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An Amino-Acid Substitution in the Influenza-B NB Protein Affects Ion-Channel Gating

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Abstract. The effects of site-directed mutations in NB, a protein encoded by the influenza B virus that has been shown to form cation-selective ion channels at pH 6.0, were studied on ion channel characteristics in artificial lipid bilayers. It was thought that the residues in the hydrophobic region of NB we selected for mutation might be involved in the transport of cations across the channel and that changes in these residues might affect channel properties such as gating and ion-selectivity. Serine residues at positions 20 and 28, threonine at position 24 and cysteine at position 26 were replaced by alanine. We found that the mutation S20A gave channels that did not gate and that remained open most of the time. Proton permeability of NB channels, as detected by fluorescence quenching, was also altered by the mutation S20A: channels were no longer proton-permeable. The other mutations, S28A, T24A and C26A, did not have any detectable effect on the activity or proton permeability of channels formed by NB. The results indicate that serine 20 may have an important role in normal function of NB channels.

Key words: Influenza-B—NB protein—Ion channel gating—Amino acid substitution—Transmembrane domain

Introduction

The influenza B viral genome encodes a small membrane protein, NB (Burmeister et al., 1993), which is similar to the M2 protein of the Influenza A virus and is probably its functional analogue. NB is a small 100 amino-acid protein with a short external N-terminus, a large cytoplasmic tail and a 22-amino acid hydrophobic membrane-spanning region. We have previously shown that purified NB from influenza B virus, when incorporated into artificial lipid bilayers, forms cation-selective ion channels at physiological pH (Sunstrom et al., 1996). A synthetic peptide corresponding to the transmembrane domain of the NB protein also formed channels in lipid bilayers (Fischer et al., 2000) and the anti-influenza-A drug amantadine (which inhibits the M2 ion channel) was reported to block the NB-peptide channels in a voltage-dependent fashion (Fischer, Pitkeathly, & Sansom 2001).

We have used site-directed mutagenesis to investigate whether certain regions of the protein are directly involved in channel properties such as gating, ion-selectivity and drug-sensitivity. The transmembrane region of NB, from amino acid 19 to 40, contains three amino acids with hydroxyl side chains: 20-serine, 24-threonione, and 28-serine. Modeling of this region as an α-helix places these polar residues along the same face of the α-helix (Fischer et al., 2000). To test whether these residues at positions 20, 24 and 28 are involved in the pore of the NB channel, they were mutated to alanine residues.

Cysteine residues in the M2 protein have been shown to be important in the oligomerization of the monomers to form a tetrameric channel (Zebedee & Lamb 1988; Sugrue & Hay 1991). The NB monomer contains 7 cysteine residues and NB runs as a dimer or tetramer on non-reducing gels (Williams & Lamb 1986; Betakova, Nermut & Hay 1996) 23, 1). In this study the highly conserved cysteine at position 26 was also targeted for site-directed mutation.

We report here that the mutation NBS20A alters the channel activity of the NB protein in artificial lipid bilayers: the channels did not gate and were essentially open all the time. The other three mutations had no observable effect on the channel activity. Fluorescence quenching experiments were also car-

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ried out to determine the proton permeability of the mutant channels. Once again the channels formed by NBS20A showed altered proton permeability, whereas the other three mutants were indistinguishable from wild-type NB.

Materials and Methods

Construction of Mutations

Using PCR, mutations were introduced into the transmembrane region of NB. The plasmid pNB-6H, containing the sequence for NB from the influenza virus B/Lee/40, was used as a template (Sunstrom et al., 1996) Four primers and two PCR reactions were used to produce each desired mutation: two complementary mutagenic oligonucleotides, and primers recognizing the 5′ and 3′ ends of the NB gene (Table 1). The full-length PCR products, containing the mutant NB, a Bg1 II site at one end and an Sph I site at the other end, were cloned into the multiple cloning site of the pQE70 vector (Qiagen), thus fusing a 6×His tag to the C-terminus of the protein. The resulting plasmid was then transformed into *E.coli* M15.

Automated DNA Sequencing

Taq DyeDeoxy Terminator Cycle Sequencing kit from Applied Biosystems (ABI) was used for sequencing double-stranded DNA. The reactions were performed as per the manufacturer's instructions. Analysis was done on an Applied Biosystem model 373A DNA sequencing system. Each mutant NB gene was sequenced to confirm the presence of the mutation and that the rest of the NB sequence was as expected.

Purification of NB Poly-HIS Fusion Proteins

Forty litre cultures of the bacterial strains expressing the mutant NB were grown to mid-log phase and protein production induced by addition of 1 mM IPTG. The cells were disrupted in a French pressure cell. NB was solublized from the membrane by 2% CHAPS in phosphate buffer ($NaPO_4$ pH 7.0, 50 mM; β-mercaptoethanol 10 mM; glycerol, 5%; phenylmethylsulfonyl fluoride, 1 mM; NaCl, 500 mM; pH 7.0). Imidazole, to a final concentration of 50 mM was added to the supernatant containing the fusion protein. A column of 2 ml packed volume of Ni-NTA resin was prepared by washing the beads in phosphate buffer and then packing them into a column. The cellular extract was then spun for 10 minutes at 16,000 ×*g* at 4°C and the supernatant was applied to the column and the flow-through collected. The column containing the bound fusion protein was washed with 500–700 ml of phosphate buffer containing 0.5% CHAPS and 20 mM imidazole followed by washes with 250 ml phosphate buffer containing 0.2 mM sucrose monolaurate and 20 mM imidazole. The final wash of the column was with phosphate buffer containing 50 mM imidazole. The protein was eluted with 1–2 M imidazole. The imidazole was then dialyzed out to a final concentration of less than 1 μM. Fractions containing the NB mutant proteins (identified by Western blotting) were then tested for channel-forming activity using a planar lipid bilayer system.

NADH-Dependent Quinacrine Fluorescence Quenching

One-liter cultures of *E. coli* containing the plasmid for the protein of interest were grown to a Klett of 150. The cells were induced with 1 mM IPTG and grown for a further two hours. Membrane vesicles were prepared by harvesting the cells and resuspending them in STEM buffer {110 mM TES (N-tris[hydroxymethyl]methyl-2-aminoethanoesulfonic acid; pH 7.0), 20 mM magnesium acetate, 0.25 mM EGTA, 8.6% sucrose}, and disrupting them in a French press. Cell debris was removed by centrifugation at 25,000 × *g* for 30 minutes. This step would also have pelleted any inclusion bodies that may have been present. The membrane vesicles were pelleted by centrifugation at 400,000 × *g* for 1 hour. The vesicles were washed and resuspended in TES buffer (50 mM TES [pH 7.0], 15% glycerol). All the buffers contained the protease inhibitors 6-amino-*n*-hexanoic acid and *p*-aminobenzamidine. The NADH-dependent quinacrine fluorescence-quenching assay was used to determine the permeability of the membranes to hydrogen ions (Haddock & Downie 1974).

Table 1. Oligonucleotide primers used in NB mutagenesis

Oligonucleotide	Mutation
GATAATAATAGCCCCCCTGATG	S20A
CATCAGGGGGGCTATTATTATC	S20A
AATTATTATCGCCATATGTGTC	T24A
TGACACATATGGCGATAATAATT	T24A
GAGGCTGACAGCTATAGTGATA	C26A
TATCACTATAGCTGTCAGCCTC	C26A
GACAATGAGGGCGACACATATA	S28A
TATATGTGTCGCCCTCATTGTC	S28A
CTATGA↓GATCTTATTAGG	5′ Primer (Bgl II)
GGCCAAGCATG↓CAC	3′ Primer(Sph I)

Underlines indicate Bgl II and Sph I.

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

A synthetic peptide mimicking the last 25 amino acids of the NB protein at the C-terminus was synthesized (Applied Biosystems, model 477A). The MAP approach was used to prepare the antibodies in rabbits (Lu et al., 1991). The NBC antibody was then purified from the serum using the peptide and an ImmunoPure Ag/Ab Immobilization kit (Pierce).

Bilayer Setup

Lipid bilayer studies were performed as described elsewhere (Miller 1986; Sunstrom et al., 1996). A lipid mixture of palmitoyl-oleoyl-phosphatidylethanolamine and palmitoyl-oleoyl-phosphatidylcholine (8:2) dissolved in *n*-decane (50 mg/ml) was used. In some cases a mixture of palmitoyl-oleoyl-phosphatidylethanolamine, palmitoyl-oleoyl-phosphatidylserine and palmitoyl-oleoyl-phosphatidylcholine (5:3:2) (Avanti Polar Lipids, Alabaster, AL) was used. The lipid mixture was painted onto an aperture of 150–200 μm in the wall of a 1 ml delrin cup. The aperture separated two chambers, cis and trans, both containing salt solutions at different concentrations. The cis chamber was connected to ground and the trans chamber to the input of an Axopatch 200 amplifier. The cis chamber contained 500 mM NaCl and the trans, 50 mM NaCl, either at pH 6.0 or pH 2.5. The bilayer formation was monitored electrically by the amplitude and shape of the current pulse generated by a voltage ramp. Potentials were measured in the trans chamber with respect to the cis. The protein was added to the cis chamber and stirred until channel activity was seen. The currents were filtered at 250 Hz, digitized at 44 kHz and stored on magnetic

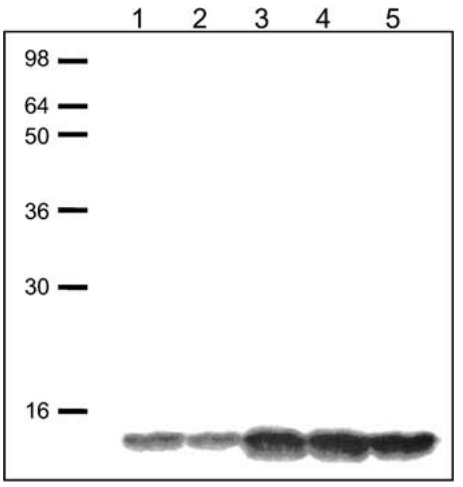

Fig. 1. Western blot of various purification fractions of NBC26A. Essentially identical results were seen for all mutants and wild-type proteins. Lane *1*, protein bound to the Ni-NTA beads. Lane *2*, beads after the elution of the protein. Lane *3*, elution with 0.5 M imidazole. Lane *4*, elution with 1 M imidazole and lane *5*, elution with 2 M imidazole. The markers on the left indicate molecular weights (kDa).

tape using a video recorder. The currents were analyzed by replaying in reverse through the same system and digitizing at 2 kHz using an A-to-D converter interfaced to an IBM-compatible computer.

Results

Wild-type and mutant NB proteins NBS20A, NBC26A, NBT24A and NBS28A, were expressed in, and purified from, *E. coli* as described above. Dialyzed fractions eluted after the Ni-NTA column chromatography, showed a single band at approximately 13 kDa on Western blots probed with the NBC antibody (Fig 1). This is consistent with the predicted molecular weight of 11.8 kDa for the NB monomer, the difference being attributed to the polyHis tag.

When 20 to 50 μl samples of these NB protein preparations were added to the solution on the cis side of the planar lipid bilayer, ion currents were typically seen after stirring for 10–40 minutes. The cis chamber contained 1 ml of 500 mM NaCl solution and the trans chamber, 1 ml of 50 mM NaCl. Both solutions were buffered at pH 6.0. The currents were recorded at a range of holding potentials between +100 mV and −100 mV and reversal potentials (E_0) measured were used to calculate the ion-selectivity of the mutant channels (Table 2). This revealed only relatively minor effects of the amino-acid substitutions on the ion-selectivity of these channels when compared with wild-type channels, which had an E_0 of 37 ± 2 mV, giving a P_{Na}/P_{Cl} ratio of 9. All four mutant proteins remained more permeable to Na^+ than Cl^- at a pH of 6.

Table 2. Reversal potentials of the different mutations at pH 6.0.

Protein	pH	E_0	P_{Na}/P_{Cl}	n
WT-NB	6	36 ± 1.9	8	4
NBS20A	6	37 ± 3.68	8	5
NBS20A	2.5	−32.14 ± 3	0.18	7
NBC26A	6	29.3 ± 13.3	5	5
NBC26A	2.5	−21 ± 2.9	0.35	5
NBT24A	6	26.5 ± 3.7	4	3
NBT24A	2.5	−29.7 ± 2.8	0.22	3
NBS28A	6	22.4 ± 6.8	3.2	4
NBS28A	2.5	−32 ± 2.7	0.19	4

The P_{Na}/P_{Cl} ratio was calculated from the Goldman-Hodgkin-Katz equation.

Channel activity of the NB mutants was also tested at low pH. Experiments were performed with the cis and trans chambers containing 500 mM and 50 mM NaCl solutions at pH 2.5, respectively. At this pH, the currents generated by all four mutants reversed between −20 mV and −40 mV (*data not shown*), indicating that the channels had become chloride-selective. This effect mirrors what we have reported previously with wild-type NB channels (Sunstrom et al., 1996).

The antibody to the C-terminus of the protein (NBC), which inhibits wild-type NB channels (Sunstrom et al., 1996) (*see* Fig. 3*B*), also inhibited the channel activity of all four NB mutants. Pre-immune serum did not affect the channels formed by NB (*data not shown*) and the NBC antibody did not have any effect on channels formed by M2 or NB lacking the C-terminus (Sunstrom et al., 1996). In addition, Western blots (*see* Fig. 1) indicate that NB is the only species in the preparations that binds to the NBC antibody. Taken together, these observations indicate that the C-terminal antibody specifically blocks channels formed by the NB wild-type and mutant proteins.

High concentrations of amantadine (2–3 mM) blocked channel activity (*data not shown*) of all four mutant NB proteins.

Mutation NBS20A Alters Channel Gating Kinetics

Although the NBS20A channels had an ion-selectivity that was similar to that of wild-type NB, their gating properties were different. Channel activity recorded in 26 bilayers was of two types. Most often (n = 21), there was a steady current without any obvious channel gating (Fig 2). This current reversed at about +31 mV, indicating five times greater permeability to sodium than to chloride ions. This steady current was not a "leak" current but was due to NBS20A. Firstly, the average conductance of bilayers after incorporation of NBS20A was 32 ± 7 pS

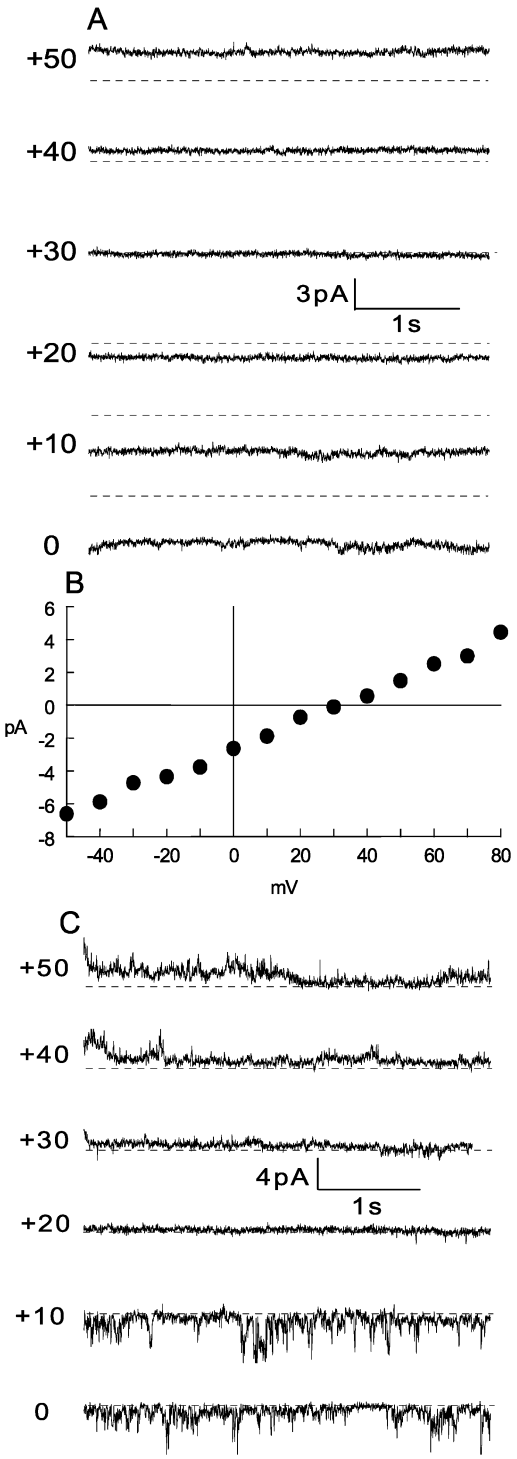

Fig. 2. (*A*) Current traces following addition of NBS20A (pH 6.0). The cis chamber contained 500 mM NaCl and the trans chamber contained 50 mM NaCl. Potentials (mV) are shown at the left of each trace. (*B*) Relationship between average current amplitude and potential. (*C*) Current traces of NBS28A, showing gating channels similar to wild-type NB. Dotted lines in *A* and *C* indicate baseline current levels.

($n = 10$). In contrast, the mean conductance of bilayers to which no protein had been added was 8.1 ± 0.98 pS ($n = 7$): the mutant NB was producing a new pathway for ions. Secondly, the NBC antibody blocked channel activity (4 experiments), as illustrated in Fig. 3*A* and *B*. Thirdly, the reversal potential of a nonspecific leak in the bilayer would be 0 mV but the reversal potential observed in the presence of NBS20A was 37.1 ± 3.7 mV. Taken together, the significant elevation of bilayer conductance, its Na^+ selectivity, and inhibition of the currents by NBC antibody, clearly indicate that the mutant NBS20A forms cation-selective channels, and, in comparison to wild-type NB channel activity, it is apparent that the NBS20A channel had markedly altered gating kinetics.

In 5 bilayers of the 26 experiments in which channel activity was seen with NBS20A, channels with gating activity similar to that of the wild-type NB channels was observed (*data not shown*). The average reversal potential of the channels was +37 ± 3.68 mV ($n = 5$), indicating a P_{Na}/P_{Cl} ratio of 8 (*see* Table 2).

At a pH of 2.5, channel activity was also observed ($n = 22$) with NBS20A. In these experiments, the reversal potential of the channels was between −20 mV and −40 mV. In a typical example, the reversal potential of the channel was −30 mV, giving a P_{Na}/P_{Cl} ratio of 0.19, making the channels 5 times more permeable to Cl^- than Na^+. Interestingly, these Cl^--selective channels showed gating behavior similar to that of wild-type NB at pH 2.5. In two experiments in which channel activity had been recorded at pH 2.5, both chambers were perfused with the same solutions, but at pH 6. After this change in pH, the gating activity of the Cl^--selective channel at pH 2.5 was observed to change to the non-gating steady current at pH 6.0 (Fig. 4) and the reversal potential returned to positive values, indicating a return to Na^+ selectivity.

In contrast to NBS20A, the three other mutations NBC26A, NBT24A and NBS28A did not show any perceivable changes in channel gating. The channel activity of the three mutations appeared similar to that of wild-type NB channels, both at pH 6 and 2.5 (*data not shown*).

Mutation NBS20A Alters Proton Permeability

NADH-dependent quinacrine fluorescence quenching has been used to show that NB channels are permeable to protons (Ewart et al., 1996). This assay determines the ability of *E. coli* membranes to maintain a proton gradient during oxidative phosphorylation. In inside-out vesicles in the presence of NADH and oxygen, the internal concentration of protons increases due to respiratory chain activity. This leads to protonation of the quinacrine dye

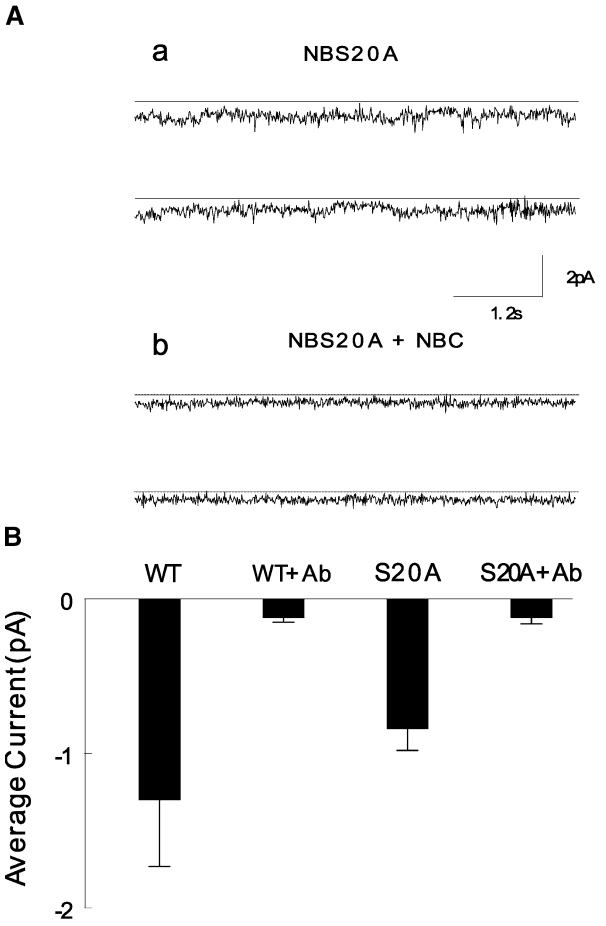

Fig. 3. (*A*) Antibody to the C-terminus of NB depresses currents generated by NBS20A.(*a*) Current traces (0 mV) before addition of NBS20A to the cis chamber. (*b*) Current traces after the addition of the antibody to the cis chamber. (*B*) *WT:* mean current (−1.3 ± 0.43 pA, $n = 3$) flowing through the bilayer with wild-type NB channels. *WT + Ab:* mean current (−0.12 ± 0.03 pA, $n = 3$) flowing through the bilayer with wild-type NB channels plus antibody (0 mV; cis chamber contained 500 mM NaCl and the trans chamber contained 50 mM NaCl). *S20A*: mean current (−0.84 ± 0.14 pA, $n = 3$) flowing through the bilayer with NBS20A channels. *S20A + Ab:* mean current (−0.12 ± 0.04 pA, $n = 3$) flowing through the bilayer with NBS20A channels plus antibody (0 mV; cis chamber contained 500 mM NaCl and the trans chamber contained 50 mM NaCl).

atebrin inside the vesicles, resulting in the quenching of fluorescence. Vesicles that are leaky to protons are unable to maintain the proton gradient (H^+_{in}/H^+_{out}), resulting in reduced fluorescence quenching. The uncoupler CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) is added to return the fluorescence back to its maximum level.

Membrane fraction of *E. coli* cells expressing mutant or wild-type NB were isolated by differential centrifugation (as describe in Materials and Methods) and used in fluorescence quenching experiments to determine the relative proton permeabilities of the vesicles. Samples of the membrane fractions were run on an SDS-Page gel and the NB proteins were detected by Western blotting using the C-terminal antibody to NB. The relative intensities of the NB bands on the blot indicated that the level of expression and incorporation into the membrane of each mutant protein was comparable (Fig. 5B).

In contrast to wild-type NB, membranes expressing the mutation NBS20A were found not to be leaky to protons. NADH-dependent quinacrine fluorescence-quenching experiments of the NBS20A membranes showed an average percentage quench of 90 ± 0.75% (Fig. 5*Ac*), which is similar to the 87 ± 2.2% quench of control membrane vesicles prepared from cells carrying the empty pQE-70 plasmid (Fig. 5*Aa*). These quenching values are significantly greater than that of the wild type NB membrane vesicles that showed an average percentage quench of approximately 60% ($n = 3$) Fig. 5*Ab*), reflecting the proton leakiness of vesicles containing wild-type NB. These experiments clearly demonstrate that NBS20A membrane vesicles are not so leaky to protons as membranes containing similar levels of wild-type NB.

The fluorescence quenching experiments carried out with membranes expressing the other three mutant proteins NBC26A, NBS28A, and NBT24A showed average percentage quenches of 46 ± 20%, 51 ± 5.8% and 67% respectively (only NBS28A shown; Fig. 5*Ad*), indicating proton permeability similar to that of wild-type NB for each of these mutants.

Discussion

In this study, we investigated the role of three hydroxyl-containing residues and a cysteine residue in the ion channel function of the NB protein from influenza B virus. All four residues are located in the N-terminal transmembrane domain of NB that forms the channel. The results indicate that mutation of the serine at position 20 to alanine (NBS20A) alters the gating properties and reduces the proton-permeability of the NB channel without affecting the relative selectivity for Na^+ over Cl^-. No significant effect of mutation of the other residues was detected.

The architecture of the pore of an ion channel governs properties of the channel such as ion-selectivity, gating, and drug susceptibility. Changing the structure of the pore region often leads to altered channel properties (Guy & Conti 1990; Galzi et al., 1992). The pores of some ion channels are surrounded by a bundle of transmembrane helices that may contain rings of hydrophilic pore-lining side chains that have a 3 to 4 residue periodicity (Kerr et al., 1994). For example, a motif of serine and threonine residues at positions "4-8-12" is seen in the second transmembrane segment of the nicotinic acetylcholine receptor superfamily (Sansom, 1992).

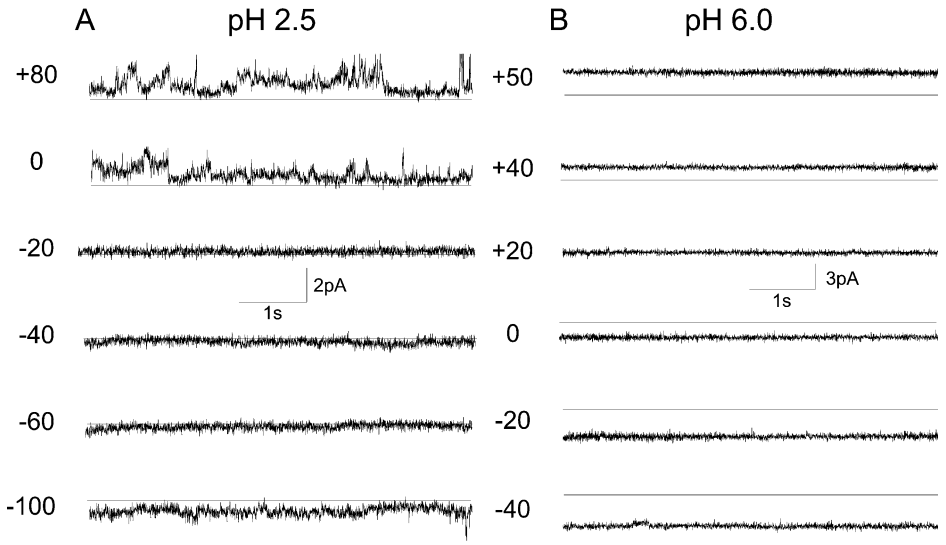

Fig. 4. Effect of pH on the ion-selectivity and kinetics of channels formed by NBS20A. Protein was added to the cis chamber containing 500 mM NaCl. The trans chamber contained 50 mM NaCl. (*A*) Channel activity at pH 2.5. Note the currents reverse at negative potentials (−40 mV). (*B*) Current traces from the same bilayer after perfusing the cis chamber with 500 mM NaCl at pH 6.0 and the trans chamber with 50 mM NaCl at pH 6.0. Note the change in channel gating and in the reversal potential (+20 mV).

These residues are highly conserved in the superfamily of receptors. It is generally accepted that hydroxyl-containing residues in transmembrane helices of ion channel groups are possible candidates for lining the pore region of the ion channel (Lear, Wasserman & Degrado, 1988) and/or for facilitating de-solvation of incoming ions by providing replacement H-bonding. From energetic considerations, hydrophilic OH groups are more likely to face the aqueous environment inside the pore or a protein-protein interface rather than the lipid phase.

As the NB transmembrane region contains three conserved hydroxyl-containing residues that are four residues apart, serine 20, threonine 24 and serine 28, we chose to investigate the role of these hydroxyl groups on NB channel function. Each of these residues was replaced with alanine, so as to remove the hydroxyl group while reducing the likelihood of having a major effect on the overall structure of the protein. Our experiments showed that channel activity was seen with all three hydroxyl-group mutations at both pH 6.0 and pH 2.5. Mutations at residues 24 and 28 had no detectable effect on NB channel properties. In contrast, the mutation S20A led to the formation of channels with an extremely long-lived open state at pH 6.0. In the majority of the experiments performed with this mutant, channel closing was rarely detected and the channels were, essentially, open all the time. If, like the M2 of influenza A virus, NB forms a homo-oligomeric structure in which the transmembrane domains have a parallel orientation in the bilayer, then it is possible that a ring of serine residues at position 20 near the mouth of the pore could form part of a gate for the channel.

Results similar to these have been seen with the nicotinic acetylcholine receptor. The nicotinic acetylcholine receptor is a large channel consisting of five subunits. Leonard et al. (1988) showed that replacement of a serine residue in all the subunits led to a reduced conductance of the channel. They also showed that there was a subtle change in the gating properties of the channel: the open time of the channel increased to double that of the wild-type channel and there were fewer closed levels per open burst.

The NB transmembrane region most likely forms an α-helix, but because the high-resolution structure of NB has not been determined, it is not known exactly how many α-helices make up the bundles that form the NB channel pore. However, computational modeling of tetrameric, pentameric and hexameric channels in 500 mM NaCl predicted conductance levels of 13, 34 and 53 pS, respectively (Fischer et al., 2000). For the majority of experiments with NBS20A in planar lipid bilayers, we observed non-gating channels with an average conductance of 32 ± 7 pS, suggestive of a 5 α-helix bundle. In a minority (5/27) of experiments, NBS20A channels with gating similar to wild type were also observed. While we do not have a definitive explanation for this, we did observe that the gating channels had a higher conductance. This raises the possibility of a different oligomeric state, forming a structure that had regained the ability to make the conformational changes responsible for gating.

In addition to gating changes, replacement of serine 20 by alanine alters proton permeability of the NB channel, as the NADH-dependent fluorescence-quenching experiments showed that NBS20A channels had reduced permeability to protons. In these experiments, membrane vesicles derived from *E.coli* expressing the wild-type NB and the mutant NB were compared for their proton permeability. The presence of the NB proteins in the membranes at similar levels was confirmed by Western blots (Fig. 5*B*). The

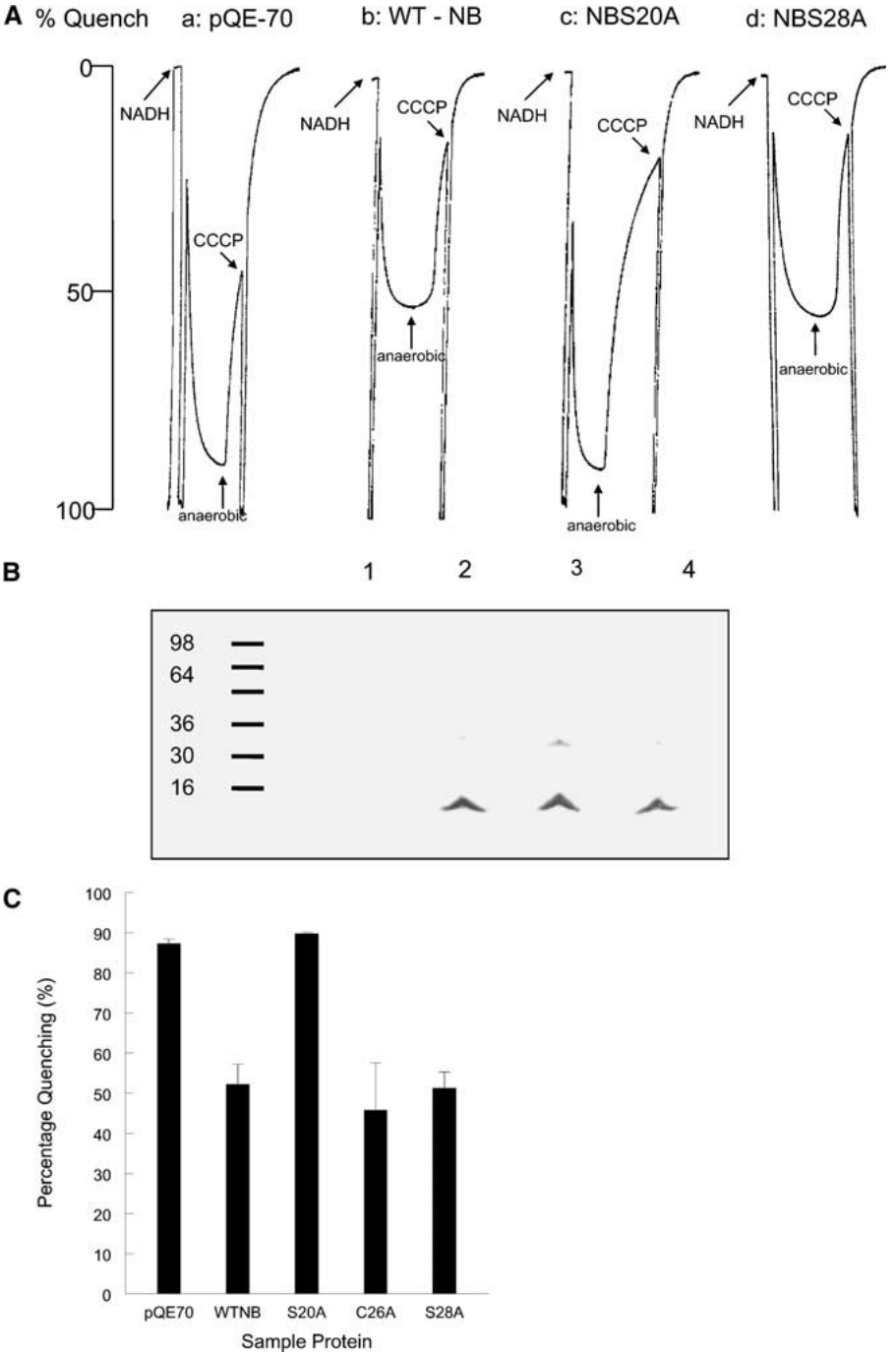

Fig. 5. (*A*) NADH-dependent atebrin fluorescence quenching in vesicles. (a) Control plasmid only -pQE-70, 87% quench.. (b) WT-NB, 54% quench; (c) NBS20A, 89% quench and (d) NBS28A, 56% quench. NADH and CCCP addition and anaerobic conditions are shown by arrows. (*B*) Western blot of membrane vesicles expressing the NB mutated proteins. Lane *1*: control vesicles carrying the plasmid pQE70. Lane *2*: vesicles expressing wild type NB. Lane *3*: vesicles from *E. coli* expressing NBS20A. Lane *4*: vesicles expressing NBS28A. The numbers on the left indicate the standard markers in kDa. (*C*) Percentage fluorescence-quenching of membrane vesicles. pQE70: control vesicles not expressing any protein; WTNB: wild-type NB; NBS20A; NBC26A; and NBS28A.

fluorescence-quenching of membranes expressing the different NB proteins shows that the wild type, NBT24A, NBC26A and NBS28A are approximately equally permeable to protons (Fig. 5*C*). In contrast, no reduction of quenching was observed in vesicles containing NBS20A (Fig. 5*Ac*), indicating that these membranes are considerably less permeable to protons.

In summary, although NBS20A forms cation-selective channels in bilayers, with conductances and P_{Na}/P_{Cl} ratios similar to those of channels formed by wild-type NB, it appears that the NBS20A mutation specifically alters channel gating and cation-selectivity so that the channels remain permeable to sodium but do not conduct protons. In conjunction with the α-helical bundle model of the transmembrane domain, perhaps this result supports the notion that a ring of OH groups from serine 20 is present at the mouth of the pore close to one side of the membrane. Removal of the H-bonding potential from this resi-

due may simultaneously affect ion selectivity as well as contribute to formation of a hyperstable conformation of the complex corresponding to an open pore. Potassium channels that do not conduct H^+ are seen in snail neurons (Byerly & Suen 1989; Decoursey & Cherny 1995).

Viral membrane proteins commonly have a number of disulfide bonds. These are usually highly conserved and may be critical in the folding and oligomerization of the protein. In a number of cases, replacing the cysteine residues that contribute to disulfide bonds reduces protein synthesis. No such observation was made with NBC26A. Replacing cysteine with alanine removes the SH group but does not discernibly affect channel properties. Therefore, it would seem that the cysteine 26 does not contribute to disulfide bonds necessary for channel formation.

It has been suggested that the ion currents originally ascribed to formation of ion channels by NB may have rather been explained as breakdown of the bilayer leading to the formation of nonspecific lipidic pores (Lamb & Pinto 1997). Therefore, a subsidiary aim of our mutagenesis study was to demonstrate that single amino-acid mutations in the putative pore-forming region of NB could alter the properties of the observed currents, thereby strengthening our conclusion that NB does, indeed, form ion channels. In this paper we show that NBS20A produced channels with altered properties, indicating that the single amino-acid change in NB was responsible. We believe that, in conjunction with the observation that the C-terminal antibody, NBC, specifically recognizes NB and blocks the channels (*see* Figs. 1 and 3), the evidence is overwhelming that these currents are due to NB and not to the development of a nonspecific leak.

It would be interesting to know the exact function of NB in the influenza B life cycle. There is every possibility that NB has a function similar to that of M2. The influenza B viruses have been known to require low pH for the dissociation of their M1 protein from the vRNPs (Zhirnov, 1990, 1992). Therefore, there is a need for a low-pH environment in the interior of the virus during the uncoating stage. We have shown that NB increases the proton permeability of *E. coli* membranes. During infection, NB may well function as a proton channel acidifying the interior of the virus leading to the dissociation of the vRNPs from the M1 protein. From the data available so far, it seems that the NB channel activity may be required during the uncoating of the virus in the early stages of infection.

Ion channels are attractive targets for the development of antiviral agents. Blocking the M2 ion channels stops viral replication by indirectly having an effect on the HA molecule and by preventing viral uncoating. The NB channel is also a prototypical homomeric channel like M2. If NB has a role similar to M2 in the viral life cycle, blocking the NB channel may arrest viral replication.

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