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THE PRION PEPTIDE FORMS ION CHANNELS IN PLANAR LIPID BILAYERS

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Abstract: One of the hypotheses concerning the pathogenic properties of the prion protein considers its influence on cellular ion homeostasis. Using the lipid bilayer technique, the influence of prion-derived peptides on the lipid bilayer conductance was characterized. To evaluate the physiological significance and possible pathological functions of the peptides, their effect on the membrane potential and respiration rate of hippocampal mitochondria was also studied. We used a peptide bearing the human prion protein sequence YSNQNNF (PrP [169-175]), and peptide SSQNNF (PrP [170-175]) bearing a naturally-occurring mutation in position 171 [N→S] linked to schizoaffective diseases in humans (Samaia, H.B., Mari, J.J., Vallada, H.P., Moura R.P., Simpson A.J.G., Brentani R.R. A prion-linked psychiatric disorder. *Nature* 390 (1997) 241). In this report, we show that PrP [170-175] N171S increases the conductance of planar lipid bilayers. Based on the conductance of single channel currents recorded in 500/500 mM KCl (cis/trans), we found a single channel conductance of 8 to 26 pS. The native prion peptide PrP [169-175] does not form ion channels in the lipid bilayer. Neither of the peptides significantly changed the membrane potential or respiration rate of isolated rat hippocampal mitochondria. We propose a possible mechanism for channel formation by aggregation of the prion-derived peptide.

Key Words: Prion, Ion Channel, Black Lipid Membrane, Mitochondria

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Abbreviations used: BLM - black lipid membrane; DASPMI - 2-(p-dimethylaminostyryl) - pyridylmethyl iodide; HOBt - 1-hydroxybenzotriazole; PrP - prion protein; TFA - tri-fluoroacetic acid.

INTRODUCTION

Prions cause fatal neurodegenerative disorders both in animals and humans. These disorders called transmissible spongiform encephalopathies, are highly infectious. The infection may be transmitted across the species barrier. In humans, such diseases include Creutzfeldt-Jacob's disease (CJD), Gerstmann-Sträussler-Scheinker disease (GSS), fatal familial insomnia (FFI) and kuru. In animals, the most frequent disease is Bovine Spongiform Encephalopathy (BSE; so-called mad cow disease) [1, 2].

It is thought that the disease is due to the conversion into its pathological form (PrP^{Sc}) of a normal glycoposphatidyloinositol (GPI) anchored glycoprotein, the so-called cellular isoform of the prion protein (PrP^C). The transformation of PrP^C into PrP^{Sc} is a process of conformational change, not a covalent modification. The cellular isoform PrP^C is rich in alpha-helices, whereas the pathological isoform PrP^{Sc} is rich in beta-sheet structures, which are insoluble and resistant to proteases [1, 2]. The physiological role of PrP is not fully understood. It has superoxide dismutase activity and binds several copper ions [3-5]. One of the theories about the physiological role of this protein is that it has a function in maintaining ion homeostasis and redox equilibrium within the cell.

It is important to know whether PrP is involved in ion transport through (across) membranes. A channel-forming role was postulated for PrP by Chapron *et al.* [6]. In fact, a peptide comprising the PrP sequence 106-126 was found to form cation channels in black lipid membranes (BLM) [7].

In this study, we examined channel formation in BLM by peptides derived from the sequence of human PrP: ¹⁶⁹YSNQNNE₁₇₅ and ¹⁷⁰SSQNNF₁₇₅ (171N-S). To evaluate the possible pathological functions of the peptides, their effect on the membrane potential and respiration rate of hippocampal mitochondria was also studied.

MATERIALS AND METHODS

Materials

Salts, sucrose and nigericin were purchased from Sigma (Deisenhofen, Germany). Asolectin from soybean (Soybean asolectin) was from Sigma-Aldrich (Poznan, Poland). Valinomycin was from Boehringer (Mannheim, Germany), and digitonin from Serva (Heidelberg, Germany). 2-(*p*-dimethylaminostyryl)-pyridylmethyl iodide (DASPMI) was from Koch-Light (Haverhill, UK). All the other chemicals were of the highest commercially-available purity grade.

Peptide synthesis

Both peptides (with C-terminal amides) were synthesized via the solid-phase method using Fmoc chemistry. A TentaGel S Ram (RAPP Polymere, Germany) was used as a support. During the synthesis, protected amino acid derivatives were coupled to the peptidyl-resin using an equimolar solution of DIC (N, N'-diisopropylcarbodiimide) and HOBt (1-hydroxybenzotriazole). After synthesis

had been completed, the peptides were removed from the resin together with the side-chain protections in a one step procedure using TFA-phenol-triethylsilane-H₂O (88:5:2:5). The peptides were purified by reverse phase HPLC in 0.1% TFA with a linear acetonitrile gradient to 95% purity. The correct molecular masses of the peptides were confirmed by mass spectroscopy.

Asolectin purification

The asolectin used for the planar lipid bilayer technique was purified according to the following procedure: 10 g of asolectin was dissolved in 50 ml of chloroform, followed by the addition of ice-cold acetone. This mixture was kept at 4°C for 1 h. The lipid precipitate was centrifuged at 2500 rpm for 15 min and the supernatant was removed. The asolectin was stored at -70°C.

Planar lipid bilayer technique

The planar lipid membrane was formed by spreading phospholipid dispersions (painted bilayer). Planar phospholipid bilayers were formed in a 250 µm diameter hole drilled in a Delrin[®] partition (Warner Instrument Corp., Hamden, CT USA), which separates two chambers (*cis* 1.8 and *trans*, 0.5 ml internal volume). Both chambers contained 100/100 or 100/500 mM KCl, 10 mM Hepes, pH 7.4 (adjusted with HCl). The outline of the aperture was coated with a lipid suspension and dried with N₂ prior to bilayer formation to improve membrane stability. Planar phospholipid bilayers were painted using a suspension of asolectin in n-decane at a final concentration of 40 mg lipid/ml. The formation and thinning of the bilayer were monitored by capacitance measurements. The final capacitance values ranged from 90 to 180 pF. Electrical connections were made using Ag/AgCl electrodes and agar salt bridges (3 M KCl) to minimize liquid junction potentials. Solutions of the peptides (4 mg/ml in DMSO) were added to the *trans* compartment. No changes in current were observed upon the addition of DMSO alone. All the measurements were carried out at room temperature. The current was measured using a Bi-layer Membrane Admittance Meter (model ID 562, IDB, Gwynedd, UK).

Data analysis

Signals were filtered at 0.1 KHz (Low Pass Bessel Filter 4 Pole, Warner Instrument Corp.), digitized at 82.5 KHz (A/D converter 1401, Cambridge Electronic Design, UK) and transferred to a PC for off-line analysis by CED Electrophysiology Package V6.41.

Hippocampal homogenates

Male Wistar rats (130-200 g) were killed by cervical dislocation, and their brains were rapidly removed, washed and placed in ice-cold 250 mM sucrose solution. The hippocampus was then isolated. For the preparation of homogenates, about 50 mg wet weight tissue was homogenized twice for 20 s at 8000 rpm using an Ultra-Turrax homogenizer T 25 (IKA, Staufen, Germany) in a 0.5 ml ice-cold

medium containing 0.25 M sucrose, 0.5 mM EDTA and 50 mM Tris-HCl (pH 7.4).

Mitochondrial membrane potential measurements

The measurements were made at room temperature in a 1 ml cuvette of a Shimadzu RF-5000 spectrofluorimeter (Tokyo, Japan) using 5 μ M of the membrane potential sensitive fluorescent dye DASPMI in a medium containing 10 mM pyruvate, 5 mM malate, 60 mM KCl, 110 mM mannitol, 10 mM KH_2PO_4 , 0.5 mM Na_2EDTA and 60 mM Tris-HCl (pH 7.4). The protein concentration of the hippocampal homogenates was 0.5 to 0.7 mg/ml. In order to permeabilize the synaptosomal membranes, 100 μ g digitonin per mg of protein was added. The samples were excited at 450 nm and the DASPMI fluorescence was registered at 520 nm.

Mitochondrial respiration

The rates of respiration of the hippocampal homogenates were measured at 30°C using a Clark type oxygen electrode (Yellow Springs Instruments, OH) in the medium described for the membrane potential measurements. Treatment with digitonin (100 μ g/mg protein) was used to reach mitochondria enclosed in synaptosomes.

RESULTS AND DISCUSSION

Several modes of action have been proposed to explain the prion-induced neurodegenerative diseases (disease mechanisms) [8], including the effects of PrP on intracellular Ca^{2+} homeostasis [9]. The prion-induced modification in Ca^{2+} homeostasis is the result of (1) prion interaction with intrinsic ion transport proteins, e.g. L-type Ca^{2+} channels in the plasma membrane and IP_3 -modulated Ca^{2+} channels in intracellular membranes [10]; and/or (2) the formation of cation channels by PrP itself [7].

Rationale for choosing the PrP fragment

Studies of the conformational transition from PrP^{C} into PrP^{Sc} are often hampered by the insolubility and infectivity of the latter. Synthetic peptides allow this obstacle to be overcome. Several peptides derived from the PrP sequence have been used, comprising sequences between residues 90 and 140. Some of these peptides revealed amyloidogenic properties [11, 12]. Among them, peptide 106-126 (human sequence) was found to form ion channels in the lipid bilayer [7], pointing to a role of this part of the PrP molecule in maintaining ion homeostasis within the cell. In this study, we decided to check the properties of another fragment of PrP, comprising amino acids 169-175 together with a peptide bearing the naturally occurring mutation 171N \rightarrow S. The rationale behind this choice was substantiated by the following facts:

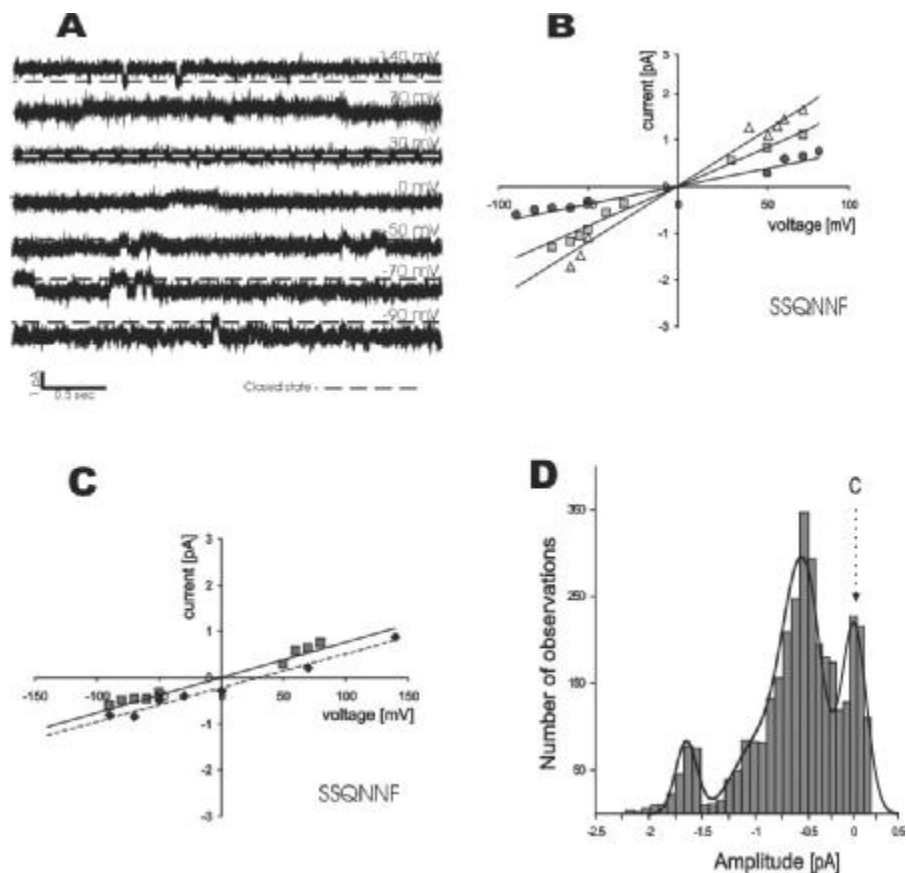


Fig. 2. The effect of prion derived peptide SSQNNF on a planar lipid bilayer composed of asolectin (lipid concentration 40 mg/ml, peptide concentration 4 μ g/ml). **A**: Current traces from a channel formed by peptide SSQNNF in planar lipid bilayer (asymmetrical KCl (100 mM/500 mM *cis/trans*) plus 10 mM HEPES, pH 7.0); the numbers over the traces represent the membrane potential applied. Filtered at 100 Hz. **B**: Current-voltage relations for channels formed in asolectin membranes by prion peptide SSQNNF (symmetrical solution of 500 mM KCl, 10 mM HEPES, pH 7.0). Solid lines are drawn to a linear fit of the data. The lines represent the simple channel conductance of 7.6 pS (filled circles), 16.8 pS (squares), 26.1 pS (triangles). **C**: Current-voltage relations for channels formed in asolectin membranes by prion peptide SSQNNF. The solid line is for symmetrical KCl (500 mM *cis/trans*) and the dashed line is for asymmetrical KCl (100 mM/500 mM *cis/trans*) plus 10 mM HEPES, pH 7.0. **D**: Amplitude histogram of the channel formed in asolectin membranes by prion peptide SSQNNF (symmetrical solution of 500 mM KCl, 10 mM HEPES, pH 7.0). The transmembrane potential was equal to -70 mV. C - indicates the baseline level. The solid line is the sum of four Gaussian functions and was calculated with pCLAMP 8 software.

channel currents recorded in symmetrical 500/500 mM KCl *cis/trans*, we found single channel conductance from 7.6 to 21.6 pS. The appearance of ion channels with different conductance reflects the fact that the channels are probably formed due to aggregation of the peptide molecules. The formation of channels with heterogeneous ion conductance by prion peptides was shown by other investigators [7] and this concurs with the aggregation properties of the peptide applied. Experiments with different salt concentration across a membrane (100/500 mM KCl *cis/trans*) allow us to trace the cation selectivity of the PrP [170-175] N171S formed channels with a reversal potential of 30 mV. This is close to the reversal potential for a K⁺-selective channel of 40.5 mV, as calculated from the Nernst equation. The native prion peptide PrP [169-175] only transiently perturbs lipid bilayer conductance without the discrete conductance changes observed for PrP [170-175] N171S (Fig. 3). Recently, it has been shown that the prion protein fragment PrP(106-126) induces apoptosis in the human neuroblastoma cell line SH-SY5Y [17]. The earliest detectable apoptotic event was the depolarization of the mitochondrial membrane occurring immediately upon treatment of cells with PrP(106-126), implicating mitochondria as a primary site of action of this peptide [17]. Hence, we have performed measurements of mitochondrial potential in isolated rat hippocampal mitochondria upon application of PrP [170-175] N171S and PrP [169-175] (Fig. 4). Neither of the studied peptides significantly changed the membrane potential or respiration rate of the mitochondria (data not shown). This observation

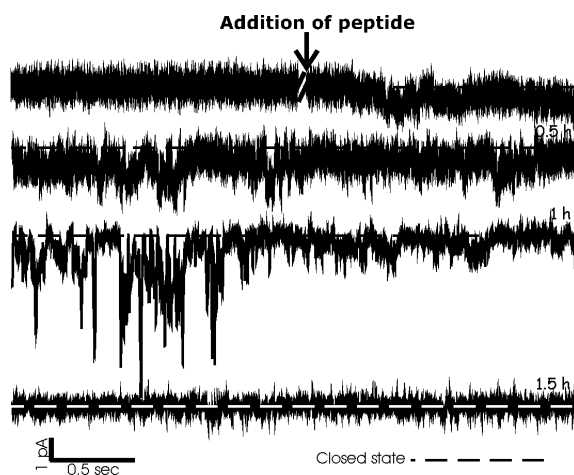


Fig. 3. Current traces from planar lipid membrane composed of asolectin after prion peptide YSNQNNF addition. Representative traces illustrate current changes induced by peptide YSNQNNF shortly after its addition and 0.5, 1, 1.5 hours thereafter. The holding membrane potential was set at -50 mV. Asolectin concentration 40 mg/ml, peptide concentration 4 μ g/ml. Asymmetrical KCl (100 mM/500 mM KCl *cis/trans*) plus 10 mM HEPES, pH 7.0. Filtered at 100 Hz.

could be attributed to the following facts. First, the peptides were added not to purified mitochondria but to hippocampal homogenates and thus the micromolar concentrations of the peptides could be absorbed by synaptosomal membranes. Second, low channel conductance may not be enough to depolarise the inner mitochondrial membrane with its high transmembrane potential and the existing mechanisms of potential maintenance in mitochondria.

Nevertheless, we believe that the channel formed by PrP [170-175] N171S can affect ionic equilibrium on the cellular plasma membrane, and the ability of the mutated peptide to form ion channels, in contrast to the non-mutated peptide, could be a reason for the corresponding neuronal disorder.

Upon insertion of a potassium selective pore into the plasma membrane, the membrane permeability to potassium is increased. Physiologically, it results in a membrane hyperpolarisation, or, at least, in the stabilization of membrane potential at the resting level, and provides an electrical driving force for calcium ion influx through calcium channels.

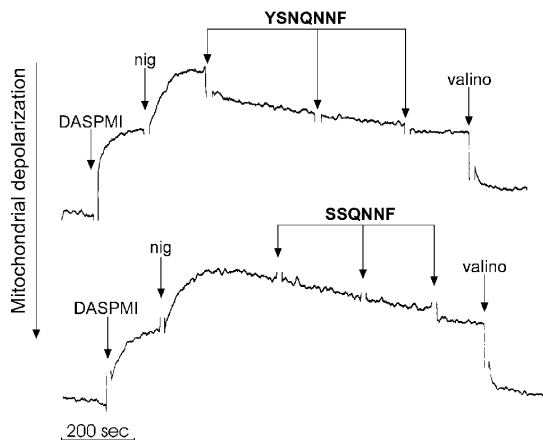


Fig. 4. The effect of prion peptides on the membrane potential of rat hippocampal mitochondria. The membrane potential measurements of hippocampal mitochondria were performed with the use of the fluorescent probe DASPMI. Mitochondrial depolarization is accompanied by a lowering of DASPMI fluorescence (in arbitrary units). Digitonin-treated rat hippocampal homogenates (0.5 mg/ml) were energized with 10 mM pyruvate and 5 mM malate. In order to maximize the mitochondrial membrane potential, 0.5 mg/ml nigericin (nig) was added followed by the additions of 4 mg/ml peptide YSNQNNF (upper trace) or SSQNNF (bottom trace) and 2 μ g/ml valinomycin (valino). The measurements were performed at room temperature.

It has been shown that, in planar lipid bilayers, PrP [170-175] N171S forms cation selective channels with conductance in the range of 8 to 26 pS. The native prion peptide PrP [169-175] only modulates asolectin planar lipid bilayer conductance without discrete conductance changes. The formation of channels

with different conductance by the prion peptide PrP [170-175] N171S could be due to different levels of peptide aggregation. Neither peptide changes the mitochondrial membrane potential or respiration rate of rat hippocampal mitochondria. The observed ion channels can probably modify (intra)cellular ionic equilibria and thus mediate neuronal dysfunctions.

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