J. Membrane Biol. 150, 127–132 (1996)

The Journal of
Membrane
Biology

Ion Channels Formed by NB, an Influenza B Virus Protein

N.A. Sunstrom, L.S. Premkumar, A. Premkumar, G. Ewart, G.B. Cox, P.W. Gage
John Curtin School of Medical Research, Australian National University, Canberra ACT 2601 Australia

Received: 6 March 1995/Revised: 17 November 1995

Abstract. The influenza B virus protein, NB, was expressed in *Escherichia coli*, either with a C-terminal polyhistidine tag or with NB fused to the C-terminus of glutathione S-transferase (GST), and purified by affinity chromatography. NB produced ion channel activity when added to artificial lipid bilayers separating NaCl solutions with unequal concentrations (150–500 mM *cis*, 50 mM *trans*). An antibody to a peptide mimicking the 25 residues at the C-terminal end of NB, and amantadine at high concentration (2–3 mM), both depressed ion channel activity. Ion channels had a variable conductance, the lowest conductance observed being approximately 10 picosiemens. At a pH of 5.5 to 6.5, currents reversed at positive potentials indicating that the channel was more permeable to sodium than to chloride ions ($P_{Na}/P_{Cl} \sim 9$). In asymmetrical NaCl solutions at a pH of 2.5, currents reversed closer to the chloride than to the sodium equilibrium potential indicating that the channel had become more permeable to chloride than to sodium ions ($P_{Cl}/P_{Na} \sim 4$). It was concluded that, at normal pHs, NB forms cation-selective channels.

Key words: Virus protein — Ion channels — Planar lipid bilayer

Introduction

The influenza A virus contains mRNA for a small protein, M_2, that is involved in replication of the virus (Lamb, 1989). M_2 is thought to form proton channels in the surface membrane of the virion and in the membrane of post-Golgi bodies within host cells (Hay, 1989; Sugrue et al., 1990; Sugrue & Hay, 1991). Proton channels in the lipid envelope of the virion would allow protons to flow from the low pH environment of the endosome into the virion leading to uncoating of virus proteins: proton channels in the membrane of post-Golgi bodies would prevent lowering of the internal pH by proton pumps so that virus proteins such as haemagglutinin would have the correct conformation for reassembly. There is now direct evidence that M_2 can form ion channels: injection of the mRNA for M_2 into *Xenopus* oocytes produces a cation-selective current that is inhibited by amantadine, a drug which interacts with M_2 to block virus replication (Pinto, Holsinger & Lamb, 1992); and a peptide mimicking the putative transmembrane sequence of M_2 forms ion channels in lipid bilayers (Duff & Ashley, 1992). More recently, M_2 expressed in Sf9 cells has been shown to form cation-selective channels in artificial lipid bilayers (Tosteson et al., 1994).

Since the influenza A and B viruses are closely related, it seemed possible that the influenza B virus might also contain a protein that forms proton channels. M_2 from the influenza A virus is a polypeptide of 97 amino acid residues containing one sequence of 19 residues with a high hydrophobic index that would comfortably span a lipid bilayer membrane (Lamb, 1989). The influenza B virus also expresses a small protein, NB, that contains a single hydrophobic sequence of 19 amino acids. Although there is no sequence homology between M_2 and NB, it seemed possible that NB might form a proton channel functionally equivalent to M_2.

We report here that NB forms ion channels when inserted in artificial lipid bilayers. A preliminary report of some of these observations has appeared previously in abstract form (Sunstrom et al., 1994).

Materials and Methods

Construction of Plasmids Encoding NB Fusion Proteins and Expression in *E. coli*

A cDNA copy of virion RNA segment 6 of influenza virus B/Lee/40 (pBSBNA) was kindly given to us by Dr. Gillian Air (Wei et al., 1987).

Correspondence to: P.W. Gage

Two different plasmid expression vectors were constructed that direct the synthesis of NB in *E. coli* as fusions; either with a C-terminal polyhistidine tag or with NB fused to the C-terminus of glutathione S-transferase (GST).

In the first method, NB cDNA was cloned in the expression vector pQE-70 (Qiagen) so that six histidine codons were added to the 3′ end of the NB open reading frame. To facilitate the cloning, the NB sequence was modified using PCR mutagenesis to introduce a Sph I restriction site at the 5′ end of the NB coding region, resulting in the recreation of the authentic ATG start codon. In addition, a Bgl II restriction site was introduced at the 3′ end of NB replacing the stop codon so that ligation of the NB-coding fragment resulted in the 6xHis tag in the vector being in frame with NB (Qiagen). The 6xHis tag is immediately followed by a translational stop codon. The resulting plasmid, pNB-6H, was used to transform *E. coli* M15 (Qiagen). The expression of NB-6H protein appeared to be toxic to *E. coli*. Therefore, expression was induced with isopropyl B-D-thiogalactoside (IPTG, Pharmacia) late in the log phase of growth. The fusion protein was located in the bacterial membrane. Bacterial membrane extract was prepared and loaded onto Ni-NTA-agarose (Qiagen) following methods described elsewhere (Waeber et al., 1993). Pooled fractions were further purified by gel filtration on a SEC-125 column attached to an HPLC. The mobile phase contained (in mM): 20 Na phosphate, 300 NaCl, 10 DTT, 0.1% Triton X-100 (BioRad), pH 4.5. Fractions containing the purified protein were identified by Western blot (Fig. 1*A*).

To construct the GST-NB gene fusion, polymerase chain reaction (PCR) primers were designed to generate BamHI sites at both termini of the NB cDNA fragment. The resultant PCR product was inserted into the Bam H1 site of pGEX-2T (Smith & Johnson, 1988), resulting in the recombinant plasmid pGEX-2T/NB in which the C-terminus of GST was fused to the N-terminus of NB. This plasmid was then transformed into *E. coli* (DH-5) and the expression of the GST-NB fusion protein was induced by the addition of IPTG to a final concentration of 1 mM. Cells were harvested and disrupted in a French pressure cell and the lysate was centrifuged (13,000 × *g*, 30 min). The fusion protein was found in the lysate pellet. Treatment with triton X-100 or sarkosyl (0.45%) (McNally et al., 1991) solubilized a low percentage of fusion protein which was capable of binding to glutathione-agarose beads. The NB protein was then cleaved from GST by treatment with thrombin by virtue of the highly specific thrombin recognition site between the fusion partners engineered to be encoded at the 3′ end of the GST gene in the pGEX-2T vector. Fractions containing the liberated NB protein were tested for channel activity by incorporation into lipid bilayers as described below.

The sequence of the NB coding region in recombinant plasmids was confirmed using the Taq DyeDeoxy Terminator Cycle Sequencing Kit and the Applied Biosystems Model 373A DNA Sequencing System.

Preparation and Purification of Polyclonal Antibodies to the C-terminus of NB

A synthetic peptide corresponding to the last 25 amino acids of the C-terminus of NB was synthesized (Applied Biosystems, model 477A). The multiple antigen peptide (MAP) approach (Lu et al., 1991) was used to prepare peptide immunogen in rabbits. Peptide-specific antibody, NBc, was purified from rabbit serum using an ImmunoPure Ag/Ab Immobilization kit (Pierce).

Recording Current in Artificial Lipid Bilayers

The methods used for forming planar lipid bilayers were similar to those described elsewhere (Miller, 1986). Bilayers were formed from a mixture of palmitoyl-oleoyl-phosphatidylethanolamine, palmitoyl-oleoyl-phosphatidylserine and palmitoyl-oleoyl-phosphatidylcholine (5:3:2) (Avanti Polar Lipids, Alabaster, Alabama) dissolved in *n*-decane (50 mg/ml). The lipid mixture was painted onto 150 μm to 200 μm diameter apertures in the wall of a 2 ml delrin cup separating *cis* and *trans* chambers containing salt solutions. The *cis* chamber was connected to ground and the *trans* chamber to the input of an Axopatch 200 amplifier (Axon Instruments) via Ag/AgCl/agar bridges. The *cis* chamber normally contained 150–500 mM NaCl and the *trans* chamber 50 mM NaCl. For experiments at pH 6, the pH buffer MES (2-(N-Morpholino)ethanesulfonic acid) was present in solutions at a concentration of 10 mM. Voltages were measured in the *trans* chamber with respect to the grounded *cis* chamber. Bilayer formation was monitored electrically from the amplitude of the current pulse generated by a current ramp. NB was added to the *cis* chamber, either directly or in liposomes formed by sonicating a lipid-protein mixture (Bear et al., 1992). After addition of NB, the chamber was stirred until channel activity was seen. Currents were filtered at 250 or 500 Hz, digitized at 44 kHz (Sony PCM 100) and stored on magnetic tape using a videocassette recorder. For analysis, currents were replayed through the same system in reverse and digitized at 1 or 2 kHz using an A to D converter interfaced to an IBM-compatible computer.

Results

A Western blot of an HPLC fraction of NB-6H probed with the NBc antibody shows a single band at approximately 12 Kd consistent with the molecular weight of 11.8 Kd expected for the NB-6H monomer (Fig. 1*A*). A silver-stained SDS PAGE of NB (Fig. 1*B*) shows NB fused to GST (lane 1) and NB cleaved from the fusion protein by treatment with thrombin (lanes 2 & 3).

The channel-forming activity of HPLC fractions containing NB was tested in lipid bilayers. These bilayers showed no channel-like activity at pHs from 6.5 to 2.5 for periods up to 10 min before addition of protein. Addition of 10 to 50 μl of an HPLC fraction shown by Western blotting to contain NB to the *cis* chamber followed by stirring resulted in the appearance of current activity within 1 to 30 min in 52 experiments with NaCl solutions at a pH of 6. Additions of fractions not containing NB gave no channel activity. Examples of currents produced by NB-6H in one of these experiments are shown in Fig. 1*C*, *a* and *b*. The *cis* and *trans* chambers contained 150 mM and 50 mM NaCl, respectively (pH 6) and the potential across the bilayer was 0 mV. The high level of channel activity illustrated in the top 2 traces was completely abolished when 10 to 20 μl of antibody was added to both chambers (traces *c* and *d*). A similar abolition of activity by the antibody was seen in 9 similar experiments. In control experiments, it was found that the antibody alone did not give channel activity.

We have also isolated NB by a completely different expression and purification system in which a GST-NB fusion protein is initially isolated and then cleaved with thrombin to separate the NB from the GST (*see* Materials and Methods). The NB produced in this way was trun-

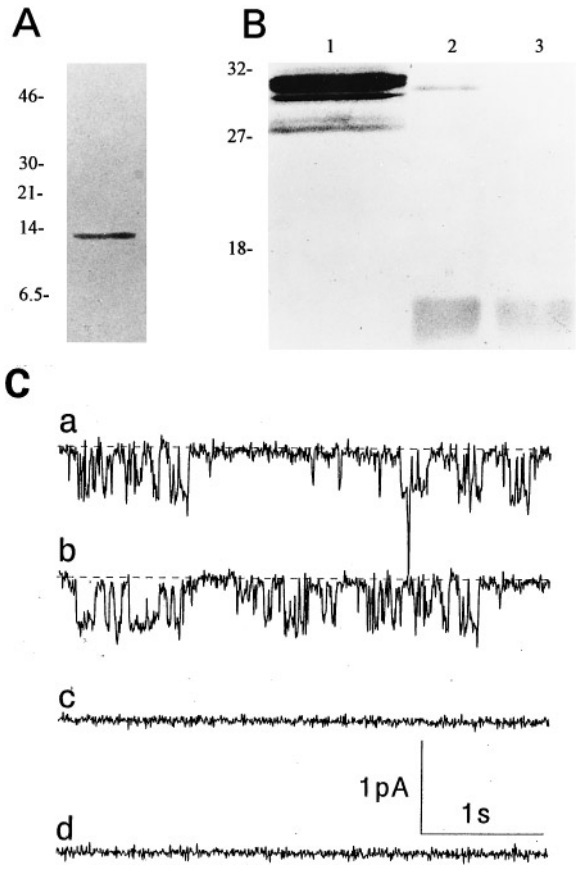

Fig. 1. (*A*) Western blot of a fraction obtained by gel filtration of a purified NB-6H bacterial membrane extract on a SEC-125 HPLC. The antibody used was antipeptide rabbit serum NBc raised against a synthetic peptide corresponding to the extreme C-terminal 25 amino acids of NB. The markers on the left indicate molecular weights (Kd). (*B*) Silver-stained SDS PAGE of partial purification of NB expressed in *E. coli* as described in Materials and Methods. Lane 1 shows fusion protein eluted with 10 mM reduced glutathione from glutathione-agarose beads. Lane 2 shows NB together with some fusion protein following treatment of the eluted fusion protein with thrombin (10 units per milligram of protein for 0.5 hr at 25°C) and removal of the GST by treatment with glutathione-agarose beads. Lane 3 shows NB cleaved from fusion protein bound to glutathione agarose beads by treatment with thrombin (40 units per milligram of fusion protein). The markers on the left indicate molecular weights (Kd). (*C*) Currents through channels formed by NB. The *cis* solution contained 150 mM NaCl, the *trans* solution 50 mM NaCl (pH 6), and the potential across the bilayer was 0 mV. (*a* and *b*) currents recorded before addition of the antibody. (*c* and *d*) traces show no channel activity after addition of NBc to both *cis* and *trans* chambers. Broken lines show the closed channel level.

cated at the C-terminus. This was indicated both by its size on SDS-PAGE and the failure of the NBc polyclonal antibody to react with the purified NB on Western blots (*not shown*). A silver-stained gel is shown in Fig. 1*B*. Coomassie and silver-stained gels indicated that 90 to 95% of the protein in fractions used was NB.

In spite of the truncation of the C-terminus, the NB prepared from the GST-NB fusion, NB(GST), gave channel activity in lipid bilayers that was indistinguishable from that produced by NB-6H, except that the NBc antibodies did not inhibit channel activity. The lack of effect of NBc was an important observation because it removed the remote possibility that a protein other than NB, that was blocked by the antibody to NB, was responsible for the channel activity. Further evidence that the effect of NBc was specific for NB was obtained in other experiments in which NBc was shown to have no effect on channel activity caused by M_2.

Channel activity caused by fractions containing NB-6H or NB(GST) could be blocked with high concentrations of amantadine. Amantadine has been reported to inhibit replication of the influenza B virus when used at more than 100 times the concentration needed to inhibit replication of amantadine-sensitive influenza A viruses (Appleyard, 1977). Although there is no evidence that this effect of amantadine is due to an effect on NB, we tested the effects of amantadine on the channels at a concentration of 2–3 mM, 200 to 300 times the concentration that blocks channels formed by M_2 (Pinto et al., 1992; Duff & Ashley, 1992; Tosteson et al., 1994). In 5 experiments with NB(GST) and 2 with NB-6H, 2–3 mM amantadine reduced channel activity. Inhibition of channel activity by amantadine in one of these experiments can be seen in the histograms in Fig. 2. Larger and more frequent currents were seen before (Fig. 2*A*) than after exposure to amantadine (Fig. 2*B*). However, although channel activity was reduced by the amantadine, it was not completely abolished.

When solutions had a pH of 5.5 to 6.5, currents reversed at positive potentials, as illustrated in Fig. 3. In this experiment, *cis* and *trans* chambers contained 500 and 50 mM NaCl, respectively. Currents are downwards (*cis* to *trans*) at potentials of 0 and +20 mV and upwards (*trans* to *cis*) at +60 and +80 mV. There was no detectable current activity at +40 mV. The average amplitude of currents recorded in another experiment is shown plotted against potential in Fig. 3*B*. The relationship between current and potential was reasonably linear and gave a null (zero current) potential at +37 mV. The average null potential measured in 4 similar experiments was +36.8 ± 1.85 mV. As the sodium equilibrium potential calculated from Na^+ activities in these solutions is +53 mV, the channels must be permeable to some other ion. Assuming that there was no appreciable proton current at this pH, the average null potential would give a P_{Na}/P_{Cl} ratio of about 9. In 3 experiments in which the *cis* and *trans* chambers contained 150 and 50 mM Na^+, currents reversed at +20 ± 2.1 mV (mean ± SEM), again giving a P_{Na}/P_{Cl} ratio of about 9. It was concluded that the channels formed by NB were much more permeable to Na than to Cl ions and that a proton current, which would have reversed at 0 mV because the pH was the same in the two chambers, was making very little contribution to the net current.

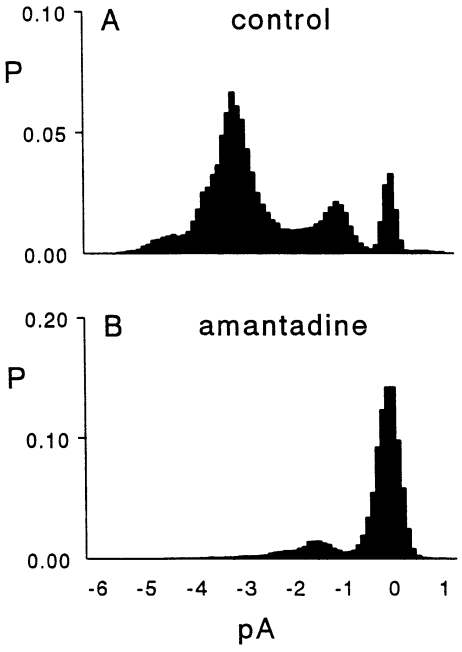

Fig. 2. Amantadine (2 mM) depresses channel activity caused by NB. All-points current amplitude probability histograms obtained before (*A*) and after (*B*) addition of amantadine (2 mM) to a bilayer showing ion channel activity caused by NB at 0 mV. (*A*) Histogram obtained from a 230-sec segment current record before addition of amantadine. (*B*) Histogram obtained from a 172-sec current record 5 min after addition of 2 mM amantadine to the *cis* chamber.

In the experiment illustrated in Fig. 3, currents were uniform in amplitude and channels had a conductance of about 45 pS. More commonly, currents displayed a wide range of amplitudes. In several experiments in which NB was incorporated in liposomes at relatively low concentration and the liposomes were then added to the *cis* chamber, channels were very small with a conductance as low as 10 pS. Increasing the NB concentration in the liposomes produced channels with higher conductance, sometimes as high as several hundred picosiemens.

It would not be surprising if a channel permeable to Na^+ were also permeable to protons. However, because of the relative concentrations of protons and Na^+ at pH 6 in the above experiments, a proton current could have been obscured by a sodium current if the channel did not have an extremely high proton/Na^+ permeability ratio. In an attempt to determine whether the channels formed by NB were permeable to protons, a glycine-HCl solution (50 mM) with a pH of 2.5 was placed in both chambers. It was thought that, under these conditions, protons would be the only cations that would pass through the channel so that any currents seen would be proton currents. The low pH was used to raise the proton concentration and increase the amplitude of any proton currents. Single channel currents were recorded in 22 experiments after addition of NB to the *cis* chamber when both chambers contained glycine-HCl (50 mM) at pH 2.5. No channel activity was seen before adding the protein during control periods of 1 to 30 min indicating that the channel activity was not a nonspecific effect of the low pH. A record of currents recorded in one of these experiments at a potential of −100 mV is shown in Fig. 4*A*. The currents reversed at a potential of 0 mV and a similar reversal potential was seen in all 22 experiments. The all-points histogram in Fig. 4*B* shows an average current of about −0.9 pA at the potential of −100 mV giving a single channel conductance of about 9 pS.

In the symmetrical glycine-HCl solutions, all ions had an equilibrium potential at 0 mV. The currents could have been carried by glycine, protons or chloride ions. However, no current activity was produced by NB in glycine-H_2SO_4 solution at a pH of 2.5 (3 experiments) although the proton concentration would have been the same as in the glycine-HCl solutions at the same pH. This observation suggested that the currents in the glycine-HCl solution at pH 2.5 (Fig. 4) were chloride currents. In support of this conclusion, currents recorded with a high concentration of NaCl in the *cis* chamber and 50 mM NaCl in the *trans* chamber at a pH of 2.5 reversed at negative potentials rather than at the positive potentials seen at higher pHs. This change in reversal potential is illustrated in Fig. 5. The currents shown in Fig. 5*A* were recorded in solutions containing 150 mM (*cis*) and 50 mM (*trans*) NaCl. The currents are upward (*trans* to *cis*) at +40 and +20 mV, essentially zero at −20 and downward at −40 mV. The shift in null potential to negative potentials was observed with both NB-6H and NB-(GST). In 5 similar experiments with NB(GST) (*cis*/*trans* 150/50 mM NaCl), currents reversed between −10 and −20 mV. The null potential was determined more accurately in 4 experiments with NB-6H (*cis*/*trans* 500/50 mM NaCl) using 10 mV changes in potential. A current-voltage curve from one of these experiments (Fig. 5*B*) shows a null potential at −22.5 mV. The average null potential obtained from 3 similar experiments was −25 ± 3.1 mV. If only Na^+ and Cl^- were making a significant contribution to the current, this null potential indicates that the bilayer was about four times more permeable to chloride than to sodium ions. It appears from these results that channels formed by NB change from cation-selective to anion-selective when the pH is lowered to 2.5.

Discussion

We have shown that HPLC fractions containing the influenza B protein, NB, give cation-permeable channels in artificial lipid bilayers at pHs from 5.5 to 6.5. Although 90 to 95% of the protein in fractions used (judged from Coomassie blue and silver-stained gels) was NB, the possibility remained that the channel activity could have been caused by a protein other than NB. The inhibition of channel activity by the purified antibody makes

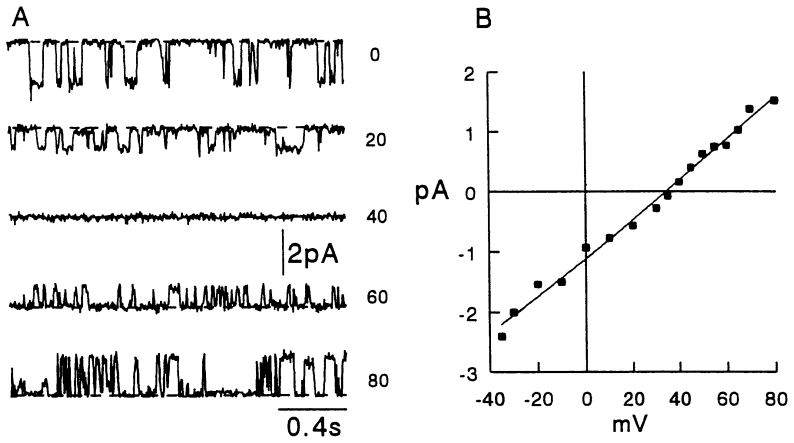

Fig. 3. Channels formed by NB are more permeable to sodium ions than to chloride ions at pH 6. (*A*) Current traces recorded at the potentials shown at the right of each trace after addition of NB to the *cis* chamber. The *cis* chamber contained 500 mM NaCl and the *trans* chamber 50 mM NaCl, both at pH 6. Broken lines denote the closed channel level. (*B*) The relationship between average current amplitude and potential recorded in another experiment in which the *cis* and *trans* chambers contained 500 and 50 mM NaCl, respectively (pH 6). The line through the data points is a second order polynomial.

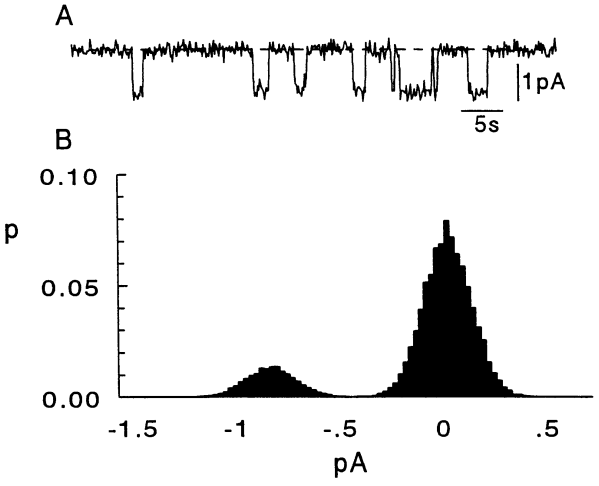

Fig. 4. Currents recorded in 50 mM glycine-HCl solutions at a pH of 2.5 (*cis* and *trans*) after addition of NB to the *cis* chamber (*A*) A current trace showing single channel openings at a potential of −100 mV. (*B*) An all-points amplitude probability histogram of current recorded at a potential of −100 mV (the trace in *A* is a segment of the same record).

this very unlikely. The assumption of specificity of the antibody effect is strengthened by the observation that the antibody did not inhibit channel activity caused by NB isolated from the GST fusion protein that had lost part of its C-terminal. This demonstrates that the antibody is not a nonspecific blocker of ion channel activity.

The block of channel activity caused by amantadine at concentrations that inhibit replication of the influenza B virus (Appleyard, 1977), provides some further evidence that the channel activity was produced by the NB. It could be argued, however, that the high concentration of amantadine that blocked channel activity could have had a nonspecific effect on a channel formed by a protein other than NB. However, we have been unable to depress the activity of channels formed by M_2 from amantadine-resistant viruses with concentrations of amantadine as high as 10 mM (*not shown*). It is interesting that amantadine was found not to block completely channel activity caused by full length M_2 in planar bilayers also (Tosteson et al., 1994).

It is possible that the 6 histidine residues added to the C-terminus might have changed the properties of channels formed by NB. However, the properties of channels formed by NB containing the added 6 histidines were no different from those of channels formed by NB obtained from NB-GST after the GST had been removed with thrombin.

The lowest single channel conductance recorded was 9–10 pS, similar to the smallest single channel conductance seen with the hydrophobic segment of M_2 in artificial bilayers (Duff & Ashley, 1992). Presumably, the higher conductance levels seen with both NB and M_2 (Duff & Ashley, 1992; Tosteson et al., 1994) in artificial bilayers are due to synchronized opening and closing of many channels.

Whether the channels formed by NB are permeable to protons remains an open question. Reversal of the currents at 0 mV in symmetrical glycine-HCl solutions at pH 2.5 was consistent with the idea that the current is carried by protons. However, the absence of current in glycine-H_2SO_4 solutions at a pH of 2.5 and the change from positive to negative reversal potentials in asymmetrical NaCl solutions at pH 2.5 indicate that the channels are permeable to chloride ions at this pH. Hence the currents in the glycine-HCl solution may have been chloride currents. Nevertheless, our results in no way prove that the channel formed by NB is not proton-permeable. What we have shown is that NB, at a physiological pH, forms cation-permeable channels that may well be permeable to protons but that the proton conductance was much smaller than the Na conductance under our experimental conditions.

Now that it has been shown that NB can form ion channels, it may be possible to find an agent that will block the channels that can then be tested on the influenza B virus. If NB, like M_2, forms ion channels necessary for replication of the virus, it should prove possible to inhibit replication of the influenza B virus with agents that block the channels. It may turn out that many

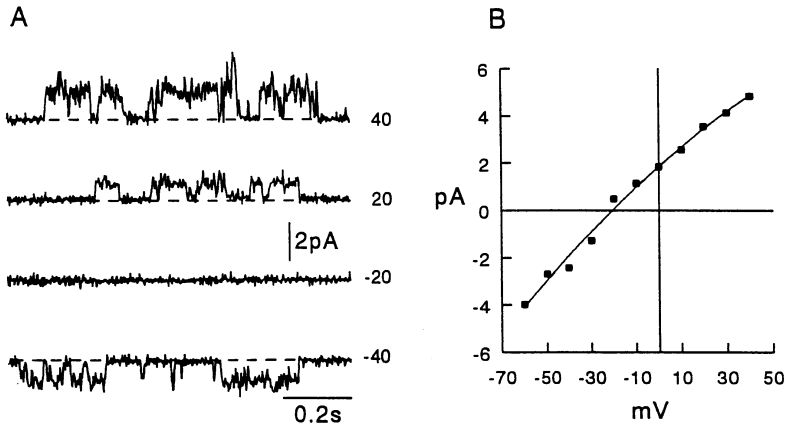

Fig. 5. Channels are more permeable to chloride than to sodium ions at pH 2.5. The *cis* chambers contained 150 mM NaCl in *A* and 500 mM NaCl in *B*: the *trans* chamber contained 50 mM NaCl (pH 2.5). (*A*) Traces recorded after addition of NB to the *cis* chamber at the potentials shown to the right of each trace. The broken lines show the closed channel current levels. (*B*) Average current amplitude plotted against potential. The line is the best fit to the data points of a second order polynomial.

enveloped viruses contain proteins that form ion channels needed for their replication. Such channels could provide targets for a new class of antiviral agents.

We are grateful to Dr. Gillian Air for providing us with the cDNA.

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