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ARTICLE

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Amantadine blocks channel activity of the transmembrane segment of the NB protein from influenza B

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Abstract NB is short auxiliary protein with ca. 100 amino acids, encoded in the viral genome of influenza B. It is believed to be similar to M2 from influenza A and Vpu from HIV-1 in that it demonstrates ion channel activity. Channels formed by the protein can be blocked by amantadine. We have synthesized the putative transmembrane segment of NB (IRG S^{20} IIITICVSL I^{30} VILIVFGCI A^{40} KIFI (NB, Lee)). Reconstituted in a lipid bilayer, the peptide shows channel activity. The addition of amantadine leads to dose-dependent loss of channel activity. Channel blocking is reversible. Channel behaviour of the peptide in the presence of amantadine is in accordance with findings for the intact channel. Thus, the synthetic transmembrane peptide captures the ion channel activity of the intact NB protein.

Keywords Viral ion channels · NB protein · Influenza B · Conductance measurements

Introduction

NB is a protein of ca. 100 amino acids encoded by influenza B. The first 18 residues on its N-terminal end are located at the extramembraneous side. About 22 residues span the membrane, leaving ca. 60 residues on the C-terminal end (Shaw et al. 1983; Williams and Lamb 1986). NB is expressed at the surface of the virion with its N-terminal site facing the outside of the capsule and its C-terminus facing the inside (Betakova et al. 1996). NB is exposed to the cytoplasm in infected cells (Betakova et al. 1996; Brassard et al. 1996; Shaw and Choppin 1984). By analogy with the M2 protein of influenza A, it is suggested that NB is a channel-forming protein. Conductance measurements with purified NB expressed in *Escherichia coli* and reconstituted in lipid bilayers reveal channel activity (Sunstrom et al. 1996). However, depending on the experimental conditions (e.g. salt concentration, pH), NB-induced channels in transfected MEL cells exhibit selectivity for either Na^+, Cl^- or H^+ ions (Chizhmakov et al. 1998). Thus, as emphasized by Lamb and Pinto (1997), it is important to firmly establish that NB is a channel protein, and that its channel inducing activity is not simply due to resolution of endogenous channels. To this end we have shown that synthetic peptide corresponding to the transmembrane helix of NB self-assembles to form ion channels in lipid bilayers (Fischer et al. 2000b).

Until recently, the only antiviral drug available was amantadine (amantadine-1) and its structural analogue rimantadine (α-methyl-1-adamantanemethanamine) (Davies et al. 1964; Hoffmann 1973). Amantadine is applied to infections caused especially by influenza A. Its application results in side effects on the central nervous system (Couch and Jackson 1976). Side effects are found to a lesser extent for the application of rimantadine (Dolin et al. 1982). Amantadine is used for preventive therapy and also during an already ongoing infection with influenza A. Amantadine selectively blocks the viral ion channel protein M2. This molecule and its derivatives have also been investigated as possible drugs effective against the human immunodeficiency virus-1 (HIV-1) (Kolocouris et al. 1996).

The conception of M2 channel blocking by amantadine is that the molecule enters the pore and prohibits an ion flux (Wang et al. 1993). It has been shown that the sensitivity to amantadine is affected by mutations of residues Val27, Ala30 and Ser31, which are

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located within the putative lipid membrane spanning (transmembrane (TM)) region of M2 (Pinto et al. 1992). Investigations using neutron diffraction data are in support of the loci for the amantadine-M2 channel interaction to be between Val27 and Ser31 (Duff et al. 1994). Experiments with M2 expressed in oocytes of *Xenopus laevis* and whole cell current recordings show that concentrations between 1 and 100 μM amantadine have an effect on the channel activity of M2. Sensitivity is different between the individual virus strands (Udorn > Weybridge > Rostock) and is due to mutations in the TM region of the protein. Synthetic peptides analogous to the TM region of M2 lose channel activity in the presence of ca. 20 μM amantadine (Duff and Ashley 1992).

Experiments with amantadine have been performed with NB expressed in *E. coli*, purified and reconstituted in a lipid membrane. Amantadine concentrations of 2–3 mM lead to reduced channel activity (Sunstrom et al. 1996). The drug lowers the current amplitude and its frequency.

We have synthesized the putative TM region of NB proposed by a computational study on the single strand embedded in a hydrated lipid bilayer (Fischer et al. 2000b). The TM region (IRG S^{20} IIITICVSL I^{30} VILIVFGCI A^{40} KIFI (NB, Lee)) exhibits channel activity (Fischer et al. 2000a). In the present study we address the sensitivity of the TM segment of NB to amantadine when reconstituted into a lipid bilayer. Amantadine is added with increasing concentration to the lipid bilayer containing the TM segments of NB.

Materials and methods

Peptide synthesis

The transmembrane segment of NB (IRG S^{20} IIITICVSL I^{30} VILIVFGYI A^{40} KIFI; NB, Lee) was synthesized using standard Fmoc methodology on an Applied Biosystems 430A automated peptide synthesizer. PAC-PEG-PS resin and the HATU coupling reagent purchased from PE Biosystems (Warrington, UK) were used. For a detailed description, see Fischer et al. (2000b). The peptide was purified by preparative HPLC using a POROS RP 4.6×100 mm column at a flow rate of 5 mL/min. The buffers used were A = water (0.1% trifluoroacetic acid, TFA) and B = acetonitrile (0.1% TFA). A gradient was run between 5% and 50% B over 20 min. Matrix-assisted laser desorption ionization-time of flight spectrometry (MALDI-TOF) was done on a Micromass TofSpec 2E mass spectrometer operating in the linear mode from an α-cyano-4-hydroxycinnamic acid matrix. For automated amino acid sequence determination, samples were adsorbed onto poly(vinylidene difluoride) (PVDF) membrane of 0.2 μm porosity using a ProSorb cartridge and following the manufacturer's protocol. The membrane-bound samples were then excised from the ProSorb cartridge and N-terminally sequenced on an Applied Biosystems 494A "Procise" sequencer.

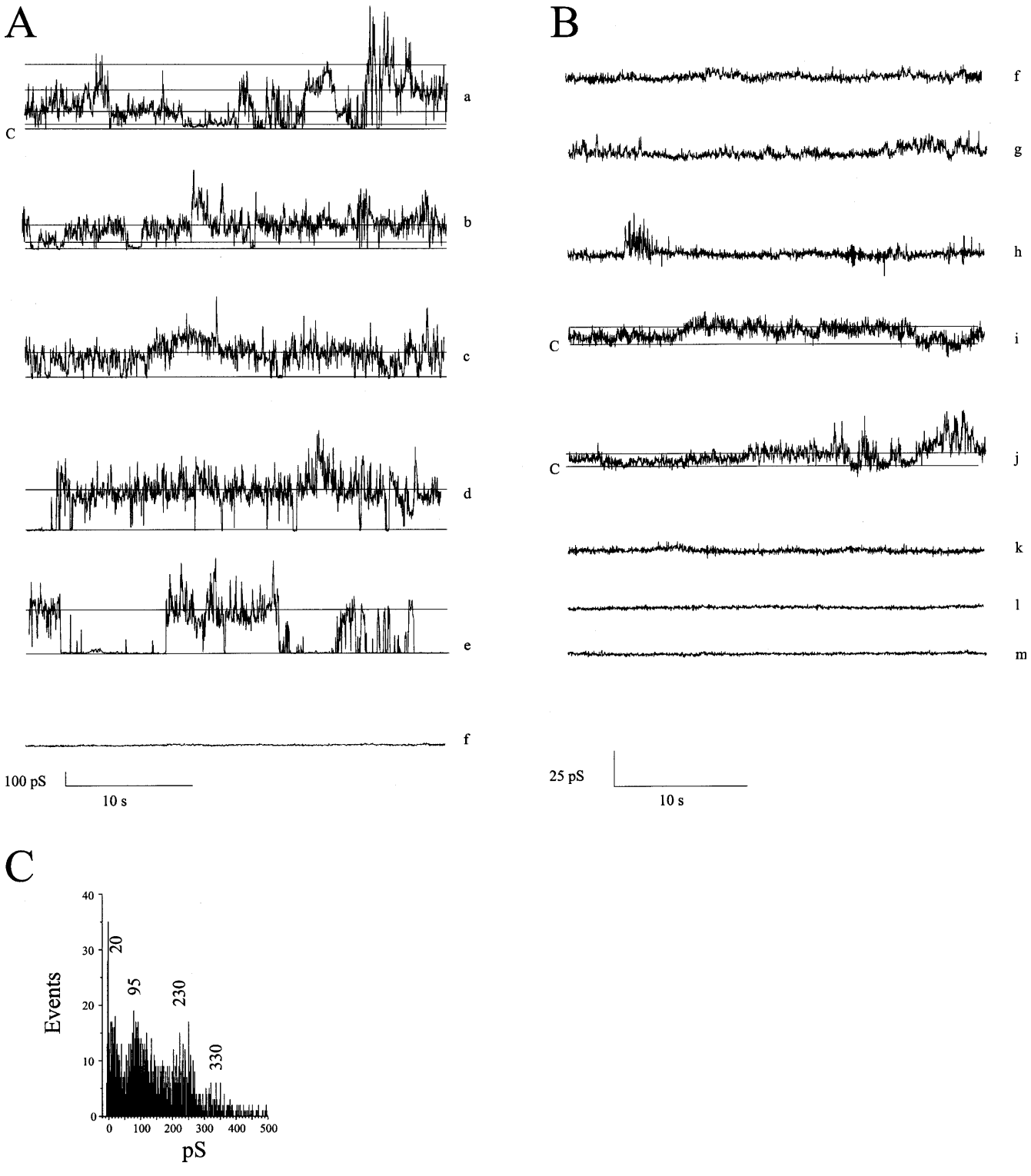
Channel recordings in planar lipid bilayers

A planar lipid bilayer was formed across an aperture (ca. 130 μm diameter on the long axis) in a thin (25 μm) Teflon film (Yellow Springs Instruments, Ohio, USA) (Montal and Mueller 1972). About 40 μL of lipid [10:1 (w/w) L-α-phosphatidylcholine (type II-s) and cholesterol, both from Sigma] in chloroform were spread on top of a buffer (0.5 M KCl, 10 mM *N*,*N*-bis(2-hxdroxyethyl)-2-aminoethanesulfonic acid (BES), pH 7.0), which was raised across the aperture. After adding the protein (dissolved in methanol) on the *cis* side (amplifier) the bilayer was formed by raising the buffer level. Amantadine (Sigma, UK) was added to the *trans* side (ground) in subsequent amounts to reach the final concentration prior to the recording. Electrical currents were measured with an Axopatch 1D amplifier at a rate of 5 kHz and filtered with 1 kHz using a Digi Data 1200 interface (Axon Instruments, Calif., USA). Currents were generated using a Function Generator TG 302 (Levell, Barnet, UK). For data analysis, Origin 5.0 was used. Data in Fig. 3 were fitted according to the relationship: integrated current = $1/\{1 + ([\text{amantadine}]/K_{app})\}$ (see Wang et al. 1993 and references therein).

Results

The upper trace of Fig. 1A shows recordings of NB peptide reconstituted in lipid bilayers at an applied transmembrane potential of –100 mV without the presence of amantadine. Analysis of the data reveals approximately four conductance levels of ca. 20, 95, 230 and 330 pS (see also Fischer et al. 2000b) (Fig. 1C). Amantadine is added on the *trans* side of the membrane. Applying a transmembrane voltage of *cis* –100 mV pulls the *trans* amantadine towards the lipid bilayer. At a concentration of 0.02 mM amantadine the channel activity remains unchanged; levels of 20 and 140 pS are observed. With increasing amantadine concentration, conductance levels of 130 pS (0.04 mM), 230 pS (0.06 mM) and 270 pS (0.08 mM) are found. The open time of the events seem to be extended over several seconds. At amantadine levels of 0.06 and 0.08 mM the open periods are interrupted by short periods of channel inactivity. Channel inactivity increases with increasing drug concentration. At 0.1 mM amantadine, channel activity is completely abolished. Addition of amantadine is continued until a final concentration of 2.1 mM is reached (Fig. 1B). Between 0.1 and 2.1 mM amantadine, events of channel activity are only observed occasionally, e.g. at 0.7 and 0.9 mM amantadine, with conductance of around 5 pS.

To address the question of whether amantadine-induced blockage of channel activity is voltage dependent, we applied +100 mV in the presence of 2.1 mM amantadine (Fig. 2A). After several seconds, channel activity is restored. The current profile is identical with the profile at –100 mV. Subsequent reversal, as shown in Fig. 2A, allows the ion flux for only a few seconds before recordings return back to the zero line (lower trace in Fig. 2A). This experiment could be repeated several times. In the presence of 4.1 mM amantadine (Fig. 2B), channel activity is blocked at –100 mV transmembrane potential. The reversed potential of +100 mV does not lead to any restoration of channel activity. Voltage as high as +170 mV is necessary to induce an ion flux (data not shown). From these data we conclude that



channel inactivity is reversible and controlled by amantadine diffusion in a dose-dependent manner. Fitting the current integrated over time from each trace in Fig. 1 with a sigmoidal dose-response curve allows an estimate of the binding constant: $K_{app} = 0.08 \pm 0.01$ mM (Fig. 3).

Discussion

Channel activity of the TM segment of NB can be blocked by the antiviral drug amantadine with an approximate binding constant $K_{app} = 0.08$ mM. Channel behaviour of the TM segment is comparable to the

Fig. 1A–C Ion channel activity of NB at –100 mV. Amantadine is added from a stock solution of 200-fold excess in such a way that on the *trans* side the final concentration as outlined is reached. **A** Channel activity without amantadine (*upper trace*) and in the presence of 0.02–0.1 mM amantadine (*following traces*). *Black lines* represent the main conductance stages: 20, 100, 230 and 330 pS (0 mM amantadine, *a*); 140 pS (0.02 mM amantadine, *b*); 125 pS (0.04 mM amantadine, *c*); 230 pS (0.06 mM amantadine, *d* and 0.08 mM amantadine, *e*). Scaling is 100 pS and 10 s. **B** Recording with 0.1 (*f*) to 2.1 mM (*m*) amantadine: 5 pS (0.7 mM amantadine, *i* and 0.9 mM amantadine, *j*). Note the change in scaling (25 pS and 10 s) compared to **A**. **C** Current histogram of trace *a* in **A**

behaviour of the complete protein in the presence of amantadine. For M2 of influenza A it has been reported that channel activity of the TM segment incorporated into planar lipid bilayers can be reversibly blocked by amantadine (Duff and Ashley 1992). In the case of the fully transcribed M2 protein and reconstitution into a planar lipid bilayer, the presence of amantadine reduces the channel opening (Tosteson et al. 1994). For Vpu it has been demonstrated that the synthetic TM segment of the Vpu protein reconstituted in a planar lipid bilayer shows similar channel activity and ion selectivity to the complete channel protein (Schubert et al. 1996). Sensitivity of the segment to amantadine has not yet been reported. However, it is striking that in the case of the viral ion channel proteins in general, simply the TM segments reflect the behaviour of the complete channel.

A possible explanation, which is compatible with our results, is that amantadine interacts via integration in the hydrophobic phase of the lipid bilayer and subsequent attachment to the helical segments. This could hamper any rotational motion of the segments necessary for channel gating. Similar rotational/screw-like motions are suggested for other short and complex ion channels (Armstrong 1992; Armstrong 1981; Guy and Seetharamulu 1986; Kerr et al. 1995; Unwin 1995). However, such motions could also be blocked if amantadine penetrates into the pore.

Amantadine by itself does not perturb the bilayer (Duff et al. 1993). Once channel activity is blocked, the peptide-containing membrane does not show any leak current up to ca. 4.1 mM amantadine (data not shown). Therefore the possibility of causing channel block by distorting the bilayer, with the consequence of disabling the peptides to assemble, might be ruled out. Corresponding effects of amantadine, as those mentioned for NB peptide, were not seen with the channel forming peptide alamethicin (data not shown). This suggests that channel block is due to specific interaction of amantadine with the NB peptide.

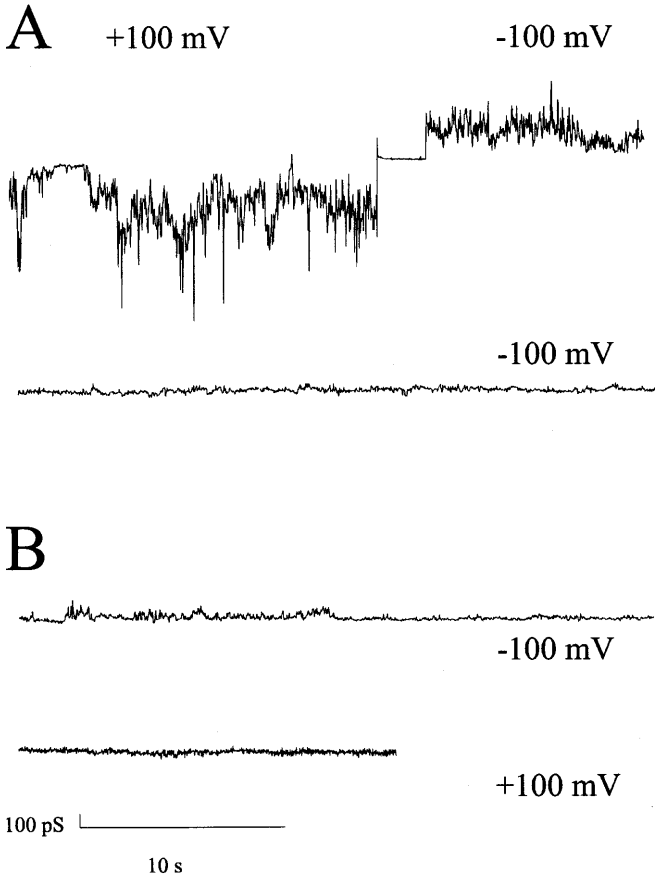


Fig. 2 NB peptide in the presence of 2.1 mM (**A**) and 4.1 mM (**B**) amantadine. Applied potentials of + 100 and –100 mV. The second *lower* curves in **A** and **B** are each recorded ca. 1 min after recording of the upper curves

Conclusion

The TM segment of NB is sensitive to the antiviral drug amantadine. Concentrations of amantadine down to 0.04–0.1 mM are detectable because of a change in the channel behaviour of the peptide induced by amantadine. We find changes in the frequency, duration and amplitude of the current. This is in accordance

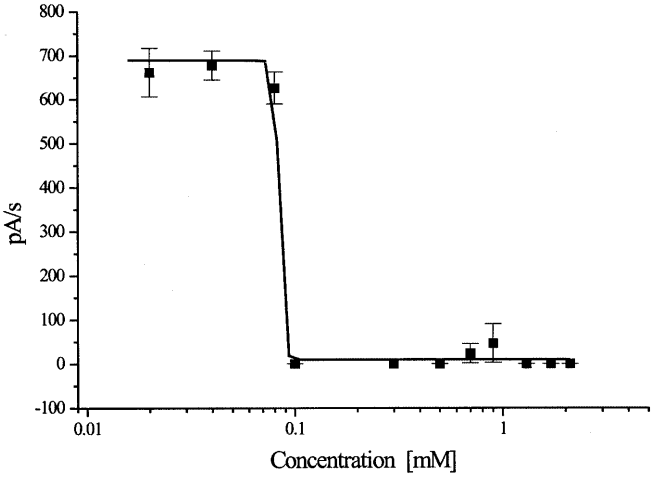


Fig. 3 Dependence of the integrated current on the addition of amantadine. Data derive from segments of continuous recordings of ca. 30 s in length (mean ± SEM). The curve represents a sigmoidal fit, resulting in $K_{app} = 0.08 \pm 0.01$ mM

with findings for the whole protein. The TM segment represents the behaviour of the whole protein in this respect. Amantadine shows reversible blocking of the channel formed by the TM segments of NB.

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