Alphavirus 6K Proteins Form Ion Channels*

Ross River virus and Barmah Forest virus are Australian arboviruses of the Alphavirus genus. Features of alphavirus infection include an increased permeability of cells to monovalent cations followed by viral budding. Virally encoded ion channels are thought to have a role in these processes. In this paper, the 6K proteins of Ross River virus and Barmah Forest virus are shown to form cation-selective ion channels in planar lipid bilayers. Using a novel purification method, bacterially expressed 6K proteins were inserted into bilayers with a defined orientation (i.e. N-terminal cis, C-terminal trans). Channel activity was reversibly inhibited by antibodies to the N and C termini of 6K protein added to the cis and trans baths, respectively. Channel conductances varied from 40–800 pico siemens, suggesting that the protein is able to form channels with a range of possible oligomerization states.

Members of the Alphavirus genus include a number of medically significant pathogens: Western, Eastern, and Venezuelan equine encephalitis viruses, Chikungunya virus, O’nyong-nyong fever virus, Sindbis and Semliki Forest viruses (SFV) (on which most of the extensive molecular characterization of alphaviruses is based), Ross River virus (RRV), and Barmah Forest virus (BFV). The latter two viruses are the etiological agents of epidemic polyarthritis in Australia.

Alphaviruses have an approx. 11.7 kb single-stranded RNA genome of positive sense. The 5’ end (7.6 kb) encodes components involved in genomic replication and mRNA synthesis. The 3’ end (4.1 kb) encodes the structural proteins of the virus. The structural proteins are translated from a subgenomic mRNA as a single polyprotein in the order capsid-PE2–6K-E1. The polyprotein is cotranslationally translocated across the ER membrane. Signal sequences alternating with stop-transfer (anchor) sequences direct the topology of the polyprotein with respect to the ER membrane (1). Cleavage occurs via cellular proteases and the autoprotease activity of the capsid protein to generate the viral capsid and membrane proteins (2). Alphavirus 6K proteins are small (58–61 amino acids), hydrophobic, and associate with membranes (3). The function of 6K protein in the virus life cycle is not fully understood. However, the 6K protein of SFV has been shown to function as a stop-transfer peptide sequence for the insertion of the E1 protein into the ER membrane (1). Additionally, expression of 6K protein causes an increase in membrane permeability in Escherichia coli and enhances both membrane permeability and viral budding in eukaryotic cells (4–8). On the basis of these observations and structural similarity with other small virus proteins, it has been suggested that the 6K protein is a virally encoded ion channel (viroporin) (9). Viroporins are short (50–120 amino acids), integral membrane proteins that enhance membrane permeability in infected cells (9). Viroporins have been shown to affect glycoprotein processing, transport of proteins through the ER, and virion budding (6, 10–13). Other proteins identified as viroporins include the M2 protein of influenza A (14, 15), the NB protein of influenza B (16), the Vpu and Vpr proteins of HIV-1 (17, 18), and the 3A and 2B proteins of poliovirus (19, 20). The members with confirmed ion channel activity are: M2, NB, Vpr, and Vpu (15–18, 21). Of these four proteins, M2 is the only one for which the role of the ion channel activity in the physiological functions of the protein is understood (22).

The topology of the structural proteins of SFV has been determined by in vitro translation-translocation assay (1). The results obtained were consistent with the 6K protein crossing the membrane twice (1). However, prediction of transmembrane regions by the method of Sonnhammer et al. (23) suggests that all mature alphavirus 6K proteins cross the membrane only once (see Fig. 1B) (23). In this paper, we present evidence that the 6K protein spans the membrane once when reconstituted in vesicles or incorporated into planar lipid bilayers.

We show that purified, recombinant 6K proteins from both BFV and RRV form cation-selective ion channels when inserted into planar lipid bilayers. Antibodies specific for each 6K species were found to inhibit channel activity. Additionally, the site-directed mutation of serine 32 to proline (S32P) in BFV 6K protein resulted in altered ionic selectivity of the channel. A synthetic peptide, corresponding to the amino acid sequence of BFV 6K protein (B6Kpep), also makes ion channels with similar properties.

EXPERIMENTAL PROCEDURES

Cloning of RRV 6K and BFV 6K—The coding sequence for the BFV 6K gene was amplified from a BFV cDNA clone (24), which contained the 6K gene region using the primers 98–16 (5’-gcgggatccgacacttta-
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Gagat), which introduces a BamHI site (underlined) and 98–17 (5'-atagcctagctagctagctag) creates, which introduces a XhoI site (underlined). The coding sequence for the RRV 6K gene was amplified from a cDNA genomic clone of RRV strain T48 (25). Primers used were 98–14 (5'-cgaggtctagctagctag), which introduces a BamHI site (underlined) and 98–55 (5'-cgaggtctagctagctagctag), which introduces a XhoI site (underlined). PCR was performed with 0.035 units/μl native Phu polymerase (Stratagene) in a Corbett Research FTS-1 capillary thermocycler with the following parameters: one cycle of 94°C for 2 min; 25 cycles of 94°C for 30 s, 45°C for 1 min, 72°C for 30 s; and one cycle of 72°C for 5 min. PCR product size was confirmed by electrophoresis on 2% TAE (40 mM Tris acetate, 1 mM EDTA)-agarose gels (6 V/cm, 1 h) followed by ethidium bromide staining (50 μg/ml) and photography. The PCR products were gel-purified with QiAquick Spin Gel Extraction Kit (Qiagen), digested with restriction enzymes (BamHI followed by XhoI), and ligated into the vector p2GEX (21), which had also been BamHI/XhoI-digested and 5'-dephosphorylated with shrimp alkaline phosphatase.

The plasmid p2GEX is based on pGEX-2T (Amersham Biosciences), and contains an additional copy of the Schistosoma japonicum GST cDNA designed to express an unfused copy of GST. Previous observations from this laboratory suggest that the co-expression of free (unconjugated) GST in addition to the GST fusion from the same plasmid can enhance cytoplasmic yields of largely insoluble GST fusion proteins representing the N terminus of BFV 6K, the N terminus of RRV 6K (α-B6N), the C terminus of BFV 6K, the C terminus of RRV 6K (α-B6N), and the C terminus of RRV 6K (α-B6N). Initial immunizations with FUS in Freund's complete adjuvant and subsequent booster immunizations at 3 and 5 weeks were in Freund's incomplete adjuvant. Blood samples (10 ml) were collected by ear vein cannulation at 6, 8, and 10 weeks postimmunization. Antisa were assayed for specificity by dot-blot analysis using unconjugated peptides. Affinity chromatography was used to purify antibodies from antiserum. Briefly, peptides were dissolved in 6 M guanidine HCl/10 mM Tris, pH 8.0, reduced with 10 mM dithiothreitol, desalted, (NAD desalting column), and covalently linked to SulfoLink columns (Pierce). Columns were gradually returned to more physiological conditions by sequential washing with PBS containing decreasing amounts of guanidine hydrochloride. Antisa were applied to the columns, and after thorough washing, a specific IgG bead was eluted using 100 mM glycine, pH 2.5.

Synthesis and Purification of a Synthetic 6K—The 58-mer peptide (DTLDDFSFLWNQNMF-WLQIAJSPVAAFLCYSNRAKNNLACMKMFGLISGLCQVQ) corresponding to the amino acid sequence of BFV 6K (referred to hereafter as B6Kpep) was synthesized manually using Boc chemistry in the Biomolecular Resource Facility at the John Curtin School of Medical Research, Australian National University. B6Kpep was dissolved in 6 M guanidine HCl/10 mM Tris, pH 8.0, reduced with 10 mM dithiothreitol, desalted, (NAD desalting column), and covalently linked to SulfoLink columns (Pierce). Columns were gradually returned to more physiological conditions by sequential washing with PBS containing decreasing amounts of guanidine hydrochloride. Antisa were applied to the columns, and after thorough washing, a specific IgG bead was eluted using 100 mM glycine, pH 2.5.

Western Blot Analysis—Proteins were transferred from polyacrylamide gels to polyvinylidene difluoride membranes with a semi-dry apparatus (Amersham Biosciences). Nonspecific protein binding sites on the polyvinylidene difluoride membranes were blocked in 10% (w/v) skim milk proteins in PBS with 0.5% Tween 20 (TPBS). Primary antibodies were either rabbit antiserum (1:100), or affinity purified antibodies in 10% (w/v) skim milk proteins in TPBS. The secondary antibody was a goat-raised anti-rabbit IgG alkaline phosphate conjugate added in PBS (Sigma), and color development was with Western Blue-stabilized substrate for alkaline phosphatase (Promega).

Protein Estimation and N-terminal Protein Sequencing—Protein estimation was using a bichinonic acid protein assay (Pierce, kit form) in a 96-well plate. Fatty acid-free bovine serum albumin was used to create standard curves. A Bio-Rad model 450 microplate reader was used to measure absorbance at 570 nm, with background at 405 nm (50 μg/ml) and used to measure absorbance at 340 nm (50 μg/ml). Mass spectroscopy, dot-blot, and Western analyses were used to confirm the identity of the eluted protein (data not shown).

Electrophysiology—Vesicles containing purified 6K protein were fused with lipid bilayers using standard techniques described previously (17, 21). Briefly, lipids (PE/PC/PS; 3:1:1) supplied in CHCl3 were dried under argon, mixed with 0.3 M NaCl (20–25°C), distilled, and painted over a 120–180-μm diameter circular hole in a Delrin cup. Both solutions contained: trans, 60 mM NaCl, 10 mM TES, pH 7.0 or 8.0, total volume 1 ml; and cis, 10 mM TES with 510 mM NaCl or 500 mM KC1 or 500 mM CaCl2 each buffered to pH 7.0 or 8.0, total volume 1.3 ml.
Currents were measured via AgCl-coated silver wire electrodes without agar bridges. This can potentially result in large changes in the junction potential at the electrodes during experiments (e.g. if the AgCl coating of the electrode becomes chipped). To control for this possibility, the current between cis and trans bath solutions was clamped, and the voltage offset adjusted to zero prior to painting a bilayer. At the conclusion of every experiment the bilayer was broken, and the current immediately clamped to zero. If the potential difference between chambers was greater than 10 mV the experiment was discarded. After a good bilayer had been established, vesicles containing 6K protein were added to the cis bath. The bath solution was stirred until channel activity was observed. Potential was controlled and current recorded with an Axopatch 200A amplifier. Currents were filtered at 500 Hz, digitized at 2 or 5 kHz by using an A-to-D converter interfaced with a desktop personal computer. The cis bath was grounded, and voltages are expressed as trans potential relative to cis.

Reversal potentials were determined by changing the holding potential in 2–10 mV steps observing the current at the cis bath. For channel inhibition experiments, antibody was added in small aliquots (<5 μl, 14 μg/μl), and bath solutions were stirred for 1 min before recording any effects. The cis bath was set up to enable perfusion by a pair of back-to-back syringes connected to tubing at the ends of which could be lowered into the cis bath.

**Ion Activity Coefficients**—Ion activities for each species were calculated from the ionic strength, \( I \), using the Guy-Chapman “Limiting Law” (31). This could not be used for the CaCl\(_2\) solution because the ionic strength was too high. Therefore, Ca\(^{2+}\) activities were determined using a calcium-selective electrode (which gave a linear response in the range of CaCl\(_2\) concentrations used in the bilayer experiment).

**Calculation of Relative Ionic Permeabilities**—Relative ionic permeabilities were determined from reversal potential data and ionic activities. Reversal potentials were determined as described above and corrected for liquid junction potential effects. Liquid junction potentials for differing bath solutions can be calculated using the Henderson equation (32). The junction potential for a given pair of dissimilar bath solutions was determined by measuring the voltage difference (without a bilayer present) between the cis and trans baths of the bilayer apparatus. This voltage was measured relative to that obtained from symmetric solutions (i.e., when the liquid junction potential equals zero). The potential difference between the two baths (when the current was clamped to zero) was measured with a 4 M KCl-containing agar bridge electrode. Experimentally measured values were not found to deviate by more than 2 mV from those predicted by the Henderson equation. As the Henderson equation is only valid for monovalent species, the liquid junction potential for the Ca\(^{2+}\)-containing cis solution was determined experimentally. Once corrected for liquid junction potentials, the sum of the currents from all ionic species is zero at the reversal potential, and the constant field equation can be solved. The ionic activities of the species present (see above for calculation) were used in conjunction with corrected reversal potential data to iteratively solve the constant field equation for \( I = 0 \) as described previously (33). Firstly, the \( P_{Na}/P_{Cl} \) ratio was determined for each channel species by optimizing the constant field equation to obtain the Na\(^{+}\) permeability (bath conditions for cis: 510 mM NaCl, 10 mM TES, pH 7.0, and for trans: 60 mM NaCl, 10 mM TES pH 7.0). \( P_{Na}/P_{Cl} \) was assumed to remain constant in the presence of other ions (i.e. when determining K\(^{+}\) and Ca\(^{2+}\) permeabilities). The TES anion was assumed not to permeate the channel. For calculating ionic activities in the CaCl\(_2\) solutions, Ca\(^{2+}\) activity was determined as described above, and the activity of Cl\(^{-}\) was derived from a table of mean activity coefficients for CaCl\(_2\) at a range of molalities (34). As the mean activity coefficient of a salt solution is the geometrical mean of the activity coefficients of its component ions, the activity of Cl\(^{-}\) in a CaCl\(_2\) solution could be derived.

**RESULTS**

Purified, recombinant 6K proteins of RRV and BFV were produced after expression in *E. coli* with N-terminal glutathione-S-transferase tags. The cDNA fragments encoding the RRV or BFV 6K proteins were cloned into the vector p2GEX (described under “Experimental Procedures”), and optimal expression and purification methods were determined.

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**Neutralization—** Alphavirus 6K proteins contain a large proportion of hydrophobic residues (see Fig. 1) and have been shown to interact with membranes in virus-infected cells (1, 3, 35). To examine potential toxicity of the RRV and BFV 6K proteins in *E. coli*, we monitored the growth of cultures expressing GST-6K fusion proteins. Induction of GST-6K expression with IPTG rapidly led to arrest of *E. coli* cell growth. Cultures of *E. coli* expressing RR 6K fusion protein, induced during the log phase of growth, continued to grow for only 10 min postinduction (Fig. 2). In contrast, control cultures (uninduced or expressing un-fused GST from p2GEX) continued to grow for over 2 h (p2GEX not shown for clarity). Fig. 2 shows combined results from seven induced cultures expressing RRV 6K compared with five
uninduced cultures. Similar depression of growth was observed in E. coli expressing BFV 6K protein (data not shown). This indicates that expression of the 6K fusion protein inhibits growth of E. coli and is consistent with the findings of Sanz et al. (4) that SFV 6K protein enhances membrane permeability in bacteria.

Purification of RRV GST-6K and BFV GST-6K Fusion Proteins—GST-6K fusion proteins of BFV and RRV were purified from E. coli extracts as described under “Experimental Procedures.” SDS-PAGE was used to assess the efficiency of different steps throughout purification. Coomassie staining of extracts from bacteria expressing RRV GST-6K that had been purified on glutathione columns revealed the major constituents to be proteins of molecular masses 28 kDa and 34 kDa (Fig. 3A). The 28-kDa protein was immunoreactive to anti-GST antibodies on a Western blot (not shown) and represents “free” GST expressed from the unfused copy of the gene in the expression plasmid. The 34-kDa protein was also immunoreactive with anti-GST antibodies, and its size corresponds to that expected for the RRV GST-6K fusion protein. Surprisingly, the 34-kDa protein was not recognized by polyclonal antibodies raised against the N-terminal 20 amino acids of RRV 6K. Rather, a single protein of a molecular mass of ~32 kDa was immunoreactive to the anti-RRV 6K N-terminal antibodies (Fig. 3B). The 32-kDa protein was of much lower abundance than the 34-kDa protein and most likely represents a truncated form of the RRV 6K fusion protein. Unfused GST was present in about 16-fold excess compared with the full-length protein (determined by Coomassie staining of serial dilutions of the RRV GST-6K fusion protein, data not shown). Fusion protein expression was thereby calculated to be ~1.7 mg/liter of LB culture after bicinechonic acid assay of total protein (data not shown). Similar results and yields were obtained from cells expressing the BFV GST-6K fusion protein, except that no truncated protein was detected.
Buffer conditions were cis: Na\(^{+}\), K\(^{+}\), Ca\(^{2+}\) and Cl\(^{-}\). A channel that was closed at 1100 mV than at 100 mV (Fig. 4 solid line). RRRV 6K protein was added to the cis chamber (Fig. 5B). A dramatic increase in channel activity was seen when added to the trans chamber (n = 5, not shown).

Conversely, antibody against the C-terminal of RRRV 6K inhibited channel openings when added to the trans bath (n = 6, Fig. 5B), but not the cis bath (n = 7, not shown). All points histograms of currents recorded before and after addition of antibody are shown in Fig. 5, C and D. This chamber-dependent effect of the antibodies demonstrates that channel inhibition was specific to the particular epitope recognized by the antibody. Further, the results indicate that the RRRV 6K protein is oriented in the vesicles with the N-terminus facing the outside and the C-terminus the inside. When placed in the cis solution, vesicles fuse with a bilayer so that the part of a transmembrane protein that faces the outside of the vesicle will face the cis solution (36).

GST and Uncut RRV GST-6K Fusion Protein Do Not Form Ion Channels—Unfused GST was obtained from a bacterial culture expressing p2GEX using the purification procedure devised for 6K. The purified GST was “inserted” into vesicles and incubated with thrombin. The GST protein did not make channels in the bilayer system (n = 5, total time waiting for channel activity = 1 h). In a similar manner, uncut RRV GST-6K fusion protein was tested and found not to have any ion channel activity (n = 5, total time waiting for channel activity = 1.5 h). The GST and uncut fusion protein (control) experiments were only performed on days that vesicles were incorporating into the bilayer quickly (consistent incorporation in under 30 s).

BFV 6K Also Forms Ion Channels—BFV 6K protein was purified and inserted into planar lipid bilayers essentially as described for RRRV 6K. Channel currents were observed in 35 experiments using protein from three separate purifications. When no protein was added to the solutions, no activity was seen (n = 20, total waiting time = 90 min). Very little protein was required to form channels (e.g. 2 ng of RRRV 6K protein introduced to the cis chamber was sufficient). Typical current traces are in Fig. 4A in which the currents generated by the RRRV 6K protein can be seen to reverse between +30 and +60 mV, indicating a preference for Na\(^{+}\) over Cl\(^{-}\). Average reversal potential data from a number of experiments are displayed in Table I.

The open probability of RRRV 6K channels was found to be voltage-dependent. Channels were much more active at ~100 mV than at 100 mV (Fig. 4B). A channel that was closed at positive holding potentials could often be reactivated by switching to negative holding potentials.

The permeability of RRRV 6K channels to cations other than Na\(^{+}\) was tested by changing the solution in the cis bath to either KCl or CaCl\(_2\) while maintaining the holding potential at ~100 mV. When the cis chamber contained potassium or calcium ions, currents were still observed. Thus, the channels were also permeable to the cations K\(^{+}\) and Ca\(^{2+}\). To determine the relative permeabilities of Na\(^{+}\), K\(^{+}\), Ca\(^{2+}\) and Cl\(^{-}\), reversal potential data were corrected for liquid junction potentials and then inserted into the Constant Field equation (described under “Experimental Procedures”). It was found that the permeability sequence for RRRV 6K channels was Na\(^{+}\) > K\(^{+}\) > Ca\(^{2+}\) >> Cl\(^{-}\). Ionic permeability ratios are given in Table I.

Antibodies Selectively Inhibit RRRV 6K Channels—Polyclonal antibodies raised in rabbits immunized with synthetic peptides corresponding to the N or C termini of RRRV 6K protein were used to confirm that the 6K protein was indeed the channel-forming molecule in preparations of the purified recombinant protein.

When antibody raised against the N-terminal 20 amino acids of RRRV 6K was added to the cis chamber, a reduction in current to baseline levels occurred (n = 8, Fig. 5A). The same antibody had no effect when added to the trans chamber (n = 5, not shown).

Lipid bilayer method (see “Experimental Procedures”). When planar lipid bilayers were exposed to vesicles containing the RRRV 6K protein, ion currents were observed in 83 experiments using protein from five separate purifications. The average incorporation time was 76 s. When no protein was added to the solutions, no activity was seen (n = 20, total waiting time = 90 min). Very little protein was required to form channels (e.g. 2 ng of RRRV 6K protein introduced to the cis chamber was sufficient). Typical current traces are in Fig. 4A in which the currents generated by the RRRV 6K protein can be seen to reverse between +30 and +60 mV, indicating a preference for Na\(^{+}\) over Cl\(^{-}\). Average reversal potential data from a number of experiments are displayed in Table I.

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BFV 6K Also Forms Ion Channels—BFV 6K protein was purified and inserted into planar lipid bilayers essentially as described for RRRV 6K. Channel currents were observed in 35 experiments using protein from three separate purifications. When no protein was added to the solutions, no channel activity was seen (n = 15, total time waiting for channel activity = 70 min). Typical traces recorded are shown in Fig. 6A. As found for RRRV 6K channels, the reversal potential of BFV 6K channels lay between +30 and +60 mV (Table I).

The current-voltage relationship for BFV 6K channels from one experiment is shown in Fig. 6B. The largest single channel currents are plotted against the potential at which they were recorded. The channels can be seen to have a linear current-voltage relationship with a reversal potential close to +40 mV.

Ionic permeability ratios for BFV 6K are given in Table I. The ion selectivity of BFV 6K channels was similar to that of RRRV 6K ion channels: Na\(^{+}\) > K\(^{+}\) > Ca\(^{2+}\) >> Cl\(^{-}\). The P_{Na\(^{+}\)}/P_{Cl\(^{-}\)} and P_{K\(^{+}\)}/P_{Cl\(^{-}\)} ratios were also similar for channels formed by BFV and RRRV 6K proteins, indicating an approximate 15-fold preference for Na\(^{+}\) over Cl\(^{-}\), and a slight (1.3-fold) preference for Na\(^{+}\) over K\(^{+}\). However, in comparison to RRRV 6K, the BFV
6K channels showed 2-fold greater selectivity for Na\(^+\) over Ca\(^{2+}\) (Table I).

It was thought that the wide range of channel conductances observed might correlate with alteration of the number of subunits in 6K channels. In an effort to create channels with different numbers of subunits, we made vesicle preparations with varying amounts of fusion protein per milligram of lipid. The conductance of BFV 6K channels was found to correlate with the amount of protein added/mg of lipid. However, there

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**Table I**

| Channel species | NaCl | KCl | CaCl\(_2\) |
|-----------------|------|-----|-----------|
| BFV 6K (wt)     | 48.3 ± 1.9 (n = 9) | 31.7 ± 1.4 (n = 8) | 4.3 ± 1.2 (n = 6) |
| BFV 6K S32P     | 46.9 ± 1.6 (n = 13) | 37.2 ± 0.7 (n = 5) | 12.7 ± 0.9 (n = 9) |
| RRV 6K (wt)     | 49.1 ± 0.7 (n = 12) | 33.2 ± 1.0 (n = 11) | 22.5 ± 2.3 (n = 7) |

| Channel species | \(P_{Na}\)/\(P_{Cl}\) | \(P_{Na}\)/\(P_{K}\) | \(P_{Na}\)/\(P_{Ca}\) |
|-----------------|-----------------|-----------------|-----------------|
| BFV 6K (wt)     | 14.6 ± 4.3      | 1.3 ± 0.1       | 6.2 ± 0.1       |
| BFV 6K S32P     | 12.2 ± 2.7      | 1.0 ± 0.03      | 3.6 ± 0.2       |
| RRV 6K (wt)     | 16.3 ± 1.7      | 1.3 ± 0.05      | 2.6 ± 0.4       |

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**Fig. 5.** Inhibition of channel activity by chamber-specific addition of antibodies. A, channel activity is shown before (control) and after (+ α-R6N) addition of anti-RRV 6K N terminus antibody to the cis chamber. B, channel activity is shown before (control) and after (+ α-R6C) addition of anti-RRV 6K C terminus antibody to the trans chamber. A dashed line represents the closed (baseline) state. Channel openings are downward deflections from the baseline. C, D, all points histograms showing the effect of antibodies on RRV 6K channels. Paired histograms are shown before (above, “RRV channel activity”) and after addition of antibody to the stated chamber (below). C, anti-RRV 6K N terminus (α-R6N) was added to the cis chamber. D, anti-RRV 6K C-terminus (α-R6C) was added to the trans chamber. Current amplitude probability histograms were generated from 30 s of record. NB, baseline current = 0 pA.

**Fig. 6.** BFV 6K ion channels. A, channel activity is shown for a range of potentials. The closed state is shown as a solid line. Openings are deviations from this line. Scale bars are 500 ms and 10 pA. Solutions contained cis: 510 mM NaCl, 10 mM TES, pH 7.0; trans: 60 mM NaCl, 10 mM TES, pH 7.0. B, current-voltage relationship for bacterially expressed BFV 6K. Largest single opening events of a single channel are plotted for each holding potential. Solutions were the same as used in A.
ograms were generated from 30 s of record. An antibody raised against the N-terminal of BFV 6K was added to bilayers as described under procedures. Channel activity is restored by perfusion of the cis chamber (washout). The channel closed state (C) is represented by a solid line. The holding potential was −100 mV. Solutions contained cis: 510 mM NaCl, 10 mM TES, pH 7.0; trans: 60 mM NaCl, 10 mM TES, pH 7.0. B, all points histograms show the effect of antibody on BFV 6K channels. Paired records were shown before (Pre-Ab) and after (+ α-B6N) addition of anti-BFV 6K N terminal antibody to the cis chamber. Current amplitude probability histograms were generated from 30 s of record. NB, baseline current = 0 pA.

was considerable overlap in the conductances of individual channels from different vesicle preparations. Channels were observed with conductances in the range 40–800 picosiemens.

Antibodies Selectively Inhibit BFV 6K Channels—When antibody raised against the N-terminal of BFV 6K was added to the cis chamber, a reduction in mean channel current occurred in nine of 10 experiments. Typical results are shown in Fig. 7. Channel activity before addition of the antibody can be seen in Fig. 7A (control). Addition of the N-terminal antibody to the cis chamber blocked all channel activity (Fig. 7, A and B). Upon washing the antibody out of the cis bath by perfusion, channel activity reappeared (Fig. 7A, washout). Similar results were obtained in three experiments. No effect on channel activity was observed when the BFV 6K N-terminal antibody was applied to the trans bath (n = 5, data not shown). All points histograms for 30 s of data are shown in Fig. 7B. Unfortunately, attempts to purify an antibody against the C-terminal of BFV 6K by affinity chromatography were unsuccessful.

Addition of a control antibody raised against a pentahistidine peptide did not result in channel inhibition when added to either bath (data not shown). This demonstrates that channel inhibition was specific to the particular antigen recognized by the antibody.

BFV 6K Synthetic Peptide Forms Ion Channels—A synthetic peptide corresponding to the entire amino acid sequence of BFV 6K protein (B6Kpep) was purified, inserted into vesicles and added to bilayers as described under “Experimental Procedures.” The peptide formed cation-selective channels with properties similar to those of the recombinant BFV 6K protein. Channels were seen in 42 experiments from seven separate purifications of B6Kpep. Typical records are shown in Fig. 8. The reversal potential of B6Kpep channels was +55 ± 1 mV (n = 12, cf. +48 ± 2 mV for BFV 6K). The channels could be blocked by addition to the cis chamber of antibody raised to the N-terminal 20 amino acids of BFV 6K. Perfusion of the cis chamber to wash out the antibody relieved channel inhibition (data not shown). As the synthetic peptide was devoid of E. coli contaminants, these results confirm that the ion channel activity seen in recombinant preparations of GST-6K was indeed due to the 6K protein.

A Point Mutation in BFV 6K Alters Ion Channel Properties—In an attempt to cause a major change in ion channel activity and thus further confirm the link between channel activity and the BFV 6K protein, we mutated serine 32 to proline. Serine 32 of BFV 6K is positioned at the C-terminal side of the predicted transmembrane region of the protein (23). This residue has a potential role in ion permeation because of its polar nature. Additionally, insertion of proline might be expected to disrupt the channel’s active conformation as proline is known to be a “helix breaker” (37). Protein from seven separate purifications generated currents in 91 experiments. When Ser-32 of BFV 6K was mutated to Pro, the reversal potential of the channels with CaCl2-containing cis bath solution changed from +4.3 to +12.7 mV. This corresponds to a significant drop (p < 0.001) in the PNa/PCa ratio from 6.2 to 3.6 (Table I). A loss of Na+ selectivity is indicated by a reduction of PNa/PCa from 1.3 in wild-type BFV 6K, to 1.0 in BFV 6K (S32P) (p < 0.01). The change of channel properties caused by specific alteration of a single amino acid in the BFV 6K sequence also confirms that the observed ion channel activity is indeed due to 6K and not a contaminant.

DISCUSSION

We have shown that the 6K proteins of two alphaviruses, RRV and BFV, form cation-selective ion channels in planar lipid bilayers. As well as the high purity of the final preparations, a number of other experiments established that the currents observed were caused by the 6K proteins and were not due to an unidentified contaminant E. coli protein. Firstly, antibodies raised against the N and C termini of the 6K proteins modulated channel activity. Secondly, a single nucleotide substitution (S32P) was sufficient to modify the calcium permeability of BFV 6K protein. Thirdly, a synthetic peptide corresponding to the amino acid sequence of the BFV 6K protein formed ion channels with similar properties. Modulation of the synthetic peptide channels by the N- and C-terminal antibodies was identical to modulation of channels formed by bacterially expressed BFV 6K protein. Finally, E. coli extracts of neither uncleaved GST-6K fusion proteins nor GST alone contained molecules that caused ion channel activity.
Alphavirus 6K Proteins Form Ion Channels

Orientation of 6K in Bilayers—The use of affinity-purified antibodies to specifically inhibit channel currents from both sides of the bilayer (Figs. 5 and 7) supports our conclusion that the 6K protein forms an ion channel. The chamber-specific effect of antibody inhibition suggests further that 6K proteins are oriented in bilayers with the N-terminal facing the cis bath, and the C-terminal facing the trans bath. Given the length of the 6K polypeptide chain, location of N and C termini on opposite sides of the membrane suggests that the hydrophobic domain consists of a single transmembrane α-helix.

Our evidence that 6K has a single transmembrane domain is in contrast with earlier results suggesting that 6K crosses the membrane twice, with both termini exposed to the lumen of the ER (1, 38). However, these data do not exclude the possibility that the C-terminus of 6K is only transiently located in the ER lumen. The C-terminus of the E2 protein of Sindbis virus has been shown to retract through the ER membrane following cleavage by the signalase enzyme (39). A similar retraction of the C-terminal of the RRV and BFV 6K proteins may occur following cleavage by signalase.

Thrombin cleavage of GST-6K fusion proteins did not occur in solutions containing detergent. Thrombin must be unable to access its cleavage site under these conditions, perhaps because it is buried in the folded conformation of the fusion protein. This steric hindrance may also explain the inability of antibodies against BFV and RRV N termini to recognize full-length fusion protein on Western blots (see Fig. 3A). Because 6K is an integral membrane protein, it is possible that it adopts its native conformation only in a lipidic environment. Indeed, association of GST-6K fusion proteins with lipid vesicles prior to the addition of thrombin resulted in successful cleavage, indicating that some conformational change had occurred in the 6K protein. Even when in vesicles, relatively large amounts of thrombin were required to cleave fusion proteins to completion (Fig. 3A), again presumably due to restricted access of thrombin to its cleavage site. However, the fact that thrombin digestion proceeded to completion, i.e. complete disappearance of the 34-kDa band corresponding to GST-6K indicates that the N termini of the 6K proteins are all located exterior to vesicles.

Comparison of 6K Channel Properties—The low sequence identity between alphavirus 6K proteins (7% by sequence alignment) is perhaps reflected in some of the differing properties of RRV and BFV channels (see Figs. 4A, 6A, and Table 1). Broader features of the 6K ion channels studied are similar, however. Both are cation channels, they have similar reversal potentials, and both show strong activation at negative voltages.

A Point Mutation in BFV 6K Alters Channel Properties—Transmembrane peptide domains are often characterized by a hydrophobic, α-helical core that is “anchored” at the membrane surface by more polar residues (40). Polar residues within transmembrane domains of ion channels are thought to form an aqueous-lined pore for ion conduction (41). In choosing to make the mutation S32P in the BFV 6K protein, we sought to introduce a major structural change within the transmembrane domain as well as removing a potential cation-interacting residue. In similarity with residue serine 24 of the Vpu protein of HIV-1, residue serine 32 of BFV 6K protein is located at the C terminus end of a putative transmembrane helix. Serine 24 of Vpu is predicted to face the channel pore and thought to have a role in gating and/or selectivity of the channel (42). BFV 6K channels containing the S32P mutation were less cation-selective than wild-type channels, and the \( \frac{P_{Na^+}}{P_{Ca^{2+}}} \) ratio was decreased (see Table 1). These changes were consistent with a gating/selectivity role for serine 32 in BFV 6K channels.

What Role Does 6K Ion Channel Activity Play in Virus Replication?—Alphavirus proteins are associated with cellular membranes at several stages of the infection cycle. It would be interesting to know the intracellular location of 6K ion channel activity. Early events in infection (binding and internalization) can be excluded as they do not require 6K protein (5, 43). The E1 protein of SFV has been shown to catalyze acid-induced fusion of membranes (43). Ion channel activity of 6K is therefore unlikely to be required for membrane fusion in the endosome (contrasting with the M2 protein of influenza A) (22). A membrane-impermeable biotinylation reagent has been used to label primary amines of the glycoproteins of SFV-infected BHK cells (44). Precipitation of biotin-labeled proteins on immobilized streptavidin-agarose suggested that 6K is associated with glycoproteins at the cell surface (44). Further indirect evidence that the 6K protein proceeds to the cell membrane is provided by immunoprecipitation of purified SINV virus with antibodies against the E2 glycoprotein; trace amounts of coprecipitated 6K suggest that the protein must have been at the plasma membrane prior to budding into virions (45). Thus, the subcellular location of 6K ion channel activity could be the ER, Golgi, trans-Golgi network, or plasma membrane.

A variety of cellular effects have been observed following site-directed mutation of alphavirus 6K protein. Defects have been found in the areas of glycoprotein processing and trafficking, virus assembly, and budding. Deletion mutants of 6K protein of Sindbis (21 amino acid deletion) and Semliki Forest viruses (full deletion) depress virus replication (10, 46), possibly by interfering with glycosylation and trafficking of proteins through the ER (10, 47). Defects at the level of budding are common: nucleocapsids accumulate at the plasma membrane of 6K-deleted SFV (6). Virions failed to fully pinch off from the plasma membrane when a 21-amino acid deletion was introduced to the 6K protein of SINV (10). In the only mutagenesis experiment on 6K that involved insertion of amino acids, cleavage of the SINV structural polyprotein precursor was retarded (48). No gross structural abnormalities have been noted in 6K-deleted virions although, in SFV, the mutant virions are more heat-labile (6). Budding defects caused by point mutations in 6K can be rescued by a second-site mutation in glycoprotein E2 (38), hinting at an interrelationship between 6K, the E2 protein, and lipid (10). Budding defects have additionally been noted when SINV 6K cysteine residues that normally acquire a palmitoyl group are mutated (8).

Following the onset of viral RNA translation in alphavirus-infected cells, the plasma membrane becomes more permeable to monovalent cations (49). This is followed by an increase in permeability to larger molecules, such as translation inhibitors (50). It is possible that the ion channels formed by 6K proteins are responsible for both of these changes. The wide range of conductance values obtained for 6K channels may reflect the protein’s ability (as infection proceeds) to form channels with larger pores and conductances. Certainly, RRV and BFV 6K channels are several-fold more permeable to monovalent cations than divalent cations, and the plasma membrane potential is several-fold less cation-selective than wild-type channels, and the \( \frac{P_{Na^+}}{P_{Ca^{2+}}} \) ratio was decreased (see Table 1). These changes were consistent with a gating/selectivity role for serine 32 in BFV 6K channels.
The 6K protein has been shown to have a role in virus replication in vitro (6, 10). A drug that blocked 6K ion channels could be used to obtain further understanding of the role and subcellular location of 6K ion channel activity in alphavirus infection.

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Julian V. Melton, Gary D. Ewart, Ronald C. Weir, Philip G. Board, Eva Lee and Peter W. Gage

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