as in PBCV-1 Kcv, IRb+/IK+ < 1); and 3) weak inhibition of inward current by Cs+ (as in PBCV-1 Kcv ICs+/IK+ close to 1). The similarity with PBCV-1 Kcv is striking but not complete; indeed, the tail currents upon return to −80 mV in K+ are more similar to MA-1D wt than PBCV-1 wt, and K+ replacement by Rb+ causes only a weak inhibition of inward current relative to PBCV-1 wt. However, when 10 mM Cs+ is added to the K+ solutions, the kinetics of the mutant channel MA-1D V19F resembles PBCV-1 Kcv. In contrast, amino acid substitution at position 54 (L54I) has no effect on the properties of the MA-1D channel (Table I). We were surprised to find that the strong effects of the V19F mutation are suppressed completely in the double mutant MA-1D V19F L54I (Table I and Fig. 4).

Substitution at residue 54 completely reverses the effects of mutation 19 on current kinetics in K+ (Fig. 4A) and on inward currents in the presence of Cs+" (Fig. 4B). Consistent with these results is the effect on inward currents in Rb+; whereas the IRb+/IK+ ratio of the mutant V19F is 0.5, that of the double mutant V19F L54I is > 1 (Table I).

The opposite single and double amino acid substitutions in positions 19 and 54 were constructed in PBCV-1 Kcv. No effect is observed with the F19V substitution, whereas an increase in Rb+ inward conductance occurs with the I54L mutation. Again,
the double mutation (F19V I54L) reverses the effect of the single mutation (I54L) (results summarized in Table I).

The results with MA-1D Kcv indicate that both positions, 19 in TM1 and 54 in the pore helix, are important in determining channel properties. In particular, position 19 in TM1 of MA-1D Kcv strongly affects events occurring in the inner pore, such as Cs⁺ block. The impact of TM1 on pore properties seems to be mediated by the pore helix through position 54, because the effects of the substitutions in positions 19 and 54 are not independent. This interaction also occurs with PBCV-1 Kcv. Furthermore, the results obtained with the double mutants indicate that these two positions, 19 and 54, are not, as we originally expected, solely responsible for channel kinetics. In fact, both double mutants (PBCV-1 Kcv F19V I54L and MA-1D Kcv V19F L54I) have kinetics similar to those of their respective wild-type channels. The lack of effect on kinetics indicates that amino acid residues 19 and 54, although responsible for channel properties, must interact with other positions to exert their effect on channel properties.

The Selectivity Filter Interacts with TM1 and the Pore Helix—Additional evidence for an interaction between different domains within the pore was obtained by analyzing channel properties in Rb⁺ solutions. As mentioned above, PBCV-1 Kcv typically shows strong inward rectification when external K⁺ is replaced with Rb⁺ (Fig. 3A). In contrast, none of the new channels, except NY-2B Kcv (Fig. 5), exhibited similar strong inward rectification.

The unique behavior of NY-2B Kcv among the new Kcv channels focused attention on position 66 in the selectivity filter. This residue is changed only in NY-2B Kcv. Although three other residues, at positions 64, 78, and 84, are also changed only in NY-2B Kcv, residue 66 is unique because it is highly conserved in all K⁺ channels. Indeed, an aromatic residue, either Tyr or Phe, is present in this position in all known “one pore” K⁺ channels. Therefore overlooking amino acids 64, 78, and 84, the only combination of amino acids conserved in all channels that do not inactivate in Rb⁺ (AN69C, AL-2A, CA-4B, MA-1D, NY-2A) is Leu-54–Phe-66 (Fig. 1). PBCV-1 Kcv and NY-2B Kcv, both of which inactivate in Rb⁺, lack one of these two amino acids (the combination is Ile-54–Phe-66 in PBCV-1 Kcv and Leu-54–Leu-66 in NY-2B Kcv). The I54L mutation that produced the Leu-54–Phe-66 combination in PBCV-1 Kcv increases the Rb⁺ current in the PBCV-1 Kcv channel (IRb⁺/IK⁺ >1; Table I) and the corresponding current/voltage relations are shown in Fig. 5. As expected, creating a Leu-54–Phe-66 combination in NY-2B Kcv abolishes its strong inward rectifying behavior in Rb⁺ (IRb⁺/IK⁺ >1; Fig. 5).

Again, the results indicate that a single amino acid does not completely determine a given channel property. Amino acid 54 in the pore helix and amino acid 66 in the selectivity filter are predicted by the model to be 13.4 Å apart with no apparent direct interaction. Nevertheless, their concerted action is required to affect Rb⁺ conductance. The view that channel properties are the result of the interaction between distantly located amino acids was strengthened by the observation that position 19, in TM1, also affects channel behavior in Rb⁺ (previously mentioned for MA-1D V19F; Fig. 3C). Indeed, the double mutant PBCV-1 Kcv F19V I54L reverses the Rb⁺ phenotype established by the Leu-54–Phe-66 pair (Fig. 3 and Table I). Hence, TM1 interferes with the Leu-54–Phe-66 phenotype and can suppress it entirely.

In summary, these results provide evidence showing the concerted effects on channel function exerted by three different domains in the viral K⁺ pore protein: the TM1 domain (amino acid 19), the pore helix (amino acid 54), and the selectivity filter (amino acid 66). The results also indicate that identical combinations of residues in these three sites, Val-19–Leu-54–Phe-66 can produce opposite behaviors; e.g. compare MA-1D wt (IRb⁺/IK⁺ >1) with PBCV-1F19V I54L (IRb⁺/IK⁺ <1). Thus, the resulting phenotype of a channel depends on the combination of these three amino acids within the context of their channel-specific background.

Amino Acids 20 and 29 in TM1 Affect Cs⁺-induced Block—We obtained additional evidence that the role of indi-
individual amino acids in the Kcv proteins depends on their global context. PBCV-1 Kcv and MA-1D Kcv sequences differ in five positions: positions 19 and 54 as discussed above, as well as three other positions in TM1: 20, 26, and 29. All channels which are completely inhibited by Cs\(^+\) (AN69C, MA-1D, NY-2A, and NY-2B Kevs) differed from PBCV-1 Kcv in these three TM1 positions. Changing these three residues in MA-1D Kcv individually into the corresponding residues of PBCV-1 Kcv produced no obvious changes in current. In contrast, changing amino acid 20 in PBCV-1 Kcv into the corresponding MA-1D Kcv residue, strongly increased the Cs\(^+\)-induced block of inward currents (60% at \(-100\) mV; Fig. 6 and Table I). The mutation MA-1D Kcv I20V Kcv also exhibited other properties, although less pronounced, typical of MA-1D Kcv, such as a tendency to inactivate upon hyperpolarization in the presence of K\(^+\) and an increase in inward current in the presence of Rb\(^+\) (Table I).

A similar increase in Cs\(^+\)-induced block (65% at \(-100\) mV) occurs in the mutant PBCV-1 K29R (Fig. 6). In this case, however, the mutation affected neither the kinetics of the channel in K\(^+\) solution (Fig. 6) nor its behavior in Rb\(^+\) solution (Table I). No effect was observed by mutating residue 26. From these results, we conclude that at least two of the three amino acids at positions 20, 26, and 29 are involved in Cs\(^+\) sensitivity.

Mutations Do Not Alter Na\(^+\) Permeability or Ba\(^{2+}\) Inhibition—All the mutants described in this report were tested for their relative permeability to Na\(^+\) compared with K\(^+\) and for their sensitivity to Ba\(^{2+}\). The wt channels transported Na\(^+\) poorly and were completely blocked by 1 mM Ba\(^{2+}\) (3). None of the mutations altered these parameters, suggesting that the overall pore architecture of the channel proteins was not changed and that structural features involved in Rb\(^+\) permeability and Cs\(^+\) inhibition differ from those involved in Na\(^+\) permeability and Ba\(^{2+}\) inhibition.

DISCUSSION

Previous structure-function studies have identified several amino acids in the pore region of K\(^+\) channels that, when individually mutated, strongly influence channel properties (e.g. Refs. 18–20). However, mutagenesis studies can never be complete because it is nearly impossible to artificially change every amino acid or combination of amino acids in a protein. In the present study, we exploited the high evolutionary pressure exerted by selection on the chlorella virus genomes to maintain a functional K\(^+\) channel. The rationale behind this approach is that amino acids change in a protein without bias during evolution. Because the virus apparently requires a functional channel for replication, only mutations that result in a functional channel survive the selection process. Indeed all of the Kcv variants, even those with as many as 12 amino acid substitutions, produce a functional K\(^+\) selective channel in X. laevis oocytes (3). However, the physiological properties of the channels differ unexpectedly from the reference channel PBCV-1 Kcv. Our long term goal is to understand the contributions of each amino acid substitution on the overall physiological properties of the channel. The correlation between amino acid changes and the physiological properties of the channels guided site-directed mutations, either singly or in combinations. Comparison of Kcv channels from viruses PBCV-1 and MA-1D was especially enlightening. These two channel proteins differ by only five amino acids and yet they differ dramatically in channel kinetics, cation permeability, and sensitivity to Cs\(^+\). Analyses identified functional sites and unexpected long-range interactions in the K\(^+\) channel proteins that were previously unknown. The results also indicate that none of the functional properties considered, such as Cs\(^+\)-induced block or Rb\(^+\) permeability, can be attributed to a single residue. Instead, the complex interplay of separate amino acids is responsible for the expression of a functional property.

A simple correlation between sequence data and functional features established that amino acids in three different domains affect essential properties of the K\(^+\) channel (positions 19 in TM1, 54 in the pore helix, and 66 in the selectivity filter). However, the most important finding in this study is that these positions are not independent. For example, in virus MA-1D, the phenotype obtained by altering amino acid 19 in TM1 is reversed by a second mutation in position 54, indicating that their effects are not additive. It is noteworthy that this occurs even though the substitution in position 54, Leu54Ile, is conservative and does not produce a phenotype when introduced on its own.

The simplest explanation for these results is that the two amino acid positions, one located in TM1 and the other in the pore helix, interact. The same conclusion can be reached for these two amino acids by the reverse experiment in which
Long Distance Interactions within the K+ Channel Pore

single and double mutations were introduced in PBCV-1 Kcv to match MA-1D Kcv. In addition, in this case, the 19–54 mutant reverses the phenotype induced by the single mutation. However, in this case amino acid 54 produces the altered phenotype.

A second example of a long-range interaction was revealed by the Rb+ conductance studies. All the Kcv channels that exhibit high conductance at negative voltages in Rb+ solutions contain Leu-54 and Phe-66 in the pore helix and selectivity filter, respectively. Substituting these two amino acids was sufficient to increase Rb+ conductance in NY-2B and PBCV-1 Kcv, two channels with a low conductance in Rb+. This result suggests an interaction between the pore helix and the selectivity filter. Position 54 in the pore helix is next to two aromatic amino acids (Tyr Phe) that are highly conserved among all K+ channels; furthermore, the KcsA crystal structure suggests that the pore helix interacts with the selectivity filter (5). It is particularly interesting that: 1) amino acids 54 in the pore helix and 66 in the selectivity filter are affected by the amino acid in position 19 in TM1 and 2) interactions between amino acid positions 19, 54, and 66 are influenced by the general background. Identical amino acid combinations result in different properties in PBCV-1 and NY-2A Kvs.

Analysis of Cs+ inhibition produces a third example of a long-range interaction. All three positions that influence Cs+ sensitivity (amino acids 19 in MA-1D, 20 and 29 in PBCV-1) are located in TM1, which presumably forms the outer helix. Therefore, a feature such as a channel block, which must occur inside the pore (21–23), is affected by a distant domain. That is, amino acid 19 (or 20 or 29) must interact with the inner pore to change the sensitivity to Cs+ binding. However, when the structure of the bacterial K+ channel KirBac1.1 is used as a reference structure for Kcv (Fig. 2B), amino acid 19 is neither near nor associated with the selectivity filter or the inner pore domain; this result suggests that amino acid 19 is coupled indirectly to the pore domain. The data as a whole predict a coupling of TM1 with the pore via a long range interaction between positions 19 and 66 mediated by position 54 in the turret.

Because the architecture of the pore is conserved among K+ channels (24, 25), it is reasonable to speculate that long-range interactions observed in Kcv also occur in other K+ channels. These interactions between TM1 and inner pore, mediated by the pore helix, may represent a more general pathway during conformational changes, such as those leading to channel activation. An example would be, in voltage-activated channels, the conformational changes transmitting the movement of the voltage sensor to the gate inside the pore (26, 27).

CONCLUSIONS

We took advantage of the natural diversity of a family of Kcv channels encoded by chlorella viruses to identify amino acid positions that affect specific channel features. The amino acid substitutions, together with the different physiological properties found in the six new Kcv channels, guided site-directed mutations, either singly or in combinations. By combinatorial analysis of the six channel variants, we identified three functional sites, amino acid positions 19, 54, and 66, that affect K+ channel properties by long distance interactions. It should be noted that a combinatorial analysis of three amino acids within a sequence of 94 amino acids would require screening an extremely large library for detection. Because libraries of this size cannot be easily produced, the present approach offers a valuable tool to identify complex interactions in a K+ channel.

Acknowledgments—We thank Jack Dainty (University of East Anglia, Norwich, UK) and Dan Minor (University of California, San Francisco) for reading the manuscript and for helpful discussions.

REFERENCES