Integration of Ion Channel Proteins into a Polymer Matrix - Investigation by the Patch-Clamp Technique

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SUMMARY: Two types of natural ion channel proteins, gramicidin A and nicotinic acetylcholine receptor, were reconstituted into an artificial lipid/polymer matrix. The functionality of the proteins was fully maintained after the integration. Particular kinds of phospholipids and of polymer matrices had been chosen to set up the artificial mimic system, which itself constitutes a novel type biosensor. The functionality of the mimic system was verified by single channel patch-clamp measurements.

Introduction

Ion channel proteins play an important role in the generation of electrical signals within the nervous system and in a variety of signal transduction systems[1]. A number of important channel proteins, including the nicotinic acetylcholine receptor (nAChR)[2] and Na[3], K[4], Ca[5] channels, were cloned and sequenced. The reconstitution of such transmembrane proteins into artificial membranes has been investigated by several groups during the last decade. The high recognition capability makes the transmembrane proteins outstanding candidates for biosensors. Moreover, the flow of up to 10^6 ions upon contact with a triggering ligand offers an intrinsic amplification effect, i.e. the ion channel sensors should not only be extremely selective but highly sensitive as well. Since the functioning of transmembrane proteins depends very much on the membrane properties, successful integration of such proteins into a synthetic environment could not be achieved previously.

The nAChR[6] is a ligand-gated ion channel selective for Na^+, K^+ and other small cations. It is found in muscle and neuronal systems as a membrane glycoprotein. nAChR is a transmembrane pentamer composed of the four types of subunits α, β, γ, δ which are arranged symmetrically around the ion pore (Fig.1A). Each subunit possesses four primarily hydrophobic α-helical transmembrane segments M1 – M4, as well as a sequence having amphipathic α -helical periodicity. The transmembrane subunits form a bundle around a central inner pore which becomes narrow from a large entrance to an apparent diameter of ~ 1.0 nm within the membrane (Fig.1B).

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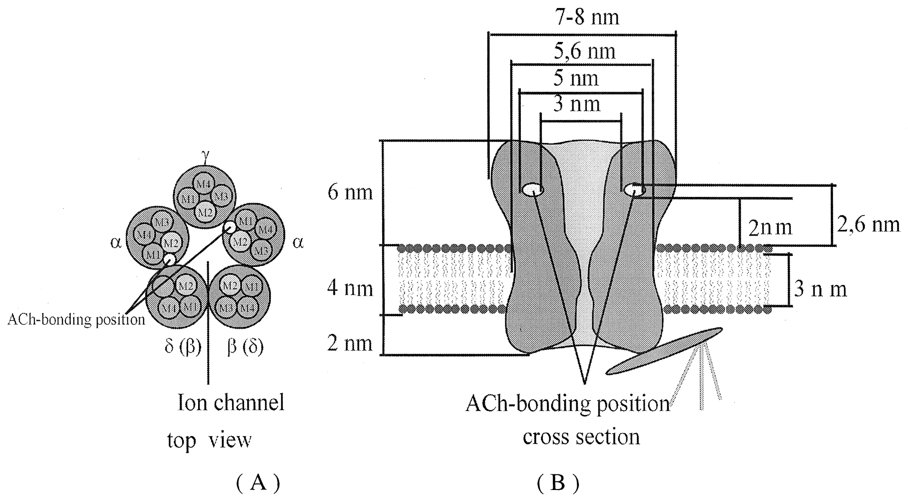

Fig. 1: Model of the nAChR[7,8]: (A) longitudinal section; (B) nAChR in lipid bilayer.

Compared to nAChR, gramicidin[9] is a much simpler, non-gated ion channel system obtained from pore-forming antibiotics. The properties of gramicidin are well known, and it can advantageously be used to establish the techniques needed for the integration of the more delicate nAChR into polymer matrices. Gramicidin A channels in lipid membranes are formed by a head-to-head ß-helical dimerisation. The polar groups of the polypeptide backbone line the lumen of the channel while the hydrophobic side chain forms the exterior surface of lumen of the channel, thereby making the secondary structure compatible with the bilayer interior (Fig.2). Our experiments were performed with natural gramicidin A which is predominant (80%) in mixture including gramicidin B, C and D. Gramicidin A channels are selective for monovalent cations.

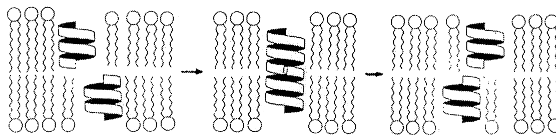

Fig. 2: Model of temporary channel formation by gramicidin A in a fluid membrane[10].

The two transmembrane proteins, gramicidin A and nAChR, were reconstituted into an artificial lipid/polymer system. The channels themselves are surrounded by a lipid bilayer,

which spans the pores of a polymer matrix. The properties of the polymer turned out to be crucial for the functioning of the ion channels. Here we report on the reconstitution of ion channels into the membrane system and show single ion channel measurements. The obtained characteristics include the duration of constant conductance level, and the life time of the channels.

Experimental Section

Chemicals. nAChR was stored at concentrations of 3.5mg/mL in PBS buffer (137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4). The material was obtained from our cooperating partner Prof. Dr. A. Maelicke (Johannes Gutenberg University Mainz, Germany). Before measurement, the material was extracted and purified in our institute[11)]. GramicidinA from *bacillus brevis* (>90%) was purchased from Fluka Biochemika (München, Germany). Acetylcholinchlorid (AChCl) (>99%), cholesterol (>99%), cesium chloride (>99.5%), potassium chloride (>99.5%), decane (>98%), hexadecane (>99%) and squalene (>99%) were obtained from Fluka Biochemika as well. HEPES biological buffer and NaN_3 were purchased from Sigma-Aldrich Chemie GmbH (München, Germany). PBS biological buffer was prepared in our lab. Ultrapure water was used for all the preparation of buffer and the measurements of the membrane currents.

Soybean lecithin, type S 75, was a product of Lipoid GmbH (Ludwigshafen, Germany). The following commercial polymer matrices have been investigated: PTFE Hostaflon© 1700 (thickness 95μm, porosity 0.26%), PTFE Zitex© G110 (thickness 262μm, porosity 40%) and PFEP Hostaflon© FEP 6107 (thickness 109μm). All products were surface modified by the cooperating group of Prof. Dr. V. Rossbach, Institute of Macromolecule Chemistry, Dresden University of Technology (Dresden, Germany). Polyethylene terephthalate (PET, thickness 10 μm, porosity 15%, pore size 10 μm) was purchased from Oxyphen GmbH (Grosserkmannsdorf, Germany).

Lipid/Polymer Matrix System. The phospholipid bilayers were formed from a mixture of 15mg/mL soybean lecithin in n-decane with 40mol% cholesterol. This solution was applied to the porous polymer, which itself was attached to a Teflon sample chamber. The polymer separated two aqueous chambers, each 1.0mL in volume. Each chamber was filled with buffer and equipped with an Ag/AgCl electrode. Currents across the bilayer were measured under

voltage-clamp conditions by means of a patch-clamp amplifier (HEKA, Lambrecht, Germany). All experiments for gramicidin A were carried out at room temperature[12)], the experimental solution used was 100mM CsCl. The nAChR was investigated in the KCl experimental solution consisted of 100mM KCl, 10mM HEPES and 0.02 % (w/w) NaN_3. The chamber which housed the measuring electrode was defined as *cis* side. The opposite side with reference electrode was called *trans*. The voltage was referenced to the *cis* side with respect to the *trans* side.

Measurement of Membrane Current. The ion channel protein nAChR, which spans both strata of the bilayer, and the agonist AChCl were added with gentle stirring to the *cis* side. Gramicidin A, which spans only one stratum of the bilayer, was added simultaneously to the experimental solution at the *cis* and *trans sides*. The incorporation of the ion channel proteins into bilayers was observed by conductance changes across the bilayer.

Results and Discussion

Recording currents across the bilayer at the single-channel level is the best way to test whether the ionic channel protein was reconstituted into lipid bilayer, and whether its functioning was maintained. Two kinds of ionic channel proteins, gramicidin A and nAChR, were added with gentle stirring. Gramicidin A, structurally simpler and experimentally less vulnerable, was used for the basic optimizations of experimental conditions, e.g. pore sizes of the polymers, lipid fluidity, bilayer stability, reproducibility of the electrical measurements. Usually, between a few tenths of a minute up to a few minutes after addition of the sample, ion channels were formed and conductance could be measured.

Gramicidin A dissolved in ethanol (10^{-9}mol/L) was added into the sample chamber symmetrically from *cis* and *trans* sides. The polymer membrane was immersed into 100mM CsCl solution. Lipid bilayers were formed across the pores of the PTFE membrane prior to immersion into the solution. The proteins diffused from both sides into the bilayer, i.e. Gramicidin A molecules went independently into the two compartments of the bilayer (cf. Fig.2). According to the fluidity of the bilayer the gramicidin A molecules are enabled to lateral diffusion. Upon accidental contact between gramicidin A molecules in the two compartments of the bilayer a temporary ion channel is formed. The mean conductance of

gramicidin A was the value of 210 $\pm$ 80 pS and with the help of the maximum likelihood method the mean life time was calculated as 8.5ms. These values and conductance state are in excellent agreement with literature data[12)].

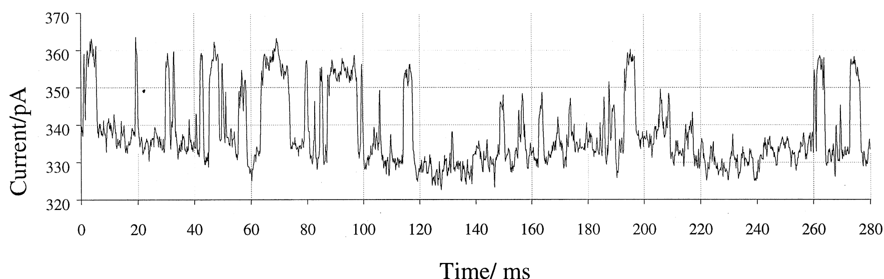

Fig. 3: Formation of gramicidin A ion channels in the lipid bilayer which spans the pores of a G110-PTFE film.
Experimental conditions: +100mV in symmetric 100mM CsCl experimental solution; molar ratio between lipid S 75 and oxidized cholesterol 3: 2, dissolved in n-decane.

The single-channel recording (Fig.3) depicts the closed state of the ion channel at a low current level of merely approx. 330 pA. A significantly higher current can be measured during the opening state of a single channel (approx. 360 pA). The distinct current levels in Fig. 3 indicate the formation of a stable bilayer. Moreover, the bilayer has an appropriate hydrophobic length of the lipid molecules and a suitable molar content of cholesterol, since open channels are merely found at the given properties. At the same time, the channels exhibit sufficiently flexible transitions between open and closed states, in good agreement to their physiological states.

The much more challenging transmembrane protein nAChR was stored in bovine serum albumin (10^{-9}~10^{-3}mol/L) and reconstituted into soybean lipid bilayer, which was set up in the pores of a polyester (PET) matrix. The protein and the agonist AChCl (10µl / 10^{-4}mol/L) were added from the *cis* side only. Three conductance levels 14pS, 26pS, 41pS, were observed, and the occurrence of each level was almost random. The ionic channels chart is shown in Fig.4.

The electrophysiological measurement by the patch-clamp technique reveals the successful integration of the nAChR protein into a lipid/polymer matrix. It could be shown by the patch-

clamp measurements, that the common fluorescence marking is not always sufficient to verify the functioning of the ion channels.

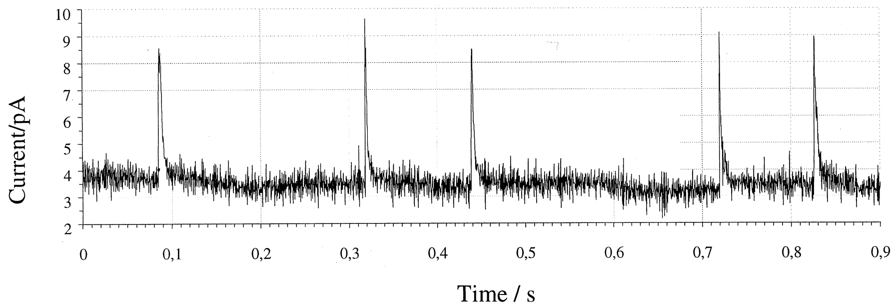

Fig. 4: Formation of nAChR ion channels in the lipid bilayer which spans the pore of a hydrophilic polyester film.
Experimental condition: +200mV in symmetric 100mM KCl experimental solution; molar ratio between lipid S75 and cholesterol 3:2, dissolved in squalen.

Single channel events could not be observed whenever the lipid solution contained quantities of common organic solvent as n-hexadecane. Under such conditions nAChR lost its channel activity. After freezing-out of the organic solvent the protein may sometimes be reactivated. The superior strategy for maintaining the channel activity of nAChR is the exchange of the solvent. We obtained the best results with squalene. As the above data reveal, we for the first time succeeded in integrating the functioning nAChR into an artificial lipid/polymer matrix. Setting up the lipid bilayer in the micropores of a polymer greatly improved the stability of the bilayer compared to the conventional black lipid membrane (BLM) technique. This substantial improvement provides the basis for technical applications of our artificial membrane system.

Conclusion

Reconstitution experiments of natural ion channel proteins into porous synthetic polymers were successfully performed. Artificial lipid bilayers were established inside the pores. We have shown that the important hydrophobic match between ion channel protein and lipid could be achieved. This opens up the possibility for the natural ion channel protein to be used in the future as a novel kind of biosensor. Model compounds of enhanced mechanical and

thermal stability and improved technical properties will have sufficient application potential in the future.

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