Volume 224, number 1, 172–176 FEB 05296 November 1987

A membrane fusion strategy for single-channel recordings of membranes usually non-accessible to patch-clamp pipette electrodes

Manuel Criado and Bernhard U. Keller

Max-Planck-Institut für biophysikalische Chemie, Abteilung Membranbiophysik, Postfach 2841, D-3400 Göttingen, FRG

Received 10 September 1987

Membranes of cellular organelles and plasma membranes of some type of cells are not accessible to the high-resolution recordings that the conventional patch-clamp technique allows. However, when these purified membranes are dehydrated together with small lipid vesicles and hydrated again, cell-size vesicles (5–100 μm diameter) are obtained, on which single-channel recordings are possible. This approach, which has been proven successful with about ten different membrane preparations of varied origin, is further illustrated with two examples. First, a known conductivity of the sarcoplasmic reticulum membrane is compared with data obtained by using other techniques. Second, a new sodium current, present at purified postsynaptic membranes from the *Torpedo* electric organ, is described.

Organelle membrane; Membrane fusion; Hydration; Patch-clamp; Ion channel

1. INTRODUCTION

Ionic channels are chemically or electrically activated macromolecules which form pores in cell membranes. They are involved in the excitation and electrical signalling in and between cells, by controlling the downhill flux of ions through cellular membranes. Although the ionic permeability of membranes has been investigated by biochemical methods, electrophysiological techniques and especially patch-clamp recording [1] constitute far more precise approaches to the study of ion channels. Thus, a wide variety of channels from the plasma membrane of many different cell types has been studied [2]. However, other ion channels from cells which cannot be patch-clamped as well as those from cellular organelles remain to be characterized. To overcome this problem we report here the fusion of membranes from different sources with small lipid vesicles to yield cell-size structures on which patch-clamp studies can be performed. All of a variety of membranes tested until now have the mechanical and electrical properties needed for the successful characterization of their ion channel proteins.

Correspondence address: M. Criado, Max-Planck-Institut für biophysikalische Chemie, Abteilung Membranbiophysik, Postfach 2841, D-3400 Göttingen, FRG

Abbreviations: FITC, fluorescein-5 isothiocyanate; TMRITC, tetramethylrhodamine-5 (and -6) isothiocyanate; AChR, acetylcholine receptor; SR, sarcoplasmic reticulum

2. EXPERIMENTAL

SR membranes (light, medium and heavy fractions) from rabbit skeletal muscle were prepared as in [3]. AChR-rich membranes from *Torpedo marmorata* electric organ were obtained as in [4]. A lysed crude synaptosomal membrane preparation was obtained from rat brain as described [5]. All membrane preparations were stored at −80°C un-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/87/$3.50

til use. Small lipid vesicles were prepared by dialysis of a solution of crude L-α-lecithin (from soybean, type II-S, Sigma) (10 mg/ml) in 1% Chaps, 100 mM NaCl, 20 mM Mops, pH 7.4. A stock solution of lecithin (100 mg/ml) was previously suspended in water using a Branson sonifier at 40 W for 5 min. After 24 and 48 h of dialysis against 500 vols of 100 mM NaCl, 20 mM Tris-Cl, pH 7.4 (dialysis buffer), the resulting liposomes were stored at $-80°C$ until use.

Membrane fusion was performed by modification of a method developed by us (Keller, B.V. et al., submitted) to obtain giant liposomes. In a standard fusion experiment purified membranes (150 μg protein) were mixed with small lipid vesicles (1.8–2.3 μmol inorganic phosphorus as determined in [6]) and centrifuged for 40 min at maximal speed in an Airfuge. The pellet was resuspended in 5 μl of 10 mM Mops buffer, pH 7.4, plus 10 μl of the same buffer containing 10% ethylene glycol, and the suspension deposited on a glass slide to form a circle of about 8 mm diameter. The membrane containing drop was dehydrated at 4°C in a desiccator with $CaCl_2$ granules for 3 h. The partially dehydrated film was rehydrated by covering it with 20 μl of 100 mM KCl (or other ions depending on the measurements to be performed later) and incubating the slide at 4°C in a closed Petri dish containing a wet filter paper pad. After 3 h, cell-size vesicles could be observed, although we typically performed patch-clamp measurements after overnight hydration. To do this 1–3 μl fused vesicles (more abundant at the edges of the hydrated film) were diluted in 300 μl filtered buffer solution (usually 50 mM KCl, 0.1 mM $CaCl_2$, 5 mM Hepes/KOH, pH 7.2) and vesicles allowed to settle on the glass surface of a small chamber. After 5–10 min the recording chamber was intensely flushed to remove vesicles not sticking to the glass surface. Single-channel recordings were carried out at room temperature using standard patch-clamp methods [1].

Membranes were fluorescence labelled by resuspending them (2 mg protein/ml) in 44 mM bicarbonate buffer, pH 8.9, containing FITC (0.25 mg/ml) or TMRITC (0.12 mg/ml). After 30 min incubation at room temperature, the membranes were sedimented in the Airfuge at maximal speed for 5 min and washed with dialysis buffer three times. Labelling was analyzed by electrophoresis [7] and membranes were fused by the standard procedure.

3. RESULTS AND DISCUSSION

A technique recently developed by us to obtain large liposomes was used in preliminary attempts to fuse native membranes which could be patch-clamped. However, the resulting material was amorphous in appearance or contained vesicles too small for our purposes. This might be due to the higher protein content of the native vesicles as compared with reconstituted liposomes. Therefore, posterior experiments were carried out by adding some exogenous liposomes to the original membranes. This strategy resulted in formation of vesicles with sizes ranging between 5 and 100 μm. Fluorescence labelling experiments, to be described below, indicated the presence of multi- and unilamellar vesicles. The latter were normally less stable during patch-clamp experiments. The protein and lipid content of the vesicles was further analyzed as a function of the amount of native membranes available to fuse with a fixed amount of exogenous liposomes. As shown in table 1, the lipid-to-protein ratio of the fused vesicles was about 2–3-times higher than that expected if all the lipid and protein available for fusion combined to form vesicles. This suggests that part of the protein is not integrated into the large vesicles. However, the more membrane protein is available to fuse, the higher is the protein content of the resulting vesicles. Therefore, by varying protein input for fusion it is possible to manipulate the final protein density of the vesicles and, consequently, as confirmed by our observations, the channel density in each pipette patch.

Membrane fusion was further visualized by using fluorescence labelled membranes. At the same time we tested the interesting possibility of integrating components of two different types of membranes into the same vesicle, by fusing them together. As shown in fig.1A, all protein components (and probably some lipids as well) of synaptosomal membranes from rat brain and AChR-rich membranes from *Torpedo* electroplax were labelled fluorescently before fusion. The homogeneous fluorescence due to both labels which can be observed in the fused vesicle of fig.1C,D suggests that components of the two

Table 1

Protein and lipid content of fused vesicles as a function of the amount of membranes available for fusion

Membranes used for fusion		Exogenous lipid added for each fusion (μg)	Lipid/protein ratio (w/w)		
Protein (μg)	Phospholipid (μg)		In original membranes	In fused vesicles	
				Theoretical	Determined
150	141	1798	0.94	13	39
300	282	1798	0.94	6.9	21
600	564	1798	0.94	3.9	8.6

The experiment was carried out with synaptosomal membranes from rat brain and similar results were obtained with different membrane types. Vesicles were recovered after fusion by collecting the liquid used for hydration but trying not to touch the lipid film, as done for a patch-clamp experiment. Protein [11] and lipid [6] contents were determined in this vesicle fraction. A molecular mass of 800 Da was assumed for each phospholipid molecule in order to express the amount of phospholipid in μg

types of membranes were integrated into the same vesicle. None of the hundreds of vesicles observed showed a particular preference for a determined type of fluorescence label and all vesicles were homogeneously fluorescent. It can be concluded that this method can be used to integrate proteins of different membrane origin in the same membrane environment.

The formation of large vesicles from isolated membranes of cellular organelles allows the characterization of single ion channels which previously could not be studied by conventional patch-clamp measurements. Previous approaches to investigate organelle ion channels were based on channel reconstitution in artificial membranes [8]. In fact, one of the most prominent features of the present approach, together with its simplicity, is that ion channels are characterized in a close to natural lipid environment and no detergent solubilization is required. In addition, patch-clamp studies of vesicles provide a higher resolution than that obtained with giant bilayers.

Fig.2A displays single-channel currents through isolated SR membranes in a symmetrical solution of 50 mM KCl, 0.1 mM $CaCl_2$, 5 mM Hepes, pH 7.2. Step-like current fluctuations at different membrane potentials correspond to openings and closings of a single K^+-selective channel in the SR membrane. The K^+ channel is voltage-dependent with negative pipette potentials V favouring channel-closing events. Three different K^+-current levels are displayed in fig.2B. The absence of dou-

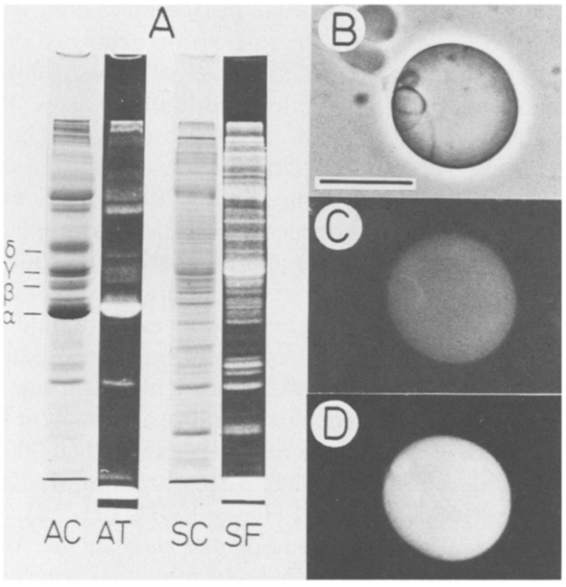

Fig.1. Fusion of membranes of different origin. (A) Electrophoretic analysis of polypeptides from AChR-rich membranes (AC, AT) and rat brain synaptosomal membranes (SC, SF). FITC (SF) and TMRITC (AT) labelling was visualized by UV transillumination and proteins by Coomassie blue staining (AC, SC). The four subunits of the AChR with approximated molecular masses of 40, 50, 60 and 66 kDa are indicated at the left of AC. (B–D) Typical cell-size vesicle prepared with membranes fluorescently labelled as shown in (A) and observed with (B) phase-contrast, (C) FITC equipment and (D) TMRITC equipment (bar, 25 μm).

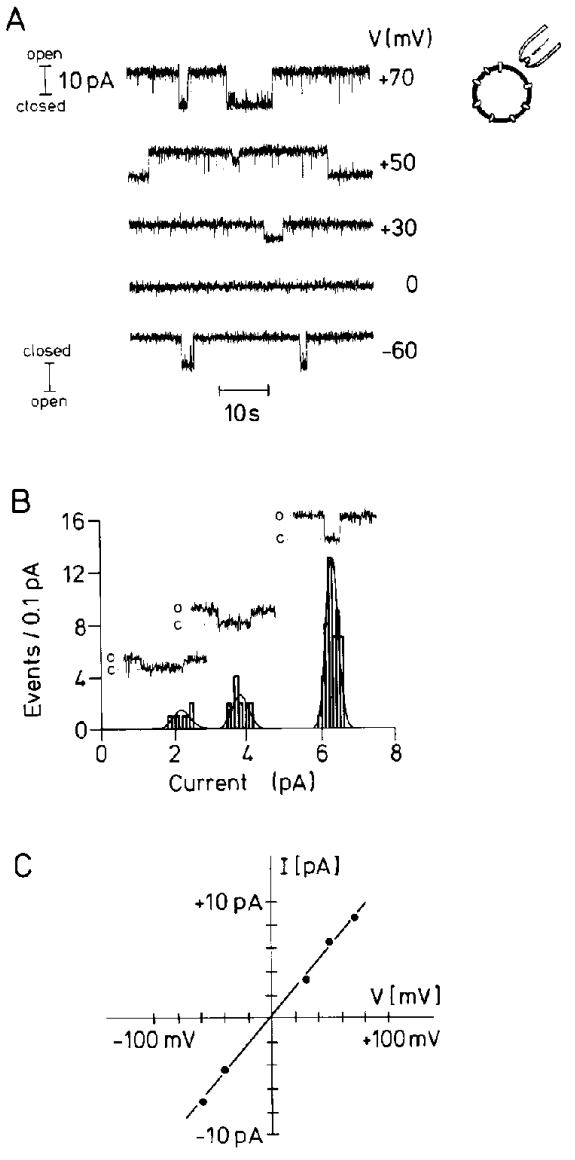

Fig.2. (A) Single-channel records of SR K^+ channels. Currents were measured in the excised patch configuration under voltage-clamp conditions. Single-channel records were filtered at 2 kHz, digitized at a sampling interval of 200 μs and plotted by using the program TAC [1]. (B) Three different levels of membrane current at $V = 50$ mV with the corresponding amplitude histogram. Solid lines are least-squares fits of Gaussian distributions with SD = 0.2 pA. (C) Single-channel current-voltage curve in a symmetrical solution of 50 mM KCl, 0.1 mM $CaCl_2$ and 5 mM Hepes/KOH, pH 7.2.

ble openings suggests that the small current steps represent partial closures of a single K^+ channel. At $V = 50$ mV, the main conductance step corresponds to 6.5 pA (fig.2B) and the distributions of two sublevels are centered at 2.1 and 3.9 pA, respectively. For the main level the single-channel current-voltage curve is linear over the voltage range -60 to $+80$ mV with a mean conductance of 126 ± 11 pS ($n = 5$) in 50 mM KCl (fig.2C). The selectivity of the channel for monovalent cations is characterized by the sequence $K^+ > Na^+ > Rb^+$. Moreover, channels show a voltage-dependent block by decamethonium at a concentration of 0.5–1 mM. These characteristic features of the K^+ channel in a close to natural lipid environment can be compared with previous results in bilayers [9].

As indicated above, another important application of the new approach reported here is the

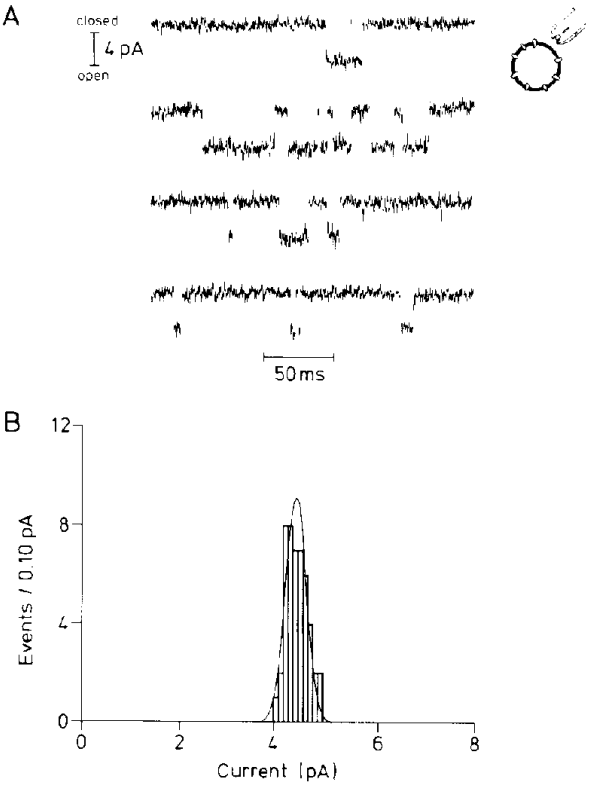

Fig.3. (A) Na^+ conductance in postsynaptic membranes of *T. marmorata*. Ionic current was carried by 50 mM NaCl, 0.1 mM $CaCl_2$ and 5 mM Hepes, pH 7.4, in the excised patch configuration. Pipette potential, $V = -40$ mV. (B) Corresponding amplitude histogram.

characterization of single channels in plasma membranes of cells not accessible to conventional patch-clamp measurements. To demonstrate this, we isolated AChR-enriched postsynaptic membranes from the electric organ of *T. marmorata* and performed fusion experiments. Patch clamp recordings of *Torpedo* membranes revealed a large Na^+-conducting channel which has not been previously reported. Fig.3A displays single-channel recordings at an applied pipette voltage of $V = -40$ mV. More negative pipette potentials prolonged channel-opening events leading to an increased opening probability. The single-channel conductance is 107 ± 9 pS ($n = 4$) in a buffer solution containing 50 mM NaCl. The reversal potential in a 5-fold NaCl gradient was found to be 36.8 mV, which is close to the reversal potential for Na (40.6 mV at 20°C). In addition to this Na^+-permeable channel, other channels typical of these membranes, such as Cl^- channels and AChR channels [10], were observed.

Finally, and in order to illustrate further the general applicability of the fusion approach described, it is important to mention additional membrane preparations which have been found to yield vesicles capable of being patch-clamped and contain several classes of functional channels: SR (heavy, intermediate and light fractions), synaptosomal membranes from rat brain, vacuolar and chloroplast membranes from plant cells (in collaboration with Hedrich, R., Göttingen), different microsomal fractions from rat hepatocytes (in collaboration with Kleineke, J., Göttingen), photoreceptor rod outer segments disks and *Chlamydomonas* eye spot membrane preparations (in collaboration with the group of Uhl, R., from this institute), and synaptic vesicle membranes (in collaboration with Stadler, H., from this institute).

ACKNOWLEDGEMENTS

We thank Dr E. Neher for his continuous support and Dr W. Vaz for his help with the fluorescence micrographs. B.U.K. was supported by a stipend from the Max-Planck-Gesellschaft.

REFERENCES

[1] Sakmann, B. and Neher, E. (1983) Single Channel Recording, Plenum, New York.
[2] Hille, B. (1984) Ionic Channels of Excitable Membranes, Sinauer, Sunderland, MA.
[3] Fernandez, J.L., Rosenblatt, M. and Hidalgo, C. (1980) Biochim. Biophys. Acta 599, 552–568.
[4] Barrantes, F.J. (1982) in: Neuroreceptors (Hucho, F. ed.) pp.315–328, De Gruyter, Berlin.
[5] Catterall, W.A., Morrow, C.S. and Hartshorne, R.P. (1979) J. Biol. Chem. 254, 11379–11387.
[6] Ames, B.N. and Dubin, D.T. (1960) J. Biol. Chem. 235, 770–778.
[7] Laemmli, U.K. (1970) Nature 227, 680–685.
[8] Miller, C. (1986) Ion Channel Reconstitution, Plenum, New York.
[9] Labarca, P. and Miller, C. (1981) J. Membrane Biol. 61, 31–38.
[10] Tank, D.W. and Miller, C. (1983) in: Single Channel Recording (Sakmann, B. and Neher, E. eds) pp.91–105, Plenum, New York.
[11] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.