Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1757 (2006) 474–479

BIOCHIMICA ET BIOPHYSICA ACTA BBA

http://www.elsevier.com/locate/bba

A new automated technique for the reconstitution of hydrophobic proteins into planar bilayer membranes. Studies of human recombinant uncoupling protein 1

Valeri Beck [a], Martin Jabůrek [b], Eamon P. Breen [c], Richard K. Porter [c], Petr Ježek [b], Elena E. Pohl [a,*]

[a] *Institute of Cell Biology and Neurobiology, Charité Universitätsmedizin, Schumannstr. 20/21, 10098 Berlin, Germany*

[b] *Department of Membrane Transport Biophysics, Institute of Physiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic*

[c] *Department of Biochemistry, Trinity College Dublin, Ireland*

Received 15 December 2005; received in revised form 7 March 2006; accepted 7 March 2006

Available online 31 March 2006

Abstract

Electrophysiological characterisation of the vast number of annotated channel and transport proteins in the postgenomic era would be greatly facilitated by the introduction of rapid and robust methods for the functional incorporation of membrane proteins into defined lipid bilayers. Here, we describe an automated technique for reconstitution of membrane proteins into lipid bilayer membranes, which substantially reduces both the reconstitution time and the amount of protein required for the membrane formation. The method allows the investigation of single protein channels as well as insertion of multiple copies (~10^7) into a single bilayer. Despite a comparatively large membrane area (up to 300 μm diameter), the high stability of the membrane permits the application of transmembrane voltages up to 300 mV. This feature is especially important for studies of inner membrane mitochondrial proteins, since they act at potentials up to ~200 mV under physiological conditions. It is a combination of these advantages that enables the detailed investigation of the minuscule single protein conductances typical for proton transporters. We have applied the new technique for the reconstitution and electrophysiological characterisation of human recombinant uncoupling protein 1, hUCP1, that has been overexpressed in *E. coli* and purified from inclusion bodies. We demonstrate that hUCP1 activity in the presence of fatty acids is comparable to the activity of UCP1 isolated from brown adipose tissue.

Keywords: Gramicidin; Oleic acid; Arachidonic acid; Brown adipose tissue; Human recombinant protein; Artificial membranes

1. Introduction

The number of membrane proteins identified from genome sequencing and cDNA library searches now greatly exceeds the number of electrophysiologically characterised transport and channel proteins, and identification of their functions based on their transport properties remains a challenge for the future. For example, a range of mitochondrial uncoupling proteins (UCP2, UCP3, UCP4, UCP5) belonging to the uncoupling protein subfamily were discovered by aimed searching of cDNA library in the last decade [2,4,13,20,31]. Their molecular transport mechanisms and, consequently, exact functions are either unknown or controversially disputed until now.

Whereas the investigation of small water-soluble proteins exhibits no essential problems, handling of highly hydrophobic membrane proteins such as UCP still requires advances in methodological approach. The most successful technique for evaluating electrophysiological properties of protein channels, a patch clamp recording of the cell membrane [19], provides an excellent signal resolution, however, it is often difficult to assign the measured channels to specific proteins. Moreover, it is inapplicable for studies of ion channels located on intercellular membranes, such as endoplasmic or sarcoplasmic reticulum. Another specific problem appearing by studies of living systems is a lack of knowledge about exact membrane properties, for example, membrane potential, which seems to be of a great

Abbreviations: AA, arachidonic acid; BAT, brown adipose tissue; OA, oleic acid; UCP, uncoupling protein

* Corresponding author.

E-mail address: elena.pohl@charite.de (E.E. Pohl).

0005-2728/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2006.03.006

importance for UCP function. As reviewed in [16], there are many pitfalls in the determination of mitochondrial membrane potential in intact cells, and there is an apparent conflict of data between results obtained by monitoring mitochondrial membrane potential with fluorescent probes and monitoring respiratory parameters with an oxygen electrode.

Protein reconstitution into artificial lipid bilayers represents an alternative method allowing the biochemical analysis of defined protein preparations in a precisely known lipid and buffer environment. Since the first description of artificial planar lipid membrane formation [15], two main approaches allowing successful reconstitution of isolated and purified protein have been developed: a "monolayer spreading" method (i) described by Schindler et al. [26] and its modification for glass patch pipettes (ii) proposed by Wilmsen and collaborators [6]. Both methods use the finding that monolayers are formed spontaneously at the air–water interface of a proteoliposome suspension [17,24]. (i) By "monolayer spreading" method two monolayers can be combined into a protein-containing bilayer by raising the solution level on both sides of a septum that divides the chamber into two parts [25–27]. At present, this method seems to be the most frequently used approach for studies of purified proteins in artificial lipid bilayers [5,21–23,30]. Disadvantages of this technique include cleaning and preparation of the chamber, as well as hole punching. (ii) The second method implies the formation of planar lipid bilayer membranes at the end of glass patch clamp pipettes [6]. Better current resolution and simple handling have been claimed as advantages of this method. However, the use of this interesting approach appears to have declined in recent years. The most evident reasons are a high membrane fragility and difficulties in control for the membrane quality because membrane capacitance and, thus, membrane diameter cannot be measured accurately due to membrane movements inside the patch pipette. The small membrane diameter, leading to the small signal to noise ratio is a further disadvantage for measurements with membrane carriers. Both methods are rather time consuming and require a well-trained researcher.

It is worth to mention that an additional technique exists, by which proteins are introduced to bilayer membrane via a proteoliposome adsorption with a subsequent membrane fusion and is applicable for measurements of ion channels [12,14], having much greater transport rates than membrane transporters. Low amount of the proteins, which can be reconstituted by this technique makes a detailed investigation of minuscule single protein conductances of proton transporters difficult.

Here, we describe essential modifications of the Wilmsen's method, allowing the formation of large planar lipid bilayer membranes at the tip of a plastic pipette. The reconstitution of hydrophilic and hydrophobic proteins is demonstrated on the examples of gramicidin, and recombinant *E. coli*-expressed recombinant human uncoupling protein-1 (hUCP1). We show that hUCP1 mediates an increase of membrane conductance only in the presence of fatty acids and this increase is inhibited by purine nucleotides in a manner identical to the native UCP1 isolated from brown adipose tissue (BAT).

2. Experimental procedures

2.1. UCP1 expression, extraction, purification and reconstitution into liposomes

E. coli strain Rosetta pLysS (Novagen) was transformed with vectors containing cDNA coding for human UCP1 [3]. The hUCP1 expression, extraction from inclusion bodies and purification followed previously described protocol [9]. The hUCP1 was reconstituted into liposomes using previously described procedures [9–11,32], employing incubation of the protein/lipid mixture in 30 mM K-TES, 80 mM K_2SO_4, 2 mM EDTA, pH 7.2, with Bio-Beads SM-2 (Bio-Rad). To introduce fatty acids, proteoliposomes were mixed with FA-containing liposomes in required proportions. The resulting multilamellar vesicles, containing FA and UCP1, were than extruded through the filter of 400 nm pore diameter using a small-volume extrusion apparatus (Avestin Inc., Ottawa, Canada) [18].

2.2. Conductance measurements

Current–voltage (I–V) characteristics were measured by a patch-clamp amplifier (EPC 10, Heka Elektronik Dr. Schulze GmbH, Germany). For conductance measurements, a ramp voltage signal operating at frequencies of 0.016 Hz was used. Membrane conductance G was determined at zero voltage from a linear fit of voltages in the interval between −50 and 50 mV. For noise reduction, the Power-Line Conditioner (Warner Instruments Inc., USA) was used. The apparatus was fixed onto a vibration-free table (Newport Corporation, U.S.A.). Data acquisition and processing were performed by Software Pulse (v. 8.65, HEKA Elektronik Dr. Schulze GmbH, Germany).

3. Results

3.1. Formation of bilayer membranes from hUCP1-containing monolayers

Bilayer membranes containing UCP1 were formed on the tip of conventional dispensable plastic pipettes (Eppendorf epT.I.P. S. Geloader, Fisher Scientific, Germany) having a diameter 150–300 μm. An electrical scheme and a common view of the experimental set-up is shown in Fig. 1A, B, C.

At the beginning of the experiment, the pipette was filled with the buffer solution and attached to the pipette holder manually. The most critical step of the membrane formation is the relative position of the pipette tip to the water surface. For successful membrane formation, the angle between the bath and pipette surfaces was held above 90° (Fig. 1D, E). To ensure this, the pipette tip was flexed as shown in Fig. 1B, C. A small plastic dispensable container with a volume of 0.75 ml and surface diameter of 12 mm (Fig. 1B) was filled with the proteoliposome suspension, permanently stirred and maintained at 37 °C. The pipette tip was placed several millimeters under the air-buffer solution surface and a pipette elevator was started. The raising of the pipette from the bath eventually leads to the formation of the first monolayer. After achieving a fix point over the surface, the pipette was lowered slowly into the solution again. As the pipette tip passes through the lipid monolayer spread on the buffer solution surface, it becomes coated with a second monolayer of lipid molecules, thereby forming the lipid bilayer. In our setup tip, elevating and lowering was carried out automatically. Typically, the self-made device operated at a frequency of 0.5–1 Hz. It was controlled by a self-made electronic controller, which stopped the up and down movement after membrane

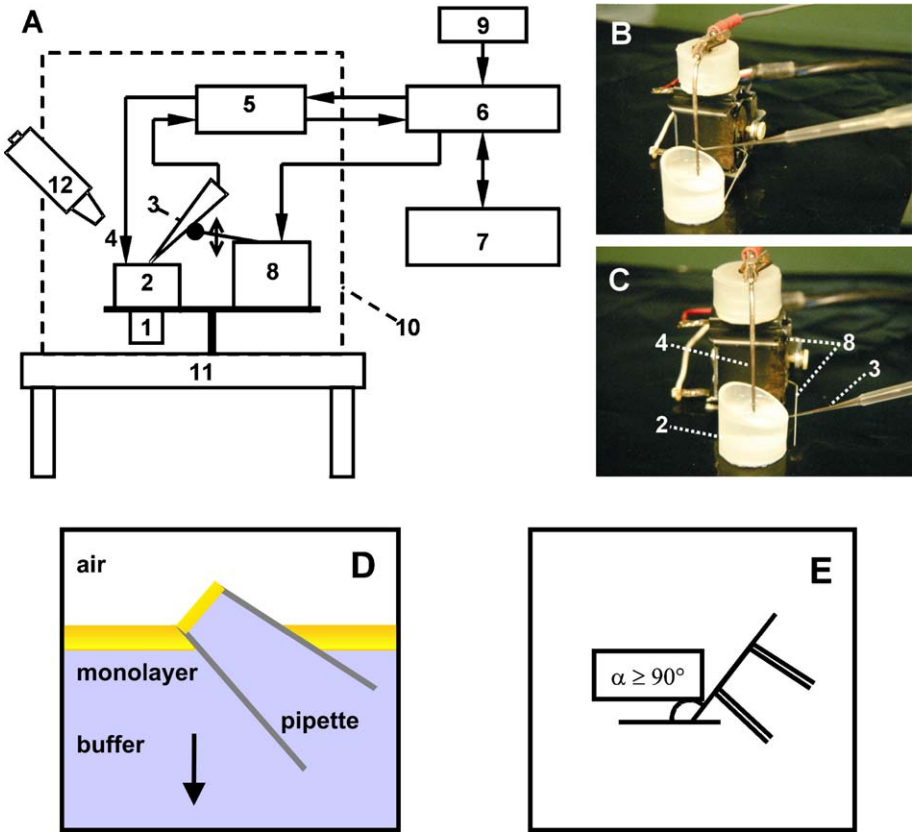

Fig. 1. Scheme (A) and common view (B, C) of the set-up. 1—stirrer, 2—thermostatic unit (not shown in B, C) with container, 3—plastic pipette, 4—reference electrode, 5—preamplifier, 6—amplifier with built-in AD converter, 7—PC, 8—pipette elevator, 9—power line conditioner, 10—Faraday cage, 11—vibration isolation table, 12—microscope or video camera. (D, E) Schemes, showing the lipid bilayer formation on the top of a plastic pipette. Arrow indicates the pipette movement direction through the monolayer. The pipette tip was flexed to hold the angle between the bath and pipette surfaces above 90° (E).

formation. Therefore, a feedback loop was installed to the analogue output of the current amplifier (Fig. 1A).

3.2. Verifying of the bilayer formation

3.2.1. Monitoring the membrane capacitance

To verify the formation of the bilayer, the capacitance of the membrane was permanently monitored (Fig. 2). For this goal, a triangle input wave with a peak to peak amplitude of 100 mV and a frequency of 5 Hz was applied to the input reference electrode after starting the pipette elevator device. A self-made electronic scheme, connected to the amplifier, was analyzing the output signal from the output reference electrode. A feedback signal was transmitted to the elevator device allowing a continuous cycle of pipette elevation followed by slow pipette lowering if bilayer formation was not indicated by capacitance measurements. In the case of successful membrane formation, the feedback signal stopped the elevation process. Specific capacitance, C_{spec}, was calculated with respect to membrane surface area without torus as determined using light microscope. It was equal to 0.95±0.05 μF/cm^2 for bilayers both with and without protein. The typical value of C_{spec}, determined based on the pipette diameter, was ca. 0.75 μF/cm^2. These values are similar to the values reported for decane-free membranes formed from proteoliposomes [28,30]. The measured capacitance remained constant during the experiment and served as a parameter for the membrane quality. In contrast, C_{spec} of decane containing membranes is between 0.4 and 0.5 μF/cm^2.

3.2.2. Reconstitution of the model peptide gramicidin

Gramicidin is known to form characteristic voltage-dependent multi-level pore fluctuations in bilayer membranes [1,7,8]. Because gramicidin exhibits these characteristic channel states

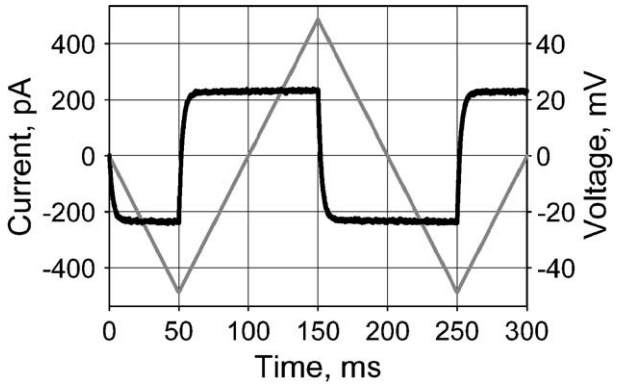

Fig. 2. Original recordings of membrane capacitance measurements. A triangle input wave (grey line) with a peak to peak amplitude of 100 mV and a frequency of 5 Hz was applied to the input reference electrode. Black curve indicates the capacitive current of the membrane.

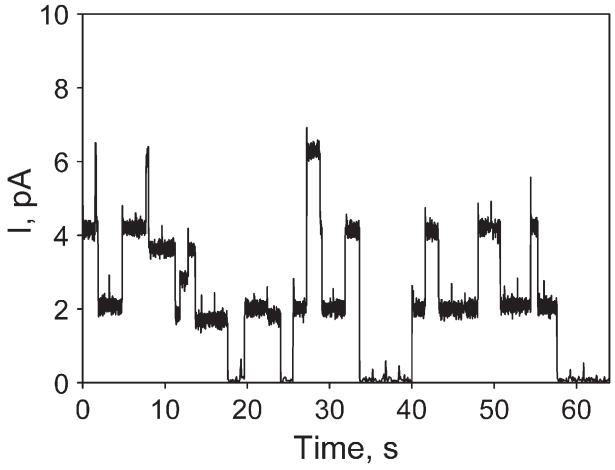

Fig. 3. Gramicidin pore fluctuation in bilayers made from E. coli lipid on the tip of the plastic pipette. Buffer solution contained 0.5 M KCl at pH 6.8. Gramicidin was added at a concentration of 5×10^{-7} mg/ml. The holding potential was 100 mV.

only in true bilayer membranes, their visualisation provides additional evidence that the pipette is sealed by a single lipid bilayer. In the described set-up (Fig. 1), the peptide gramicidin (Fluka Chemie GmbH, Buchs, Switzerland) was added from an ethanolic stock solution to the buffer solution. Fig. 3 illustrates the typical gramicidin channel activity measured in the described set-up. Current flow through the bilayer was monitored using the same amplifier (EPC 10, HEKA). The chamber was clamped at virtual ground while the pipette was clamped to the desired holding potential of 100 mV.

3.3. Conductance measurements of the membrane, containing recombinant human UCP1 in presence of long chain fatty acids

In the absence of fatty acids, the specific conductance G_0 of planar lipid membranes reconstituted with hamster UCP1 (BAT), recombinant hUCP1 and without protein was (19±6) nS/cm^2. This confirms our previous data that uncoupling protein is incapable of increasing conductance in sulphate medium in the absence of fatty acids. To be able to compare results obtained with UCP1 [30], we have first measured the activity of native UCP1 isolated from hamster BAT and reconstituted using a new approach. The introduction of the native hamster UCP1 from BAT, to the membrane, containing oleic acid in concentration 15 mol% led to the increase of the membrane conductance up to 89.5±10.5 nS/cm^2. This conductance was comparable to the values previously measured with monolayer elevation technique. Fig. 4 shows current–voltage relationships of recombinant hUCP1-containing membranes measured in the absence (white circles) and in the presence of oleic acid (OA, black circles), or in the presence of oleic acid and 2 mM ATP (grey circles). The inhibition by ATP demonstrates the sensitivity of the protein-mediated conductance to purine nucleotides. Using the advantages of the system described above, we have confirmed that the recombinant hUCP1 behaviour is qualitatively identical to the behaviour of native UCP1, purified from hamster BAT. Fig. 5 shows the original recordings and fitted curves of current–voltage dependence measured from the membranes, containing recombinant hUCP1 only (A) and membranes, containing recombinant hUCP1 and 10 mol% arachidonic acid (B). The application of

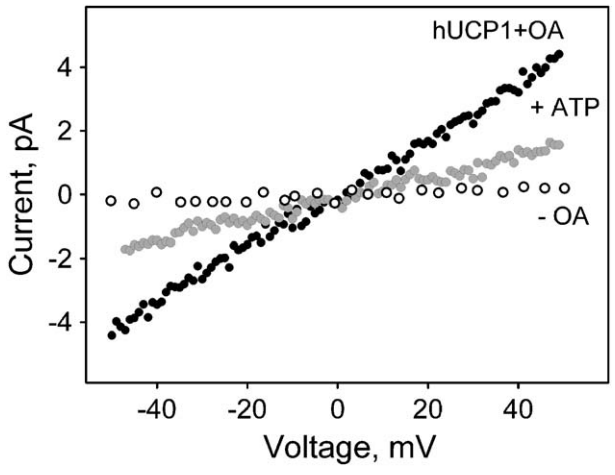

Fig. 4. Current–voltage relationships of hUCP1-containing membranes measured in the absence (white circles) and in the presence (black circles) of 20 mol% OA and after addition of 2 mM ATP (grey circle). Lipid content was 1.5 mg/ml, the content of hUCP1 ~0.7 μg/mg of lipid. The buffer solution contained 50 mM K_2SO_4, 25 mM TES, 0.6 mM EGTA at pH 7.35 and T=37 °C.

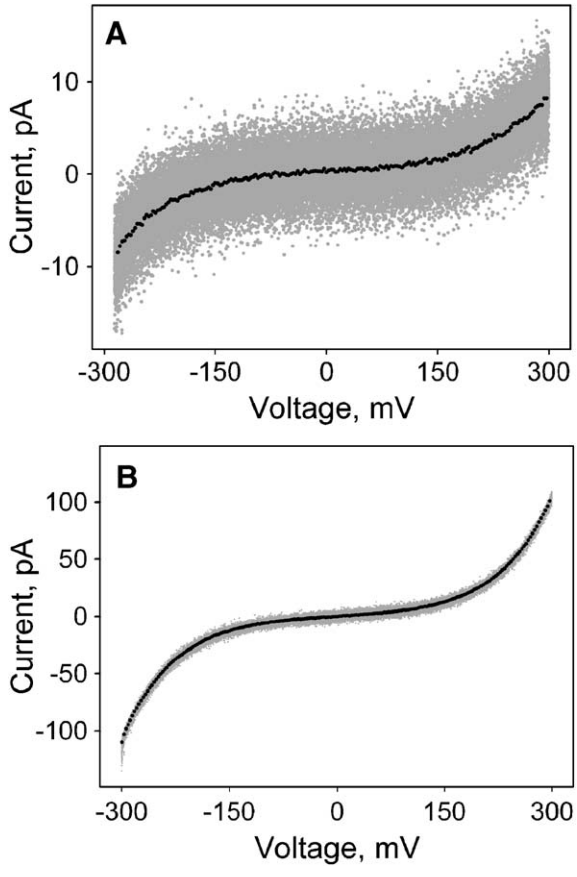

Fig. 5. Original recordings and averaged curves of current–voltage dependences measured from the membranes, containing UCP1 only (A) and membranes, containing UCP1 and 10 mol% arachidonic acid (B). Note that a voltage up to 300 mV was applied. The concentration of *E. coli* polar lipid extract was 1.5 mg/ml, the content of hUCP1 ~0.7 μg/mg of lipid. Buffer contained 50 mM K_2SO_4, 25 mM TES, 0.6 mM EGTA at pH 7.35.

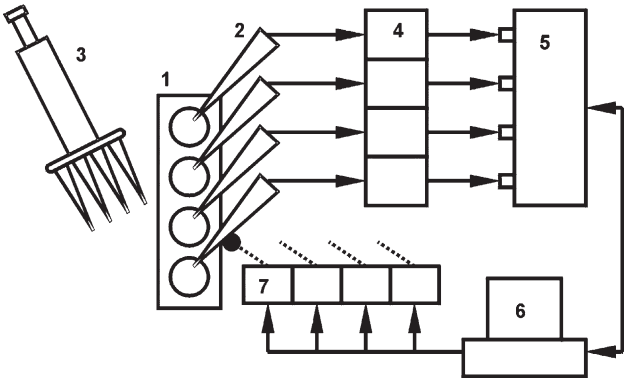

Fig. 6. Scheme of the multi-channel set-up. 1—thermostatic unit with 4 containers, 2—a set of plastic pipettes with electrodes, 3—multi-channel pipetors, 4—a set of amplifiers, 5—AD converter, 6—PC, 7—a set of pipette elevators.

voltages up to 300 mV reveals the high nonlinearity of the current at voltages exceeding ca. 120 mV.

4. Discussion

We have developed an automatic technique, which reduces substantially the efforts of any researcher and allows the usage of dispensable material for the reconstitution of membrane proteins in artificial membranes. In comparison to known reconstitution methods, the described system has several important advantages:

(i) Conventional plastic pipettes with a diameter 150–300 μm were used. Because such membranes are much larger than membranes formed on the tip of the glass patch pipette (ca. 0.5–5 μm), much more protein can be reconstituted. As a result the total signal is increased that enables the detailed investigation of the minuscule single protein conductances typical for all proton transporters in comparison with relative high transport rate of ion channels [29]. The latter can be studied in the membranes, where protein is reconstituted by fusion of proteoliposomes with planar bilayers [12,14]. Albeit the membrane surface is bigger, the amount of proteoliposomes is reduced because less buffer solution is required (50–100 μl). In contrast to the glass pipette method, the specific membrane capacitance, C_{spec}, can be measured exactly, because the diameter of the membrane on the pipette tip can be precisely measured by microscope. Thus, an important criterion for the verification of bilayer formation is fulfilled. A further advantage of the proposed set-up is worth mentioning. Conventional plastic pipettes and container are cheap dispensable objects and can be easily replaced. No additional expensive devices are necessary for pipette pulling.

(ii) The automation of the membrane formation process makes the method especially useful for researchers having little experience with the model membrane techniques. The automatic approach allows future extensions to simultaneous formation of several independent membranes, for example, in well plates (Fig. 6). Conceivably, it enables high throughput screening of ion channels and carriers.

(iii) The stability of the membranes made by this technique is significantly increased that makes it possible to apply voltages up to 300 mV. The latter is especially important for studies of mitochondrial uncoupling proteins, whose function or regulation may be dependent on the membrane potential. The present setup affords preconditions for the future study of voltage dependence of their nucleotide gating mechanism.

Acknowledgments

The financial support of the Deutsche Forschungsgemeinschaft (Po-524/2-2, 436 TSE 113/44/0-1) to EEP and of the Grant Agency of the Academy of Sciences of the Czech Republic (No. A5011106 and 1P05ME794 (AV0Z50110509)) to PJ is greatly acknowledged. We thank Kimberly Rosegger and Gregory Wulczyn for editorial assistance.

References

[1] E. Bamberg, P. Lauger, Channel formation kinetics of gramicidin A in lipid bilayer membranes, J. Membr. Biol. 11 (1973) 177.
[2] O. Boss, S. Samec, A. Paoloni-Giacobino, C. Rossier, A. Dulloo, J. Seydoux, P. Muzzin, J.P. Giacobino, Uncoupling protein-3: a new member of the mitochondrial carrier family with tissue-specific expression, FEBS Lett. 408 (1997) 39.
[3] E.P. Breen, S.G. Gouin, A.F. Murphy, L.R. Haines, A.M. Jackson, T.W. Pearson, P.V. Murphy, R.K. Porter, On the mechanism of mitochondrial uncoupling protein 1 function, J. Biol. Chem. 280 (2005) 15534.
[4] C. Fleury, M. Neverova, S. Collins, S. Raimbault, O. Champigny, C. Levi-Meyrueis, F. Bouillaud, M.F. Seldin, R.S. Surwit, D. Ricquier, C.H. Warden, Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia, Nat. Genet. 15 (1997) 269.
[5] T. Gutsmann, J.W. Larrick, U. Seydel, A. Wiese, Molecular mechanisms of interaction of rabbit CAP18 with outer membranes of gram-negative bacteria, Biochemistry 38 (1999) 13643.
[6] W. Hanke, C. Methfessel, H.U. Wilmsen, E. Katz, G. Jung, G. Boheim, Melittin and a chemically modified trichotoxin form alamethicin-type multi-state pores, Biochim. Biophys. Acta 727 (1983) 108.
[7] S.B. Hladky, D.A. Haydon, Discreteness of conductance change in bimolecular lipid membranes in the presence of certain antibiotics, Nature 5231 (1970) 451.
[8] S.B. Hladky, D.A. Haydon, Ion transfer across lipid membranes in the presence of gramicidin A. I. Studies of the unit conductance channel, Biochim. Biophys. Acta 274 (1972) 294.
[9] M. Jabůrek, K.D. Garlid, Reconstitution of recombinant uncoupling proteins: UCP1, -2, and -3 have similar affinities for ATP and are unaffected by coenzyme Q10, J. Biol. Chem. 278 (2003) 25825.
[10] M. Jabůrek, M. Varecha, R.E. Gimeno, M. Dembski, P. Ježek, M. Zhang, P. Burn, L.A. Tartaglia, K.D. Garlid, Transport function and regulation of mitochondrial uncoupling proteins 2 and 3, J. Biol. Chem. 274 (1999) 26003.
[11] M. Jabůrek, M. Varecha, P. Ježek, K.D. Garlid, Alkylsulfonates as probes of uncoupling protein transport mechanism. Ion pair transport demonstrates that direct H(+) translocation by UCP1 is not necessary for uncoupling, J. Biol. Chem. 276 (2001) 31897.
[12] Y. Jiang, A. Lee, J. Chen, V. Ruta, M. Cadene, B.T. Chait, R. MacKinnon, X-ray structure of a voltage-dependent K+ channel, Nature 423 (2003) 33.
[13] W. Mao, X.X. Yu, A. Zhong, W. Li, J. Brush, S.W. Sherwood, S.H. Adams, G. Pan, UCP4, a novel brain-specific mitochondrial protein that reduces membrane potential in mammalian cells, FEBS Lett. 443 (1999) 326.
[14] J.H. Morais-Cabral, Y. Zhou, R. MacKinnon, Energetic optimization of ion conduction rate by the K+ selectivity filter, Nature 414 (2001) 37.

[15] P. Mueller, D.O. Rudin, H.T. Tien, W.C. Wescott, Methods for the formation of single bimolecular lipid membranes in aqueous solution. J. Phys. Chem. 67 (1963) 534.
[16] D.G. Nicholls, M.W. Ward, Mitochondrial membrane potential and neuronal glutamate excitotoxicity: mortality and millivolts, Trends Neurosci. 23 (2000) 166.
[17] F. Pattus, P. Desnuelle, R. Verger, Spreading of liposomes at the air/water interface, Biochim. Biophys. Acta 507 (1978) 62.
[18] E.E. Pohl, U. Peterson, J. Sun, P. Pohl, Changes of intrinsic membrane potentials induced by flip-flop of long-chain fatty acids, Biochemistry 39 (2000) 1834.
[19] B. Sakmann, E. Neher, Patch clamp techniques for studying ionic channels in excitable membranes, Annu. Rev. Physiol. 46 (1984) 455.
[20] D. Sanchis, C. Fleury, N. Chomiki, M. Goubern, Q. Huang, M. Neverova, F. Gregoire, J. Easlick, S. Raimbault, C. Levi-Meyrueis, B. Miroux, S. Collins, M. Seldin, D. Richard, C. Warden, F. Bouillaud, D. Ricquier, BMCP1, a novel mitochondrial carrier with high expression in the central nervous system of humans and rodents, and respiration uncoupling activity in recombinant yeast, J. Biol. Chem. 273 (1998) 34611.
[21] S.M. Saparov, M. Borgnia, P. Agre, P. Pohl, Properties of *Escherichia coli* water channel (AQPZ) reconstituted into planar lipid bilayers, Biophys. J. 78 (2000) 389A.
[22] S.M. Saparov, D. Kozono, U. Rothe, P. Agre, P. Pohl, Water and ion permeation of aquaporin-1 in planar lipid bilayers. Major differences in structural determinants and stoichiometry, J. Biol. Chem. 276 (2001) 31515.
[23] S.M. Saparov, P. Pohl, Beyond the diffusion limit: water flow through the empty bacterial potassium channel, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 4805.
[24] H. Schindler, Autocatalytic transport of the peptide antibiotics suzukacillin and alamethicin across lipid membranes, FEBS Lett. 104 (1979) 157.
[25] H. Schindler, Formation of planar bilayers from artificial or native membrane vesicles, FEBS Lett. 122 (1980) 77.
[26] H. Schindler, Planar lipid–protein membranes: strategies of formation and of detecting dependencies of ion transport functions on membrane conditions, Methods Enzymol. 171 (1989) 225.
[27] H. Schindler, U. Quast, Functional acetylcholine receptor from *Torpedo marmorata* in planar membranes, Proc. Natl. Acad. Sci. U. S. A. 77 (1980) 3052.
[28] H. Schindler, J.P. Rosenbusch, Matrix protein from *Escherichia coli* outer membranes forms voltage-controlled channels in lipid bilayers, Proc. Natl. Acad. Sci. U. S. A. 75 (1978) 3751.
[29] F.J. Sigworth, K.G. Klemic, Microchip technology in ion-channel research, IEEE Trans. Nanobiosci. 4 (2005) 121.
[30] E. Urbankova, A. Voltchenko, P. Pohl, P. Ježek, E.E. Pohl, Transport kinetics of uncoupling proteins:Analysis of UCP1 reconstituted in planar lipid bilayers, J. Biol. Chem. 278 (2003) 32497.
[31] X.X. Yu, W. Mao, A. Zhong, P. Schow, J. Brush, S.W. Sherwood, S.H. Adams, G. Pan, Characterization of novel UCP5/BMCP1 isoforms and differential regulation of UCP4 and UCP5 expression through dietary or temperature manipulation, FASEB J. 14 (2000) 1611.
[32] M. Zackova, P. Ježek, Reconstitution of novel mitochondrial uncoupling proteins UCP2 and UCP3, Biosci. Rep. 22 (2002) 33.