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Transmembrane Peptides for applications in biosensors: NB from influenza B.

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Abstract

NB from influenza B is a short auxiliary protein 100 amino acids in length. It is believed to have a similar role in the virus life cycle to the channel forming protein M2 from influenza A. The latter channel can be blocked by amantadine. We have synthesized the putative transmembrane segment of NB: IRGS[20] IIITICVSLI[30] VILIVFGCIA[40] KIFI (NB, Lee). Reconstituted in a lipid bilayer the peptide shows channel activity (Fischer *et al*. Biochemistry 39, 12708-12716 (2000)). In the presence of amantadine channel activity is lost reversibly. Channel activity *per se* and in the presence of amantadine is similar to the behaviour of the complete NB protein (Sunstrom *et al*. J. Membr. Biol. 150, 127-132 (1996)). The synthetic transmembrane fragment resembles the characteristics of the intact NB protein.

Introduction

Biological lipid membranes separate the interior of a cell from the 'out-side world'. Sub-cellular compartments are also separated by lipid membranes. The amphiphatic nature of the lipids assembles these molecules in such a way that a large hydrophobic barrier is created. The hydrophobic barrier prevents the flow of charged molecules across the membrane. However, to allow for a facilitated passage of ions or molecules from one side of the compartment to the other the lipid membrane is spanned by particular proteins adapted perfectly to the hydrophobic lipid environment. These proteins are called integral membrane proteins (here referred to as membrane proteins for simplicity). Some examples are: ATPases, G-protein coupled receptors, light harvesting membrane proteins, ion channels. Approximately 30 – 40 % of the coding region of each organism's genome encodes for membrane proteins, at least for organisms whose genomes have been sequenced to

date. From a medical point of view it is therefore not surprising that about half of the drugs currently available target membrane proteins [1]. Consequently there is an enormous demand for analytical techniques detecting protein-drug interactions for the screening of new drugs and there will be even so more in the future.

There are a variety of options to initiate the transfer of information across the membrane e.g. by light, through ions, *via* hormones. For each option specific membrane proteins have evolved. For shuffling ions along the electrochemical gradient across the membrane the cells use ion channels. Ion channels form water filled pores across the lipid membrane (Fig. 1) which can, depending on the particular channel type, be opened on command (triggered by ligands or by an altered electrochemical gradient) or statistically. Also viruses, especially enveloped viruses use ion channels to survive. However, the length of these proteins is about five times less than that of ion channels found in cells. Thus, the viral channels represent miniaturized channel systems made by nature.

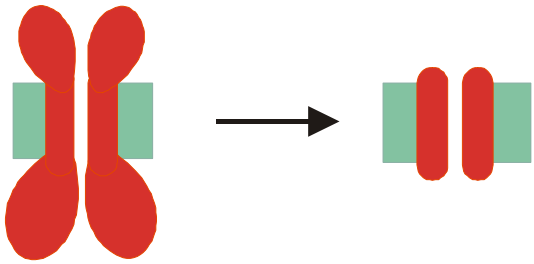

Fig. 1: *Cartoon of the channel protein NB from influenza B (red) in a lipid bilayer (green) (left) consisting of an extramembraneous (above the bilayer), a transmembraneous (TM) (within the bilayer), and a cytoplasmic (below the bilayer) segment. Two peptides are shown. In vivo more segments have to assemble to form a pore. Solely the TM segment of NB (NB peptide) in a lipid bilayer is shown on the right. Like bigger channels (e.g. nAChR) the viral ion channel forming protein has also to assemble to form functioning channels.*

Investigations on the potential use of ion channels in biosensor devices have been carried out for the last 15 years. The first report of the use of an ion channel, the glutamate receptor (a channel which is triggered by the ligand glutamate), was in 1990 [2]. The receptor was reconstituted in an artificial lipid membrane and signals were recorded electrically. Fig. 2 shows a cartoon of a sampling unit to apply the technique developed by Montal & Müller [3] (see figure legend for details). In another study a voltage dependent anion channel (VDAC) was transferred onto a platin surface and the results indicate that changes in the conductance behaviour are to be expected in such a measurement unit [4, 5]. Recently a potential sensing system has been introduced by the use of gramicidin, a statistically opening antibiotic pentadecapeptide channel [6]. The challenge for the use of large membrane proteins immobilized on surfaces is to control their orientation (the binding site of a receptor needs to be accessible for the analyte). Antibodies will play a major role for the proper alignment in the future [7]. Optical spectroscopy (fluorescence) has been used to detect the activity of a surface immobilized 5-HT_3 serotonine receptor [8]. Investigations are also being undertaken to study a suitable polymer support material for immobilization of the ion channels. It has been shown that polyimides can be covered with a lipid membrane ready for the use with micro chip devices [9].

Measurements with alamethicin, a channel forming peptide antibiotic, and the nAChR showed that the membrane was stable for ca. 50 hours. 3-Mercaptopropionic acid [10] and porous layers of polyethylene terephtalate (PET) [11] also seem to be suitable materials for coating with ion channel containing lipid bilayers .

Membrane proteins in general, and ion channels in particular, are difficult to express in large amounts suitable for e.g. structural and functional studies. This might be a bottleneck for the large-scale production of biosensors. An alternative route could be the use of fragments of the complete membrane protein (peptides). Fragments of Na^+ and K^+ channel have been immobilized on gold surfaces and used to detect an analyte [12, 13]. Another potential segment for the use in sensor devices is the TM segment responsible for the ion flow in the channel protein. For the TM segment of the nAChR it has been proved that this segment exhibits channel activity similar to the full length nAChR [14]. Usually the length of a TM segment does not exceed 30 amino acids, which means that peptides can easily be obtained by solid phase peptide synthesis or adjusted expression systems. As a case study for such investigations we use ion channel forming membrane proteins from viruses.

Three small auxiliary proteins, all of around 100 amino acids in length, coded for by different types of influenza virus are called: M2 (influenza A), NB (influenza B), and CM2 (influenza C) (Fischer & Sansom; Viral ion channels: Structure and function, review in Biochim. Biophys. Acta - Membranes, accepted 2001). A similar short protein, Vpu, is found in the genome of the human immunodeficiency virus type-1 (HIV-1) (*Retroviridae*). Ion channel activity is confirmed for M2, NB, and Vpu. Recently it has been found that the plant virus *Paramecium bursaria chlorella* virus (PBCV-1) encodes an ion channel which resembles a miniaturised K^+-channel [15]. In all cases the short proteins have to assemble to form functional channels. Solution and solid state nuclear magnetic resonance (NMR) [16-21], Fourier transform infrared (FTIR) [21-24], and circular dichroism (CD) [17,25,26] spectroscopy have now been used to resolve the structure of the influenza and HIV-1 ion channel proteins. We have also previously studied the putative single TM segment of the viral ion channel proteins by computational methods [27].

NB from influenza B is 100 amino acids long. The first 18 residues of NB from influenza B are located at the extramembraneous side (N-terminal). Ca. 22 residues span the membrane leaving ca. 60 residues on the C-terminal end [28, 29]. NB is expressed at the surface of the virion with its N-terminal site facing the outside of the capsule and its C-terminus the inside [30]. NB is exposed to the cytoplasm in infected cells [30-33]. 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 [33]. Depending on the experimental conditions (e.g. salt concentration, pH) NB-induced channels in transfected mouse erythroleukemia (MEL) cells exhibit selectivity for Na^+, Cl^-, and H^+ ions [34]. Thus, as emphasized by

Lamb and Pinto [35], 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 [26].

Until recently the only anti viral drug available was amantadine (amantadine-1) and its structural analogous rimantadine (α-methyl-1-adamantanemeth-anamine) [36,37]. Amantadine is applied to infections caused especially by influenza A. Its use results in side effects on the central nervous system [38]. Side effects are also found, to a lesser extent, for the application of rimantadine [39]. Amantadine is used for a 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 HIV-1 [40].

The model of M2 channel blocking by amantadine is that the molecule enters the pore and prohibits an ion flux [41]. It has been shown that the sensitivity to amantadine is affected by mutations of residues Val-27, Ala-30, and Ser-31 which are located within the putative lipid membrane spanning TM region of M2 [42]. Investigations using neutron diffraction data are in support of the loci for amantadine – M2-channel interaction being between Val-27 and Ser-31 [43]. 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 channel activity of M2. Sensitivity is different between the individual virus strains (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 [44].

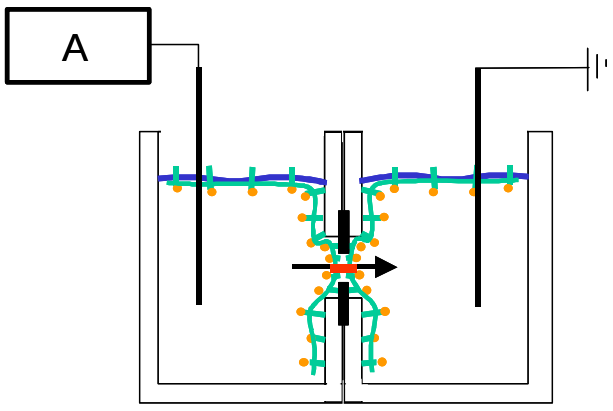

Fig. 2: *Bilayer set up used for channel recordings. The lipids are put onto an aqueous subphase (blue) in both sides of the chamber and raised to cover a small aperture in a Teflon film (thick black bars between the two chambers). Inserted ion channels (red) allow the ion flux (arrow) which can be registered with the electrodes. The left electrode is connected to the amplifier (A). This side is called the cis side in this study. The other chamber is grounded and called trans side.*

Experiments with amantadine have been performed with NB expressed in *Escherichia coli*, purified and reconstituted in a lipid membrane. Amantadine concentrations of 2 - 3 mM lead to reduced channel activity [33]. The drug lowers the current amplitude and its frequency.

We have synthesised the putative TM region of NB proposed by a computational study on the single strand embedded in a hydrated lipid bilayer [27]: Ile-Arg-Gly-Ser^{20}-Ile-Ile-Ile-Thr-Ile-Cys-Val-Ser-Leu-Ile^{30}-Val-Ile-Leu-Ile-Val-Phe-Gly-**Tyr**-Ile-Ala^{40}-Lys-Ile-Phe-Ile (NB, Lee, with Cys-38 replaced by Tyr (bold); sequence using the single letter code: IRGS^{20} IIITICVSLI^{30} VILIVFGCIA^{40} KIFI). The TM region exhibits channel activity [26]. 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 [45]. The outcome of this work is interpreted in terms of a possible use of these systems in biosensors.

Materials and Methods

Peptide synthesis:

The transmembrane segment of NB (IRG S^{20} IIITICVSL I^{30} VILIVFGYI A^{40} KIFI; NB, Lee) was synthesised using standard Fmoc methodology on an Applied Biosystems 430A Automated Peptide Synthesiser. Peptide acid linker (PAC) – poythylene glycole (PEG) – polystyrene (PS) resin (4-hydroxymethyl-phenoxyacatic acid attached to polyethylene glycol-polystyrene (MBHA) graft support) and HATU (N-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridino-1-ylmethylene]-N-methylmethanaminium hexafluorophosphate N-oxide) coupling reagent purchased from PE Biosystems were used. For a detailed description see Fischer ***et al***. 2000 [26]. The peptide was purified by preparative HPLC using a POROS RP 4.6 x 100 mm column at a flow rate of 5 ml/min. The buffers used were: A = water (0.1 % TFA (trifluoroacetic acid)) and B = acetonitrile (0.1 % TFA). A gradient was run between 5% and 50 % B over 20 min. Matrix assisted laser desorption ionisation - 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 PVDF membrane (polyvinylidene diflouride - 0.2 μm porosity) using a ProSorb cartige (PE Biosystems, Warrington, UK) and the manufacturer's protocol was followed. The membrane-bound samples were then excised from the ProSorb cartridge and N-terminally sequenced on an Applied Biosystems 494A "Procise" sequencer (PE Biosystems, Warrington, UK).

Channel recordings in planar lipid bilayers:

Planar lipid bilayers were formed across an aperture (ca. 100 μm diameter) in a thin (25 μm) teflon film (Yellow Springs Instruments, OH, USA) [46] (Fig. 2). 40 μl of lipid (10 : 1 (w/w) L-α-phosphatidylcholine (Type II-s) and cholesterol, both from Sigma) in pentane were spread on top of a buffer (0.5 M KCl, 10 mM BES (*N,N*-bis(2-hydroxyethyl)-2-aminoethane-sulfonic acid), 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 (Adamantan-1-amine

hydrochloride, 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, CA, USA). Currents were generated using a Function Generator TG 302, LEVELL, Barnet, UK. For data analysis Origin 5.0 was used.

Results

The upper trace of Fig. 3,I 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 4 conductance levels of ca. 20, 95, 230 and 330 pS (see also [26]). 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 (Fig. 3,II). 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. Fitting the current integrated over time from each trace in Fig. 3 with a sigmoidal dose-response curve allows an estimate of the binding constant to be K_{app} = 0.08 ± 0.01 mM.

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. 4,I). After several seconds channel activity is restored. The current profile is identical with the profile at -100 mV. Subsequent reversal, as shown in Fig. 4,I allows the ion flux only for a few seconds before recordings return back to the zero-line (lower trace in Fig. 4,I). This experiment could be repeated several times. In the presence of 4.1 mM amantadine (Fig. 4,II) 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.

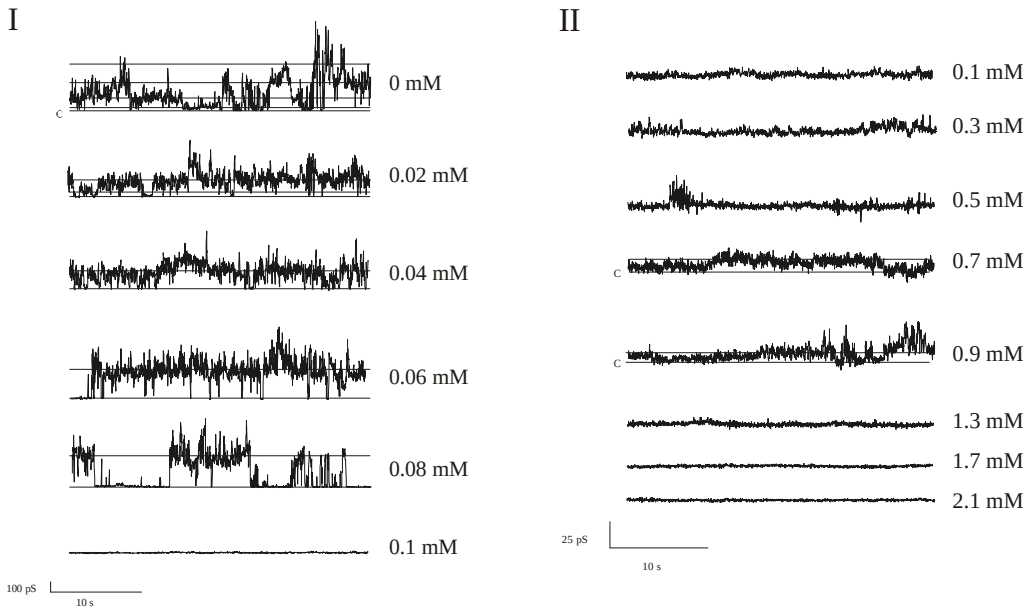

Fig. 3*: Ion channel activity of NB at –100 mV. Amantadine is added from a stock solution of 200 fold excess in such a way, that on the trans side the final concentration as outlined is reached. I: (scaling is 100 pS and 10s) channel activity without amantadine (upper trace), and in the presence of amantadine of 0.02 – 0.1 mM (following traces). Black lines represent the main conductance stages: 20, 100, 230, und 330 pS (0 mM amantadine); 140 pS (0.02 mM amantadine), 125 pS (0.04 mM amantadine); 230 pS (0.06 and 0.08 mM amantadine). II: Recording with 0.1 – 2.1 mM amantadine: 5 pS (0.7 and 0.9 mM amantadine). Please note the change in scaling (25 pS and 10s) compared to I (100 pS and 10s).*

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 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 [44]. In the case of the fully transcribed M2 protein and reconstitution into a planar lipid bilayer the presence of amantadine reduces the channel opening [47]. 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 [48]. Sensitivity of the segment to amantadine has not yet been reported. However, it is striking that in the case of 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 [49-53]. However, such motions could also be blocked if amantadine penetrates into the pore.

Amantadine by itself does not perturb the bilayer according to neutron diffraction data (Duff *et al.* 1994 [43]). Once channel activity is blocked the peptide containing membrane does not show any current leakage up to ca. 4.1 mM amantadine (data not shown). This would suggest that channel blocking through distortion of the bilayer, with the consequence of disabling the peptide to assemble, might be ruled out. However, electrophysiological data indicate that rimantadine:- a derivative of amantadine - changes the dynamics of artificial lipid bilayers [54]. The presence of rimantadine leads either to a 'rigidification' or 'fluidization' of an artificial lipid membrane in dependence of lipid composition. Rimantadine leads also to an increased aggregation of erythrocytes. These data suggest that any membrane protein might be affected by the altered fluidity of the lipid membranes caused by the drug. Rimantadine penetrates through the lipid membrane if given only to one side of an artificial membrane [55]. As a consequence the pH of the side to which the drug diffuses changes.

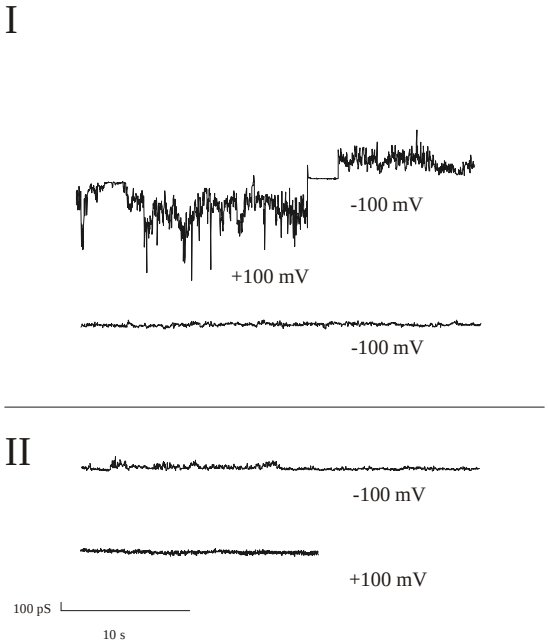

Fig. 3: *NB peptide in the presence of 2.1 mM (I) and 4.1 mM (II) amantadine. Applied potentials of +100 and –100 mV. Second curves in I and II are recorded ca. 1 min after recording of the upper curves.*

The excess of rimantadine on one side of the membrane and the increasing pH difference between the two sides alters the membrane potential. This could affect the conductance behaviour of membrane-embedded pore forming proteins such as e.g. NB. As a conclusion, future measurements have to address indirect affects such as (i) altered membrane potential or (ii) fluidity of the membrane on the pore blocking behaviour of amantadine and its derivatives on NB.

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.

Conclusion

The TM segment of NB is sensitive to the anti viral 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 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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