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Bioelectrochemistry 57 (2002) 97–106

Bioelectrochemistry

www.elsevier.com/locate/bioelechem

Mitochondrial porin incorporation into black lipid membranes: ionic and gating contribution to the total current

Silvia Micelli [a,*], Enrico Gallucci [a], Daniela Meleleo [a], Valentina Stipani [a], Vittorio Picciarelli [b]

[a] *Dipartimento Farmaco-Biologico, Facoltà di Farmacia, Università degli Studi di Bari, v.E. Orabona 4, 70125, Bari, Italy*
[b] *Dipartimento Interateneo di Fisica, Università degli Studi di Bari, 70125 Bari, Italy*

Received 12 September 2001; received in revised form 17 December 2001; accepted 21 December 2001

Abstract

We present a new ac device useful for simultaneous measurements of ionic charge movement (conductance) and gating charge displacement (capacitance) in mitochondrial porin channels incorporated in two kinds of black lipid membranes (BLMs), made up of phosphatidylinositol (charged surface) and oxidized cholesterol (neutral surface). In particular, we investigated the conductance/capacitance variations during the process of porin incorporation (VDAC) at different porin concentrations. While conductance variations are present throughout the porin concentration range investigated, a threshold value seems to be necessary in order to detect a significant capacitance variation. A clear steady state in both conductance and capacitance is reached for the phosphatidylinositol bilayer, while for the oxidized cholesterol membranes, the steady state is reached only for the conductance. The dependence of capacitance characteristics on the membrane applied voltage V_m is investigated before porin incorporation and at the ionic current steady state. The results obtained confirm that before porin incorporation, there is a small dependence on V_m^2, while afterwards we find evidence of a dual exponential voltage dependence (a result similar to that found for conductance). Finally, we investigated the capacitance dependence on the radius of the hole separating the two compartments of the cell used in the measurements. In this study, performed only with oxidized cholesterol, the radius was varied from 200 to 1050 μm. We observed a significant variation in the specific capacitance in particular for smaller radii. The results were interpreted by a simple geometrical model taking into account the influence of the torus.

Keywords: Capacitance; Phosphatidylinositol; Oxidized cholesterol; Gating mechanism

1. Introduction

As is well known, black lipid membranes (BLM) used as model membranes can be characterized by two electrical parameters: conductance and capacitance. The first is related to the ionic current through the bilayer, while the second has been associated to the gating current due to charge rearrangements in the membrane when voltage-dependent ion channels have been incorporated [1,2]. While conductance, especially when related to incorporation of different proteins, has been widely investigated due to its evident relation to channel formation, there is much less data on capacitance, even though this would provide important new information on functional properties at a molecular level.

Capacitance measurements, based on the discharge through a known resistor, have been mainly limited to black lipid membranes and used to gather information on thickness, dielectric properties, capacitance voltage dependence and capacitance dependence on the radius of the BLM [3–17]. In the latter case, a simple model, based on the two contributions from the bilayer in the center of the aperture and the surrounding torus, has been proposed to interpret the data [17].

New techniques for capacitance measurements have been developed and used to collect data on the capacitance variation during membrane bilayer formation [18].

Interest in the simultaneous measurements of conductance/capacitance has been growing recently and data have been collected on various characteristics of gating mechanism as a function of the applied voltage in the auditory cells [19], of the Shaker K^+ channel in *Xenopus oocytes*

Abbreviations: BLM, black lipid membrane; VDAC, voltage-dependent anion-selective channel; ac, alternating current; PI, phosphatidylinositol; OxCh, oxidized cholesterol.

* Corresponding author. Tel.: +39-80-5442-775; fax: +39-80-5442-796.
E-mail address: micelli@farmbiol.uniba.it (S. Micelli).

1567-5394/02/$ - see front matter
PII: S1567-5394(02)00003-8

[1,2], of the onset of Alzheimer's disease's amyloid β-peptides [20] and to investigate vesicle fusion events occurring during exocytosis [21].

Chanturiya [22], and Chanturiya and Nikoloshina [23] separate the capacitive and the reactive component of the current by using a compensating capacitor. Such an approach was used for investigating the correlation between change in the membrane capacitance induced by changes in ionic environment and the conductance of channels incorporated into the membranes due to toxins.

As far as we know, no simultaneous measurements exist of the capacitive and reactive components of the total current during protein incorporation.

In the current study, we briefly discuss a new method for simultaneously measuring the conductance/capacitance using an ac device and we report on the results obtained in the following investigations:

(1) the conductance/capacitance variations during mitochondrial porin (VDAC) incorporation and the capacitance dependence on porin concentrations. More data on the conductance characteristics have been previously published [24];

(2) the capacitance dependence on membrane applied voltage under steady state conditions for the total current;

(3) the dependence of the capacitance, before and after VDAC incorporation, on the radius of the hole separating the cell used for the measurements. Such a study could shed some light on the role played by the torus in the bilayer's capacitive characteristics and may add more information to the result of our previous investigations into conductance. In fact, we have demonstrated that the torus acts as a sort of reservoir, which is important to reach the steady state conductance [24].

The first two investigations were performed with two kinds of BLM, namely phosphatidylinositol (PI) and oxidized cholesterol (OxCh), which were chosen for their different physico-chemical characteristics: (i) OxCh is hydrophobic and has a rigid structure with a small neutral polar head, while PI has a negative polar head; (ii) moreover, from the functional point of view, it must be considered that PI is present in the outer mitochondrial membrane [25] but at low concentrations, while sterols are fundamental components with which porin is associated [26–28] and are basically suitable for the proper folding of the mitochondrial

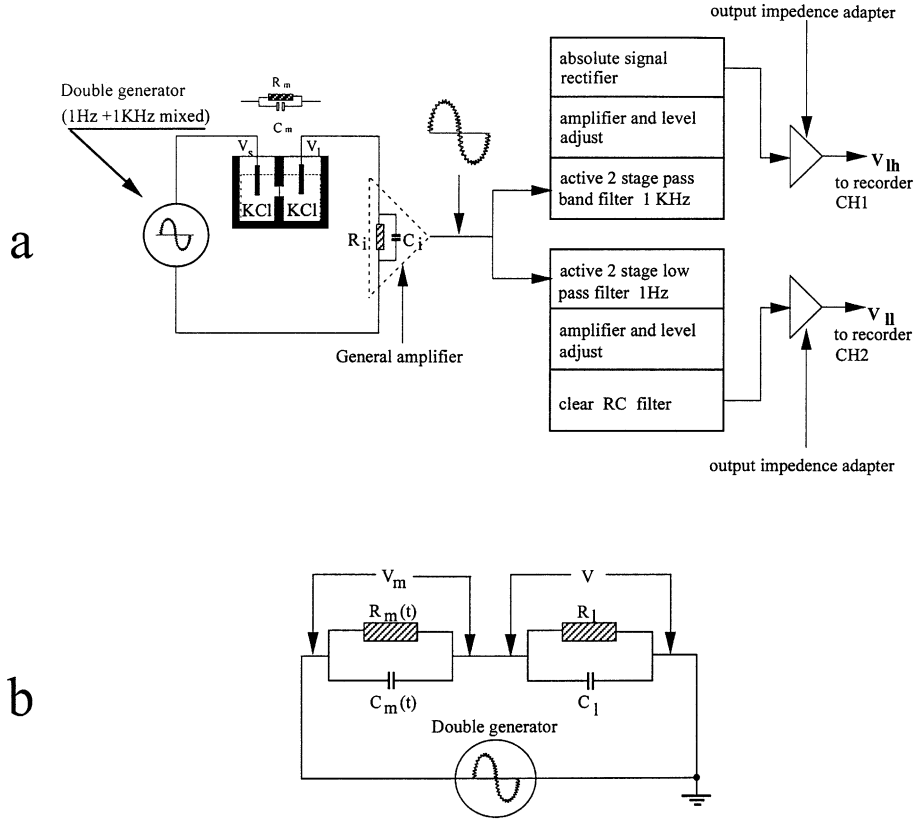

Fig. 1. Layout of the set-up used for the measurements. (a) A signal generator mixing 1 Hz of variable V_s amplitude and 1 kHz of 2-mV signals applies the voltage to a Pt electrode situated on one side of the cell. The output voltage, acquired through a second Pt electrode, was electronically amplified and filtered in order to separate the two frequency components V_{ll} (1 Hz) and V_{lh} (1 kHz). These quantities are stored in an on-line computer and used for further analysis. (b) The electrical circuit representing the membrane components (R_m, C_m) in series with the (R_l, C_l) amplifier elements, V_l (V_m) represents the amplified output voltage (membrane voltage). A full discussion of the reasons for justifying the absence of the electrical contribution of aqueous phase is reported in Ref. [31].

porin [28]. The third investigation was performed only with the OxCh bilayers.

2. Experimental procedures

2.1. Lipids and other chemicals

PI was extracted from ox brain according to Folk's method [29] and the purity was chromatographically checked using TLC with chloroform/methanol/acetic acid/water (100:50:16:2) as the solvent system. OxCh was obtained following the method used by Tien et al. [5]. Porin purified from bovine heart mitochondria [30] was kindly furnished by Prof. Ferdinando Palmieri (Dipartimento Farmaco-Biologico, University of Bari, Italy). *n*-decane and octane were from Fluka (Puriss, Buchs, Switzerland), squalene and *n*-pentane (Sigma). KCl, chloroform, methanol, acetic acid (RPE) from Carlo Erba (Milano, Italy). TLC from Merck, (Kielsegel 60 Darmstadt). Water was double-distilled in a commercial apparatus.

2.2. Preparation of thin lipid bilayers and electrical measurements

The thin lipid bilayers in *n*-decane were made up as described in our previous paper [31]; the solvent-free bilayers were formed as described by White [32]. A dual-compartment Teflon cell similar to that described by Läuger et al. [8], was filled with a KCl (1 M) solution and mechanically stirred. The bilayer membrane was formed across a circular aperture separating the two compartments.

A generator mixing 1 Hz of variable amplitude (V_s) and 1 kHz of 2-mV signals applied the voltage to a Pt electrode situated on one side of the cell. The output voltage, acquired through a second Pt electrode, was electronically amplified and filtered in order to separate the two frequency components. The data were collected via PC computer interfaced with two voltage-frequency converters and stored on floppy disk for further analysis (Vernier software, http://www.vernier.com). The layout of the set-up and the equivalent circuit used as an electrical model are reported in Fig. 1a–b. The 1-Hz (1 Kz) component of the V_1 output signal called V_{ll} (V_{lh}) is mainly associated with membrane conductivity (capacitance). The relation between V_{lh} and the capacitance was experimentally obtained by simulating the membrane capacitance with a discrete set of capacitances of known values, C_n, which were measured with a precision of 3% and measuring the corresponding V_{lh}. The data obtained (reported in Fig. 2a) were then fitted by the formula:

$$Y = A^*X/(B+X) \quad (1)$$

with Y, X corresponding to V_{lh} and C_n, respectively, while A, B are free parameters to be estimated by the fitting procedures.

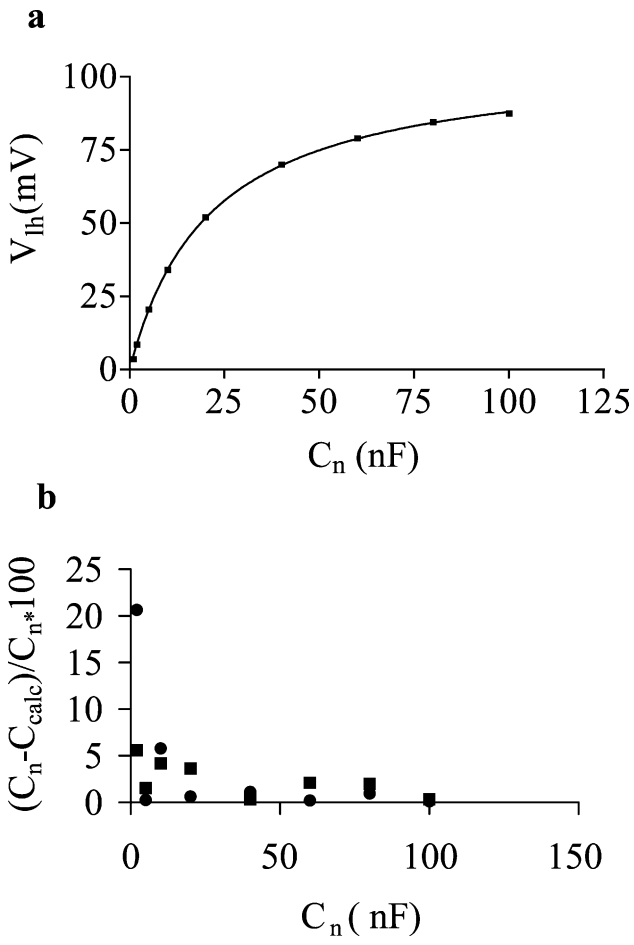

Fig. 2. (a) Sample of the data obtained during the calibration. The continuous line is the result of the best-fit discussed in the text in order to relate the V_{lh} values to the capacitance (C_n). (b) An example of discrepancy ($(C_n - C_{calculated})/C_n$) as a function of C_n.

Finally, the values of parameters A and B were used during on-line measurements of the membrane capacitance to transform the V_{lh} values into capacitance data.

By using $A = 105.4$ (mV) and $B = 20.63$ (nF) we were able to reproduce the known values of C_n for all the R_m values in the range 0.05–20 MΩ at a level better than 6%: Only for $C_n = 2$ nF and $R_m = 0.05$ MΩ (a situation never present in our bilayer) we reach a discrepancy of 20%. An example of the discrepancy obtained (discr.=$(C_n - C_{calculated})/C_n$) is represented in Fig. 2b. It is evident that for C_n larger then 2 nF, we can be confident of our measurement approach of capacity.

The above-mentioned ranges for the C_n and R_m are typical of what is found with the real bilayer under different experimental conditions of porin incorporation. This sort of calibration was repeated frequently during the measurement phase in order to check the stability of the apparatus. The system proved to be very stable. When we know the value of membrane capacitance, C_m, we can estimate both the voltage applied to the membrane, V_m and the membrane conductance (see Refs. [24,31] for details on the formula used).

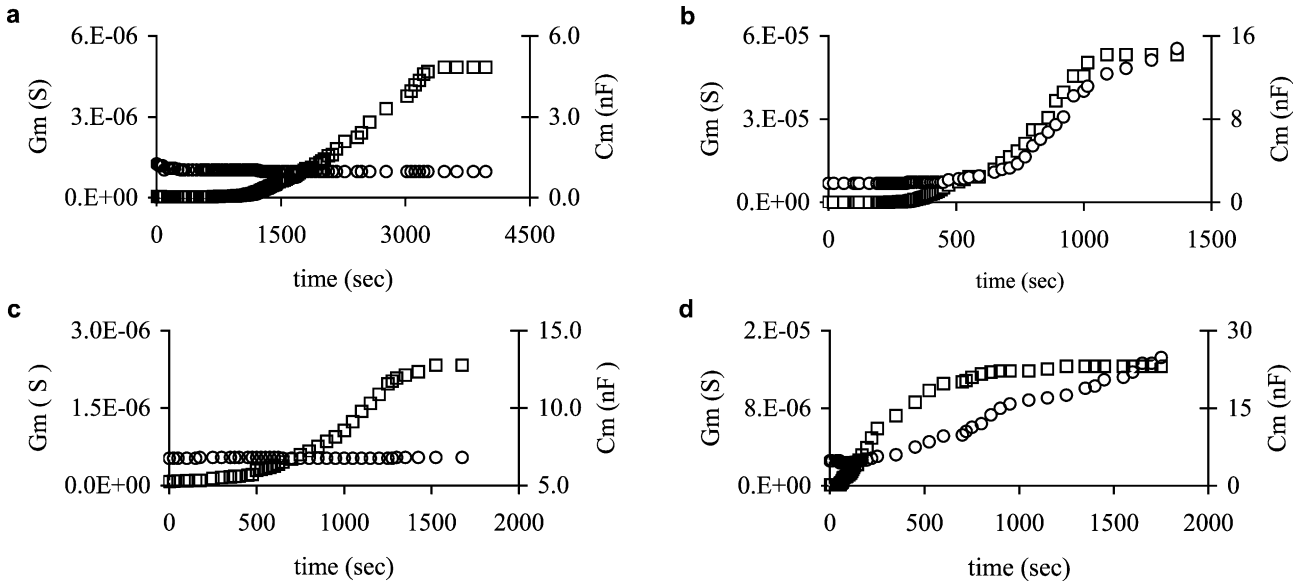

Fig. 3. Time course of the membrane conductance (G_m, □) and capacitance (C_m, ○) during porin incorporation into bilayer lipid membranes. PI membranes, porin concentration 62.7 ng/ml (a) and 600 ng/ml (b). OxCh membranes, porin concentration 1.25 ng/ml (c) and 5 ng/ml (d). Electrical resistance and capacitance of the measuring circuit were $R_l = 1$ MΩ and $C_l = 20$ nF, hole diameter $\phi = 1300$ μm, KCl = 1 M, temperature 24 ± 0.5 °C. Source voltage 80 mV.

2.3. Data analysis

The data were fitted by means of GraphPad Prism 2 (GraphPad Software; http://www.graphpad.com) software. Results are expressed as mean ± SE. Statistical significance (P) was determined by Student's t-test, performed by means of the Instat program (GraphPad).

3. Results

By using the above apparatus, we performed different kinds of measurements:

(1) Simultaneous investigation of conductance/capacitance during the process of porin incorporation and its dependence on porin concentration.

The kinetics of porin incorporation were investigated with both bilayers (hole diameter of 1300 μm) and, owing to its relatively different affinity, a porin concentration ranging from 0.67 to 600 ng/ml (PI membranes) and from 0.3 to 62.7 ng/ml (OxCh membranes).

In Fig. 3, we report an example of the data obtained during the process of VDAC incorporation.

In all cases, the time course of the conductance presents an increase due to channel formation, and this increase shows a co-operative behavior with a clear evidence of steady state level, which was explained through an equilibrium between channel incorporation and channel migration in the torus [24]. These steady state characteristics seem to be independent of the porin concentration and the bilayer used. Moreover, the OxCh membrane shows a larger affinity for the porin, as the process of incorporation is more rapid even at lower porin concentrations.

During porin incorporation, when the porin concentration is greater than the threshold value, both capacitance and conductance increase; however, when the conductance

Table 1
Membrane capacitance variation as a function of porin concentration in PI and OxCh membranes

Porin (ng /ml), (Experiment No.)	C_{m0} (μF/cm^2)/ G_{m0} (μS/cm^2)	C_{mf} (μF/cm^2)/ G_{mss} (μS/cm^2)
PI membranes		
1.25 (8)	0.30 ± 0.04/3.29 ± 0.53	0.32 ± 0.04/4.25 ± 0.59
12.5 (3)	0.24 ± 0.03/2.10 ± 0.70	0.32 ± 0.04/3.20 ± 0.80
37.6 (2)	0.25 ± 0.01/2.30 ± 0.20	0.29 ± 0.03/17.8 ± 3.70
62.7 (41)	0.23 ± 0.01/2.70 ± 0.30	0.30 ± 0.04/138.0 ± 26.3
94.1 (5)	0.21 ± 0.03/3.30 ± 0.07	0.29 ± 0.04/768 ± 59.5
200 (4)	0.22 ± 0.02/2.11 ± 0.17	0.29 ± 0.03/1310 ± 314
300 (10)	0.25 ± 0.02/2.00 ± 0.10	0.68 ± 0.02* /1170 ± 242
450 (5)	0.28 ± 0.04/3.10 ± 0.78	0.64 ± 0.01** /1500 ± 234
600 (12)	0.23 ± 0.01/1.80 ± 0.01	1.08 ± 0.17*** /1260 ± 237
OxCh membranes		
0.3 (7)	0.46 ± 0.02/2.20 ± 0.50	0.47 ± 0.03/114 ± 17
1.25 (17)	0.49 ± 0.02/3.73 ± 0.43	0.57 ± 0.03/445 ± 86.4
6.3 (12)	0.41 ± 0.01/5.00 ± 0.35	0.55 ± 0.09/702 ± 135
10.0 (9)	0.41 ± 0.01/5.76 ± 0.24	0.43 ± 0.02/845 ± 151
12.5 (9)	0.44 ± 0.02/4.73 ± 0.44	0.56 ± 0.04^{o}/1650 ± 490
37.6 (7)	0.41 ± 0.04/5.05 ± 0.58	1.02 ± 0.24^{oo}/1440 ± 298
62.7 (8)	0.36 ± 0.03/5.11 ± 0.63	1.29 ± 0.36^{ooo}/1960 ± 30.5

C_{m0} = bare membrane capacitance; C_{mf} = membrane capacitance at the last values of the conductance monitored. To allow comparison, we reported the conductance values of bare membrane (G_{m0}) and at the steady state of the total current value of porin incorporation (G_{mss}).
Experimental conditions: $V_s = 80$ mV; $\phi = 1300$ μm; $R_l = 1$ MΩ; $C_l = 20$ nF; $F = 1$ Hz. Values are the mean ± SEM. * $P = 0.019$; ** $P = 0.020$; *** $P < 0.0001$; $^{o}P = 0.017$; $^{oo}P = 0.029$; $^{ooo}P = 0.022$. In all the other cases, the variations are not statistically significant.

has reached a steady state, a slight increase in capacitance could still be observed (Fig. 3b,d), probably due to a delay in the conformational change of the protein.

This aspect is reported in more detail in Table 1, where we quote and statistically compare the capacitance variation at the very beginning of the incorporation process (C_{m0}) and when a stable steady state of the total current had been reached (C_{mf}). With our apparatus, porin concentrations of about 250 ng/ml (about 12.5 ng/ml) are required to detect a moving charge process during porin incorporation into PI (OxCh) bilayers.

As the same behavior is shown by porin incorporation in the "solvent-free" membranes of PI, we can assume that solvent leakage can be excluded as a primary cause of the capacitance increase observed. In five experiments with such membranes, an increase of $C_m = 114.4 \pm 40.1\%$ is already observed at 250 ng/ml of porin concentration. In Fig. 4, we reported a representative experiment of porin incorporation in solvent-free membrane.

VDAC is a voltage-gated protein and the membrane potential is a pre-eminent factor conditioning the different states. The open state is the preferred state at zero membrane potential, while higher membrane potentials determine channel closure and evidence exists that the structural changes during state variations are large [33,34].

In order to evaluate if in the abovementioned conditions (high porin concentration), the capacitance variation is due to protein charge movements, we performed experiments on PI membranes at a threshold concentration of porin (250 ng/ml) and at different external applied voltages.

In Table 2, it can be observed that the main capacitance variation (C_{mf}), at the steady state of total current flowing through the membrane, is obtained at applied voltages of 20, 40, 60, 80 and 100 mV. We believe that at higher applied voltages ($V_s > 100$ mV), the potential on the membrane (V_m) is also high and will impede conformational variation of VDAC responsible for the capacitance variation. However, further experiments at the current steady state of porin

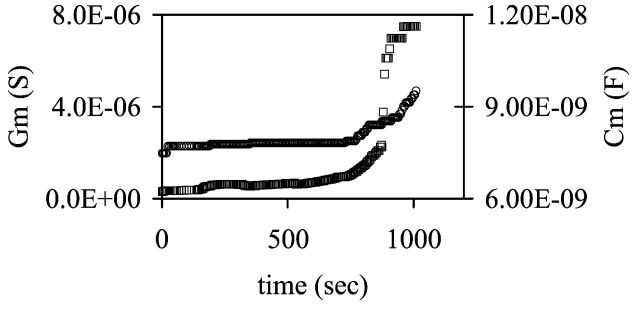

Fig. 4. A representative experiment of time course of the membrane conductance (G_m, □) and capacitance (C_m, ○) during porin incorporation into solvent-free PI membranes. Experimental conditions were: KCl = 1 M, $R_l = 1$ MΩ, $C_l = 20$ nF, $V_s = 80$ mV, porin = 250 ng/ml, hole diameter $\phi = 1300$ μm, temperature = 24 ± 0.5 °C.

Table 2
Membrane capacitance variation as a function of external applied voltage in PI membrane

V_s (mV) (Experiment No.)	C_{m0} (μF/cm^2)/ G_{m0} (μS/cm^2)	C_{mf} (μF/cm^2)/ G_{mss} (μS/cm^2)
20 (10)	0.24 ± 0.04/1.22 ± 0.09	0.58 ± 0.16* /2077 ± 461
40 (5)	0.20 ± 0.03/1.26 ± 0.20	0.38 ± 0.06** /1940 ± 507
60 (4)	0.18 ± 0.03/1.18 ± 0.23	0.55 ± 0.13*** /2069 ± 773
80 (7)	0.26 ± 0.03/1.64 ± 0.21	0.42 ± 0.07**** /1211 ± 114
100 (9)	0.21 ± 0.03/1.93 ± 0.64	0.33 ± 0.04***** /1063 ± 271
120 (5)	0.24 ± 0.03/1.43 ± 0.38	0.30 ± 0.03/836 ± 370
140 (8)	0.20 ± 0.04/1.17 ± 0.18	0.22 ± 0.05/1373 ± 377
160 (5)	0.22 ± 0.18/1.10 ± 0.18	0.26 ± 0.01/897 ± 238
200 (4)	0.20 ± 0.02/1.14 ± 0.32	0.43 ± 0.18/1073 ± 551

C_{m0} = bare membrane capacitance; C_{mf} = membrane capacitance at the last values of the conductance monitored. To allow comparison, we reported the conductance values of bare membrane (G_{m0}) and at the steady state of the total current value of porin incorporation (G_{mss}).
Experimental conditions: PI Membranes; porin = 250 ng/ml (PI); $\phi = 1300$ μm; $R_l = 1$ MΩ; $C_l = 20$ nF; $F = 1$ Hz. Values are the mean ± SEM. * $P = 0.041$; ** $P = 0.023$; *** $P = 0.037$; **** $P = 0.042$; ***** $P = 0.032$. In all the other cases, the variations are not statistically significant.

incorporation on voltage-dependent capacitance are reported below.

(2) Voltage dependence capacitance of black lipid membranes before porin incorporation.

In Fig. 5a–b, we report an example of the data obtained when different voltages, V_m, are applied (hole diameter 1300 μm) to BLM of PI and OxCh. To allow comparison with previous investigators [10,35,36] we fitted the data with the equation: $C_m = C_o + \beta V_m^2$.

Excluding the first data points (V_m 3–16 mV), we obtained data which can be fitted by a simple linear dependence with small slope values of 0.0051×10^4 μF/cm^2 × mV^2 (0.048×10^{-4} μF/cm^2 × mV^2) and intercept values of 0.31 μF/cm^2 (0.61 μF/cm^2) for PI (OxCh), respectively. These results are compatible with AA [10, 32,35–42] findings that have been interpreted by means of a simple model including electrostriction force.

(3) Voltage dependence capacitance of black lipid membranes after porin incorporation.

In order to study the voltage-dependent capacitance of VDAC, the experimental conditions used and the measurement approach are the same as those reported in our previous study on voltage-dependent conductance at the steady state [24], because, as has been mentioned, our apparatus is able to simultaneously monitor both capacitance and conductance.

The porin concentration was 0.67 ng/ml (PI membranes) and 0.3 ng/ml (OxCh membranes) and the hole radius of 900 μm.

The total current flowing through the membranes was monitored on-line until a steady state value was reached. We then switched the V_s to a maximum value (220 mV). By applying a series of decreasing voltages, each lasting 5 min,

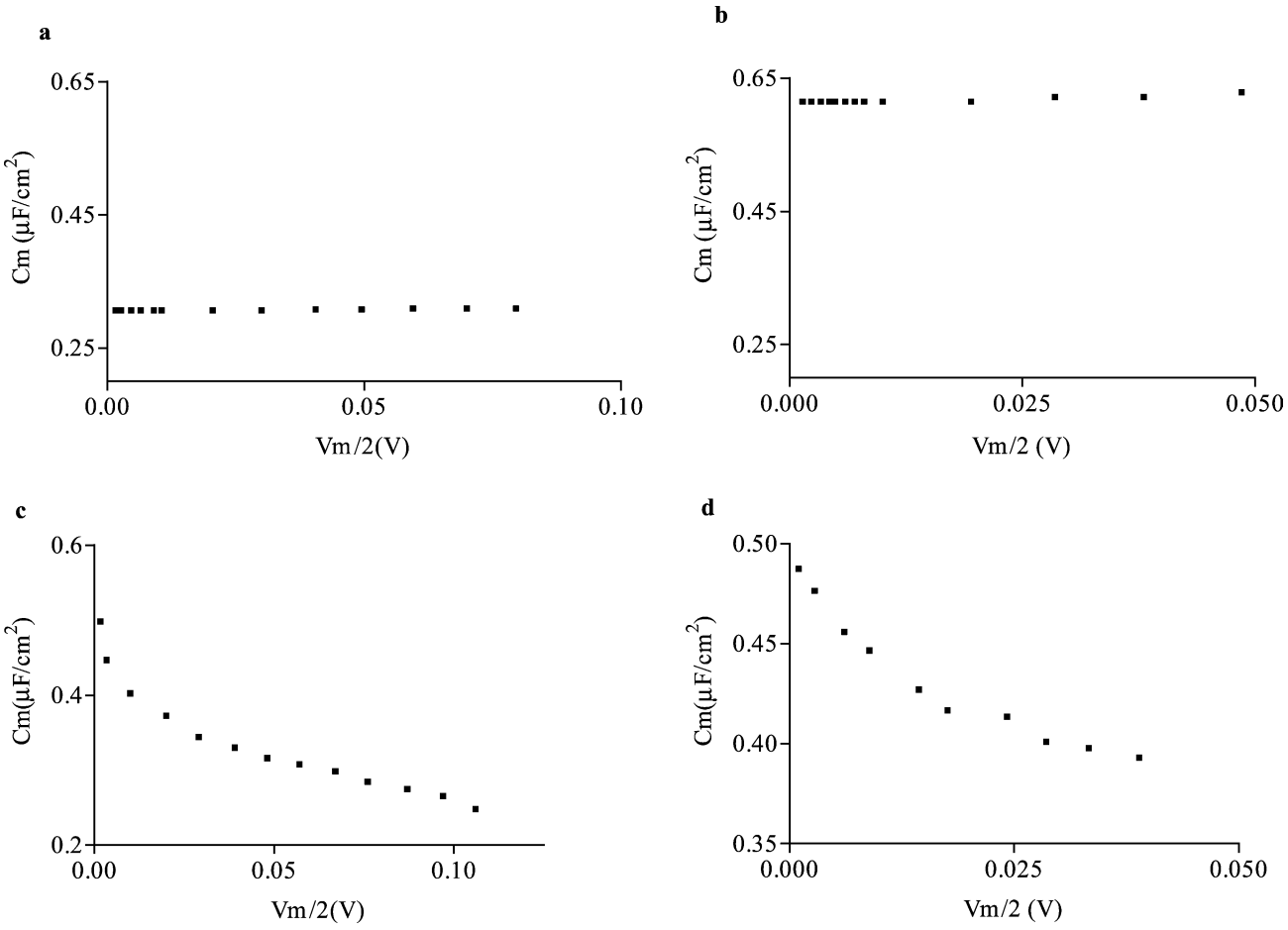

Fig. 5. A representative experiment of specific capacitance (C_s) as a function of the membrane voltage in bare membranes (a and b) and at the steady state of the total current value of porin incorporation (c and d) of PI and OxCh membranes, respectively. Experimental conditions: KCl = 1 M, R_l = 1 MΩ, C_l = 20 nF, temperature 24 ± 0.5 °C, porin concentration 0.67 ng/ml and 0.3 ng/ml for PI and OxCh, respectively.

very low values of membrane applied potential, V_m, were reached. This timeframe was considered long enough to allow the system to reach a new stable steady state. The results are reported in Fig. 5c,d.

Following this procedure, we studied the C_m dependence on the membrane applied voltage V_m. The results relative to the capacitance ratio

$$Y = (C_m - C_o)/(C_h - C_m) \quad (2)$$

(where C_h = the initial capacitance and C_o = the capacitance for the highest voltage value) are reported in Fig. 6 and show a pattern similar to those obtained in the conductance measurements [24].

The data have been parameterized with two exponential forms:

$$Y = A \times e^{-\alpha_1(V_m - V_o)} + (1 - A) \times e^{-\alpha_2(V_m - V_o)} \quad (3)$$

where V_o indicates the potential at which the capacitance ratio is equal to 1. The A parameter allows an estimation of the contribution of the two mechanisms.

The value obtained for the two exponential coefficients are $\alpha_1 = 64.7 \pm 17.5\ V^{-1}$ and $\alpha_2 = 753.3 \pm 174.3\ V^{-1}$ ($\alpha_1 = 118.1 \pm 37.3\ V^{-1}$ and $\alpha_2 = 549.4 \pm 350.8\ V^{-1}$) for PI (OxCh) for a total of five (four) experiments, respectively. We suspect that when VDAC is inserted, it is subjected to a polarization, induced by membrane applied potential V_m, which in turn is responsible for the capacitance variation. It is worth mentioning that applying tension or potential to the outer hair cell (OHC) from the mammalian cochlea, has been found to be responsible for the observed exponential capacitance variation and explained as a charge movement across the membrane [19].

We point out that in the same voltage range (< 100 mV for PI and < 40 mV for OxCh membranes, respectively) of Fig. 6, the variation of the bare BLM is less than 1% for PI and less than 2.3% for OxCh membranes, respectively.

(4) Capacitance dependence on the radius of the hole separating the two compartments.

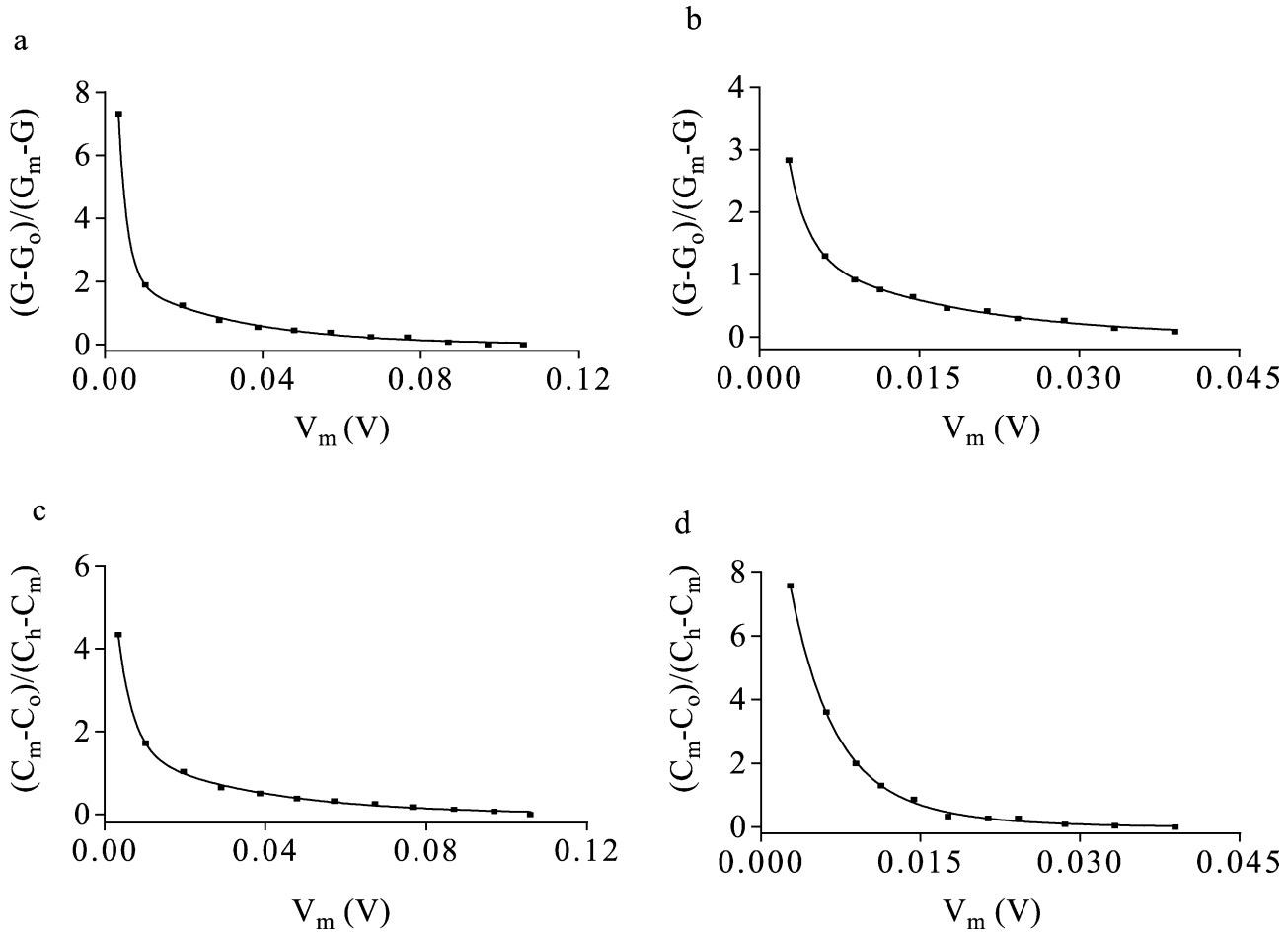

Fig. 6. A typical example of the conductance/dependence ratio $Y=(G-G_o)/(G_m-G)$; (G_m = the initial conductance and G_o = the conductance for the highest voltage value) and of the capacitance/dependence ratio $Y=(C_m-C_o)/(C_h-C_m)$; (C_h = the initial capacitance and C_o = the capacitance for the highest voltage value) on the effective potential V_m acting on the membranes. Data were parameterized with the two-exponential equation $Y=A\times e^{-\alpha_1(V_m-V_O)}+(1-A)\times e^{-\alpha_2(Vm-V_o)}$. PI (a, c) and OxCh (b, d) membranes. Experimental conditions were: porin concentration 0.67 ng/ml and 0.3 ng/ml for PI and OxCh, respectively, KCl = 1 M, R_l = 1 MΩ, C_l = 20 nF, temperature = 24 ± 0.5 °C.

In Fig. 7a, we show the data obtained with different hole radii in the range 200–1050 μm. The data were collected only for OxCh BLM since, as suggested by AA [26–28], this BLM shows a stronger affinity for VDAC. In this case, in order to highlight a dependence on the radius, we report the values of the specific capacitance C_s (i.e. the capacitance per unit area) normalized to the highest value of radius utilized (1050 μm). There is evidence, in the range of radius investigated, of a significant variation with low radius values. These results can be interpreted by means of the simple geometrical model proposed by Seydel et al. [17]. In the model, it is assumed that the bilayer is made up of a central region, allowing ion movements (i.e. a KCl flow) between the two compartments, surrounded by a zone that is a few orders of magnitude thicker (i.e. annulus or torus). Therefore, the effective bilayer area proves to be smaller than the geometrical area associated with the hole diameter. From this geometrical consideration, it is expected that the specific capacitance dependence on the hole radius is approximately given by:

$$C_s = C_\infty*(1-2*\delta r/r+\delta r^2/r^2) \quad (4)$$

where C_∞ is the true specific capacitance when the effect of torus is negligible, r is the hole radius and δr can be considered as a parameter representing the width of the torus. In our case, the data have been fitted (see the continuous curve in Fig. 7a) by Eq. (4) with a $\delta r = 0.055 \pm 0.008$ mm. A visual check of the torus width through a microscope confirms this estimate.

We also investigated, for OxCh membranes, whether capacitance at steady state of the total current of the porin incorporation, for fixed membrane applied potential V_m, was dependent on hole radius. The results shown in Fig. 7b

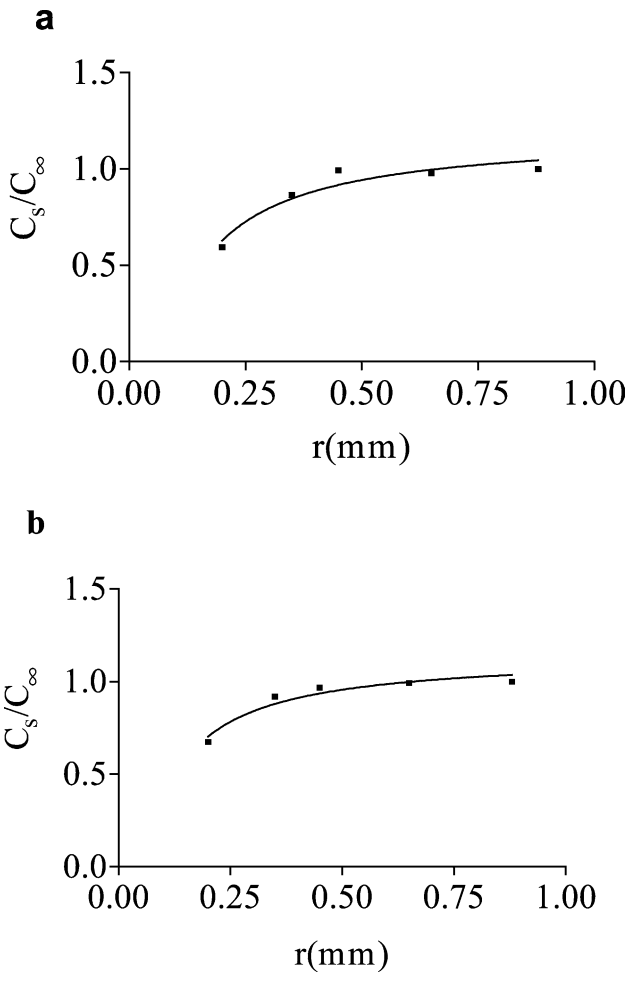

Fig. 7. Dependence of the specific capacitance C_s on the radius of the hole before (a), and after porin incorporation (b). The data are relative to OxCh membranes. Experimental conditions were: KCl = 1 M, R_l = 1 MΩ, C_l = 20 nF, porin = 1.25 ng/ml, temperature = 24 ± 0.5 °C. C_∞ corresponds to the experimental mean value obtained at r = 1050 μm. The continuous lines are the results of the best-fit obtained by using formula (4) (see text). Each point represents the mean of at least four experiments.

are similar to those reported in Fig. 7a before porin incorporation and have been fitted by formula (4) with $\delta r = 0.063 \pm 0.005$ mm.

4. Discussion

In this work, we present results obtained by means of an instrument that has proved very useful in investigating membrane conductance/capacitance simultaneously. The technical aspect of our device was verified with VDAC, a protein that when switching from an open to a closed state undergoes conformational rearrangement associated with pore dimension changes from 3 to 1.8 nm [43,44]. In fact, recent studies have demonstrated that in the mechanism of voltage gating a positively charged domain that is part of the channel wall is pulled out of the channel by the electric field created by potential variation [43,44,46]; besides, such a sensor is influenced by ionic flow [47].

Moreover, the capacitance parameter is considered to be the best tool for probing the stability and formal goodness of the lipid bilayer membrane before any experiment concerning the incorporation of different substances such as proteins, peptides or drugs can be conducted.

Due to the fact that any protein or peptide incorporation will bring charges into the bilayer or when already incorporated, potential-induced conformational variation of proteins will take place, membrane capacitance assumes a further role. In this contest, it can be considered as a useful probe for monitoring dynamic phenomena such as conformational variation in channel gating, or protein insertion into the bilayer [1,2,20,48].

The results concerning bare BLM (before porin insertion) indicate a small capacitance variation for PI membranes and for OxCh membranes as a function of applied voltage, V_m. So we can be confident that any geometrical variation at different membrane voltages is limited. This conclusion is in agreement with the results reported in previous investigations in the black lipid bilayer [10,32,35–42].

For each membrane used (PI and OxCh) a relation exists between the porin concentration and capacitance variation. In particular, a sort of threshold effect is evident: significant capacitance variations start at porin concentrations of 250 ng/ml (12.5 ng/ml) for PI (OxCh), respectively. The capacitance variation could be due to:

(1) dielectric change by the water flow through channels inserted; but the small number of the channels incorporated could only account for a small capacitance variation;

(2) to solvent leakage "squeezing" during protein insertion, but this alternative hypothesis seems unlikely, owing to the similar results obtained with "solvent-free" membranes;

(3) protein-induced polarization during fast insertion into the bilayer. The observed capacitance variation at high concentration of VDAC could indicate a voltage-dependent conformational variation of the protein during the dynamic insertion process, as suggested by Ghosh et al. [48].

Another example of voltage-induced translocation across the membrane can be seen in the long stretches of channel-forming colicin Ia [49,50]. Peptides such as alamethicin have been found to induce capacitance changes in phospholipid membranes [51]. Preliminary experiments of incorporation of the amphipathic peptide, magainin 2, into bilayers utilizing our device are giving indications of capacitance variation, a finding that may help to shed light on their mechanism of action.

Moreover, we believe that a certain protein/lipid ratio must be reached in the bilayer before the phenomenon can be observed.

The results after mitochondrial porin incorporation, under steady state conditions for the total current, indicate a relative variation of the capacitance on the membrane applied voltage (around 30–40%). It must be remarked that this variation is of substantial consistency as compared to

undoped membrane and can be related to the presence of protein. Furthermore, the capacitance variation found is in the same range as that obtained for conductance. Is worth mentioning that Iwasa [19] reported a similar non-linear variation in outer hair cell during application of membrane tension or voltage, and suggested that this behavior may be due either to a polarization effect or to a hydrophobic-induced charge movement into the bilayer [52], or to a charge movement in the protein channel triggered by conformational change in their cross-sections [19]. We believe that the change in membrane capacitance found in our experiments on voltage dependence capacitance has the same origin and depends on the protein charges moving during voltage variation. We can assume that the domain contributing to the voltage sensor in VDAC in its movement triggered by voltage change may involve a large portion of the protein forming the wall of the pore; this result supports AA findings [45]. Another classical well-known example of a voltage-sensing domain moving in the electric field is represented by S4 regions of plasma membrane cation channels (Na, K, Ca) in which the sequence of events leading to the opening of the channel is associated with the movement of the charged groups which sense the electric field applied to the membrane. In particular, when ionic and gating currents from Shaker B K^+ channels were studied, at the steady state of the total current, to establish the voltage dependence capacitance, two components in the charge movement were found and fitted by means of a double exponential [1,2].

While we are aware of the different complexities of this channel as compared to VDAC, the correlation capacitance/conductance-voltage dependence fitted by a double exponential equation could be indicative of a similar underlying conformational rearrangement mechanism. This aspect can be of practical importance from the biotechnological point of view, as for example in electroporation.

Moreover, the capacitance dependence on hole radius may be of greater importance when smaller hole radius are used, since the torus' contribution has been proved not to be negligible. This result, together with that previously discussed on the role of the torus in membrane conductance [24], calls for more investigations, both theoretical and experimental, on the problem connected with interpreting electrical parameters in measurements with a reconstituted membrane bilayer.

Finally, our results stress the fact that important information on gating charge movement into the membrane can be obtained from capacitance measurements, an aspect which is regaining interest, and that a device for simultaneously measuring conductance and capacitance (as is the case in our study) may be of use.

Acknowledgements

We wish to thank Anthony Green for helping to improve the English in this paper. This work was supported in part by MPI 40% (EG) and CNR (92.02202.Cr14;91.2524) grants.

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