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Oscillating Ca^{2+}-Induced Channel Activity Obtained in BLM with a Mitochondrial Membrane Component

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Oscillations in ion fluxes and membrane potential may be observed in cells and in mitochondria as well. We obtained Ca^{2+}-induced oscillations in channel activity in black-lipid membranes reconstituted with hydrophobic components extracted from mitochondria. Mitoplasts prepared from purified rat liver mitochondria were extracted with ethanol followed by Folch extraction and further partial purification by silicic acid chromatography. Channel activity was measured in lipid bilayers formed from bovine brain lipids and 10% cardiolipin with addition of the purified fractions. The conductance with 10 mM Ca^{2+} was 100 pS or its multiples. Ca^{2+} gradients of 4 : 1 induced oscillating channel activity for several hours, with initial open states of 40 s and closed states of 56 s; the open times gradually decreasing to 8.6 s. No channel activity was seen without added fractions. The channel activity was associated with a Ca^{2+}-binding lipid, nonpolar, low-molecular-weight fraction that in gel electrophoresis was not stained with Coomassie Blue and did not contain carbohydrate-staining material. ^{1}H-Nuclear magnetic resonance spectra of the substance showed the presence of aliphatic chains and carbonyls, but the detailed structure remains to be elucidated.

KEY WORDS: Black-lipid membrane; ion fluxes; oscillations; phase transitions; lipids.

INTRODUCTION

Oscillatory phenomena may occur in complex systems in which one state becomes unstable and changes into another state that in its turn becomes unstable and reverts to the first state. There is a multitude of factors creating such instabilities and nonlinear responses. Of special interests are those occurring in membranes and membrane-like interfaces, in which electric, diffusional, hydraulic, or chemical systems including enzymatic reactions may be involved (Franck, 1973). Oscillations of cytosolic [Ca^{2+}] occur frequently in signal transduction and are believed to result from Ca^{2+}-induced Ca^{2+} release from intracellular stores followed by their reuptake (Jafri and Keizer, 1994, Reiser *et al.*, 1992) but may also reflect periodic oscillations in the plasma membrane potential and Ca^{2+} fluxes (Berridge, 1990; Friel, 1995). Often they are associated with oscillations in ionic channel activities modulated by Ca^{2+} (Angstadt and Friesen, 1991; Enomoto *et al.*, 1991; Lakatta *et al.*, 1992; McGeoch and Guidotti, 1992; Tsunoda, 1993).

Mitochondrial oscillations involve, in addition to oscillatory ion fluxes, also cyclic changes in the respiratory rate and the red/ox state of respiratory chain components, transmembrane potential, volume, and as a consequence stretching of the inner membrane (Gooch and Packer, 1974). Oscillations of Ca^{2+} fluxes are

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[5] Abbreviations: BLM, black-lipid membranes; planar lipid bilayer; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TLC, thin-layer chromatography; Tris, Tris(hydroxymethyl) aminomethane.

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damped after a few cycles (Carafoli *et al.*, 1966), which usually is the case also for fluxes of monovalent cations in the presence of valinomycin (Gooch and Packer, 1974), but continuous oscillations have been reported for Sr^{2+} (Gylkhandanyan *et al.*, 1976). In these suspensions of mitochondria the fluxes must be synchronized to be observable by the techniques used. Nonsynchronized fluctuations of individual mitochondria may be observed in intact neuroblastoma cells viewed by confocal microsopy (Loew *et al.*, 1994). Here we report that a fraction extracted from rat liver mitochondria is able to form a regularly opening and closing channel that is operating continuously for extended periods in a phospholipid bilayer. This provides a model for studying oscillatory channel activity in a simple system.

MATERIALS AND METHODS

Rat liver mitochondria were prepared by a standard procedure as described elsewhere (Wikström and Saris, 1969) with an additional washing of the mitochondria with the isolation medium (1:20 v/v) in order to reduce the contamination by microsomes. This was followed by a discontinuous sucrose gradient centrifugation (80,000 *g* for 3 h) using 10% stepwise increases of sucrose from 10 to 60%. The harvested mitochondrial band was suspended in 5 volumes of 20 mM Tris-buffer, pH 7.5, and centrifugated at 5500 *g* for 15 min. Mitoplasts were then prepared by suspending the mitochondrial pellet in 8 volumes of hypotonic medium containing 20 mM Tris buffer, pH 7.5, and stirring during 20 min at 4°C, followed by centrifugation at 10,000 *g* for 20 min.

Lipid extract of the inner membrane was prepared from mitoplasts by treatment by cold (–20°C) 60% ethanol (4 mg mitoplast protein/ml). After stirring for 30 min, the suspension was centrifuged at 7000 *g* for 15 min, the supernatant was evaporated under vacuum, and the lipids were extracted by the Folch procedure (Folch *et al.*, 1957). The chloroform phase was evaporated to dryness and the residue dissolved in chloroform:methanol (2:1).

For testing the purity of cell organelle fractions, the activities of the membrane marker enzymes cytochrome *c* oxidase (EC 1.9.3.1) (mitochondrial inner membrane), monoamine oxidase (EC 1.4.3.4) (mitochondrial outer membrane), and glucose-6-phosphate phosphatase (EC 3.1.3.9) (endoplasmic reticulum) were assayed as described elsewhere (Gasnier *et al.*, 1988).

For electron microscopy, mitochondria and mitoplasts were fixed for 2 h in 2% glutaraldehyde and 100 mM phosphate buffer, pH 7.4. Electron microscopy was performed with the CM-120 instrument (Philips, NL) at the Centre de Microscopie Electronique Appliquée à la Biologie, Claude Bernard University, Lyon. ^{1}H-NMR spectra were obtained with a Bruker AMX-400 appa-ratus using $CDCl_3$ as solvent (Pollesello *et al.*, 1991). Molecular weight estimation was performed by using HPLC (Waters, USA) and a gel-filtration column equipped with a microcylinder refractometer index detector with automatic calculator and recorder. Two types of columns were employed: a V-Styragel linear column for M_R from 0.1 to 10.000 kDa and a V-Styragel 500 A for oligomers (M_R from 0.1 to 10 kDa).

The extract of the inner mitochondrial membrane extract was tested for the presence of sugar residues on nitrocellulose dot-blot using digoxigenin-labeled plant lectins which bind specifically to special terminal sugars. Staining was performed using 5-bromo-4-chloro-3-indolylphosphate and 4-nitroblue tetrazolium (Haselbeck and Hösel, 1990).

TLC was performed on silica gel plates (Art. 5626, Merck, Darmstadt, D) with different solvents. Staining was performed with anisaldehyde, copper acetate, rhodamine 6B, and iodine vapors as described elsewhere (Kates, 1972). SDS-PAGE was carried out according to Laemmli (1970), using 14% polyacrylamide gel. Gel patterns were visualized with Coomassie Brilliant Blue R-250 (Haselbeck and Hösel, 1990).

Binding of Ca^{2+} by various fractions was studied by two procedures. PAGE gels were washed with 20 mM Tris-HCl, pH 7.5, for 30 min, by overnight incubation of the gel with ^{45}Ca-labeled 10 μM $CaCl_2$ in the wash buffer. After removing excess radiocalcium by washing with calcium-free buffer for 30 min, the gel was dried and allowed to expose Kodak film for 2 h. In the second method we applied drops from the various fractions to PVDF membranes followed by 5-min incubation with 20 mM Tris, pH 7.4, 1 h incubation in ^{45}Ca at a concentration of 10 μM, and 0.5 h washing in 20 mM Tris, pH 7.4. After drying, the radioautography was performed as described above.

Measurement of channel activity was carried out as described elsewhere (Mironova *et al.*, 1994) using an operational amplifier (OPA 101, Burr-Brown Corp., Tucson, Arizona) connected to a Tectronix 2211 digital storage oscilloscope to record the *trans*-membrane current at various clamped voltages. BLM was formed from 1.2 mg brain lipid + 100 μg cardiolipin and extract from 5 mg mitoplasts, dried and diluted in 60 μl

n-decane. The conductance of the BLM without extract was 3–10 pS/mm^2 in 10 mM $CaCl_2$.

All reagents were of analytical grade. Polyvinylidene difluoride membranes and silicic acid were purchased from Bio-Rad laboratories (Hercules, California). Digoxigenin-labeled lectins and polyclonal sheep antidigoxigenin Fab fragments conjugated with alkaline phosphatase were from Boehringer (Mannheim, D).

RESULTS

Ca^{2+}-Induced Channel Activity of the Lipid Extract

Channel activity in the presence of 0.5–10 mM Ca^{2+} was obtained with the mitoplast extract when reconstituted into a bilayer formed from brain lipid extract and cardiolipin; Fig. 1 shows one representative experiment. In the absence of Ca^{2+}, the permeability of the reconstituted membrane was the same as that of the control membrane. When K^+ (or Na^+) was used as the cation in the absence of calcium, no channel activity was seen until Ca^{2+} was added to activate the channel (not shown). The Ca^2-activated channel was poorly selective. In the presence of high concentrations of $CaCl_2$, the channel was more anion- than cation-selective (the reversal potential in gradient 1:4 was from +18 to +22 mV) in contrast to the behavior at 1 mM, when it was more Ca^{2+}-selective (the reversal potential then was from –8 to –12 mV). The voltage–current characteristics shows an increase in permeability on increasing the voltage from 40 to 100 mV (Fig. 2).

The Intracellular Localization of the Studied Component

The localization of the channel-forming component in mitochondria was ascertained by the use of specific membrane marker enzymes. Table I shows that the mitoplast fraction had negligible activity of the microsomal marker enzyme glucose-6-phosphate phosphatase but still considerable monoamine oxidase activity, a mitochondrial outer membrane marker enzyme.

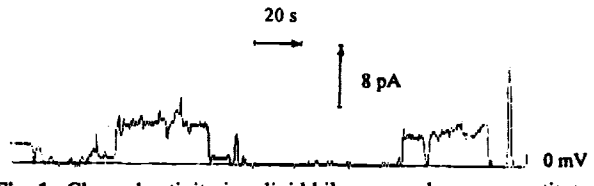

Fig. 1. Channel activity in a lipid bilayer membrane reconstituted with a mitochondrial membrane extract. The chamber solution contained 20 mM Tris-HCl buffer, pH 7.4, and 1 mM $CaCl_2$; the applied voltage was 40 mV. For the formation of the lipid bilayer and for the reconstitution, see Materials and Methods.

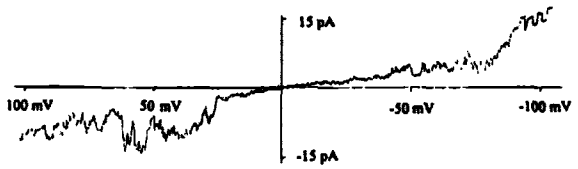

Fig. 2. Voltage–current characteristics of the channel activity. Experimental conditions were as in Fig. 1.

Electron microscopy confirmed the purity and integrity of the mitochondrial fraction after purification and the absence of microsomal material (Fig. 3A). Moreover, the mitoplast fraction contained both typical mitoplasts and also swollen mitochondria (Fig. 3B).

Partial Purification and Characterization of the Channel-Forming Component

Figure 4 shows the SDS-PAGE of the mitoplast extract after staining with Coomassie Blue, Schiff's reagent, and ^{45}Ca-binding radioautography, respectively. Of special interest is that a low-molecular-weight component was made visible by all these staining procedures. The Ca^{2+}-binding was assayed at pH 7.4; there was no binding at pH 6.0 (not shown). The presence of carbohydrate-containing material was further confirmed by chemical detection of sugars by binding to specific lectins. Indeed, several lectins were found to be bound to the components in the extract. These data point to the presence of glycolipid in the extract.

Step 1. Silicic acid chromatography of the extract. The extract from 300 mg mitoplasts dissolved in 1 ml chloroform was applied to a chloroform-washed column formed from 2 g silicic acid. The elution was carried out at room temperature with a flow rate of 60 ml/h with solvent mixtures as described in Table II. The eluate fractions were evaporated to dryness and dissolved in 0.5 ml chloroform:methanol (2:1). Aliquots, 10 µl, were applied to TLC plates and TLC carried out as described in Materials and Methods using chloroform:methanol:water (65:25:4). Figure 5 shows the TLC plate stained with anisaldehyde. The main material in the extract, first lane on the left, consisted mainly of phospholipids with some cholesterol and free fatty acids present. Eluate fractions

Table I. Specific Activities of Marker Enzymes[a]

	Monoamine oxidase	Cytochrome *c* oxidase	Glucose-6-phosphate phosphatase
Crude mitochondria	32	19	26 (4%)
Purified mitochondria	94	37	12 (1.8%)
Mitoplasts	49	37	12 (0.3%)
Microsomes	—	—	640 (100%)

[a] The specific marker enzymes activities are expressed as nmol · min^{-1} · mg^{-1} protein for monoamine oxidase and glucose-6-phosphate phosphatase activities and as δd_o · min^{-1} · mg^{-1} protein for cytochrome *c* oxidase activities. Glucose-6-phosphate phosphatase activities are also expressed as percent of the activity in the microsomal fraction.

1, 4, and 6 possessed Ca^{2+}-binding components (Table II). Fraction 1 bound lectins GNA from *Galanthus nivalis* and WGA from *Triticum vulgaris*. This indicates the presence of terminal mannose residues and *N*-acetylglucosamine in components of this fraction. The Ca^{2+}-stimulated channel activity was also found in fraction 1, Table II. Thus, phospholipid-containing fractions were devoid of channel activity.

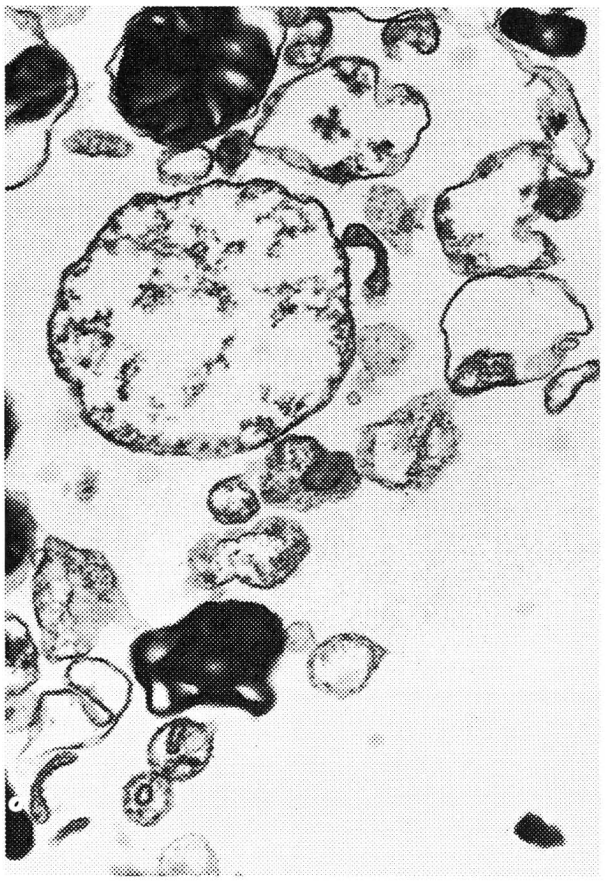

Fig. 3. Electron micrographs of mitochondrial (**A**) and mitoplast (**B**) preparations. Magnification 30.000 ×.

It can also be noted that fractions 4 and 6 had Ca^{2+}-binding acitivity but no channel activity.

Step 2. Silicic acid chromatography of the active fraction 1. In TLC the fraction 1 showed the presence of three bands, of which the uppermost appeared likely to contain more than one component (Fig. 5, Lane 1). In order to obtain a better separation of these components the silicic acid column chromatography was repeated reducing the elution rate to 20 ml/h and collecting smaller volumes. The eluted fractions were again analyzed by TLC using the solvent system chloroform:methanol:water (90:10:0.5); see Fig. 6. In this system the first 1–4 ml of the eluate contained the uppermost fractions (Lane 2), while the middle fraction was eluted with 5–8 ml (Lane 3), and the third with 9–12 ml (Lane 4). By using marker lipids and by NMR analysis it was found that band c corresponds to cholesterol, and d to fatty acids. Lectin was bound to

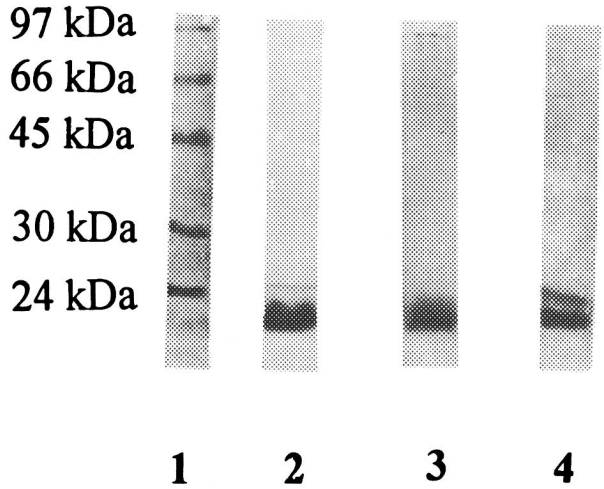

Fig. 4. PAGE of lipid extract of mitoplasts. Lane 1, molecular mass standards; Lane 2, staining with Schiff's reagent; Lane 3, stained with Coomassie Blue; Lane 4, Ca^{2+} binding visualized with the aid of incubation with ^{45}Ca and radioautography.

Table II. Ca^{2+} Binding in Fractions Obtained by Silicic Acid Chromatography of the Lipid Extract of Mitoplasts[a]

Fraction number	Eluant	Elution volume (ml)	Ca^{2+} binding
1	Chloroform	20	+
2	Chloroform:methanol (9:1)	20	–
3	Chloroform:methanol (4:1)	10	–
4	Chloroform:methanol (2:1)	10	+
5	Chloroform:methanol (1:1)	10	–
6	Chloroform:methanol:H_2O (30:60:20)	10	+

[a] For experimental details, see Step 1 in Results.

the component (s) in band b. Fractions from Lanes 3 and 4 in Fig. 6 possessed Ca^{2+}-binding and channel activity (data not shown).

Step 3. Silicic acid chromatography of active fractions from Step 2. In the active fractions (Lanes 3 and 4, Fig. 6) there was one component that was not stained by anisaldehyde or copper acetate. This component, which was localized below the fatty acid band, could be stained with iodine vapor (Lane 8, Fig 6) and gave fluorescence with rhodamine 6B under wet conditions in UV. In order to separate this component from fatty acids we used the same column as in Step 2 but the temperature was reduced to 4°C. The Ca^{2+} binding and the channel activity resided in the component of Lane 7 in Fig. 6. As seen in this figure (Lanes 7 and 8), the component of interest was close to the fraction of free fatty acids with a slight overlap.

Preliminary characterization of the purified component. The presence in ^{1}H-NMR of a strong peak at 1.3 ppm (not shown) may be assigned to $-CH_2-$ groups, and a peak at 2.45 ppm indicating the presence of carbonyls. It is thus likely that the component contains aliphatic chains with some carbonyls. There was some free carboxyls seen in the area 9–10 ppm, presumably due to some contaminating free fatty acids; see above. Since these did not bind Ca^{2+} in the binding assay, they are not due to the component of interest. Since the area 4–5 ppm was virtually empty, there is no evidence for ester bonds. The molecular weight estimation by gel filtration in HPLC showed the presence of two peaks corresponding to approximately 750 and 1500 Da which could be due to Holigomerization of the same component.

Oscillatory Channel Activity

In the presence of 10 mM Ca^{2+} the conductance of the channel was 100 pS or multiples thereof (Fig. 7). In Fig. 1 it is seen that the channel has a tendency to remain either open or closed for some time. In the pres-

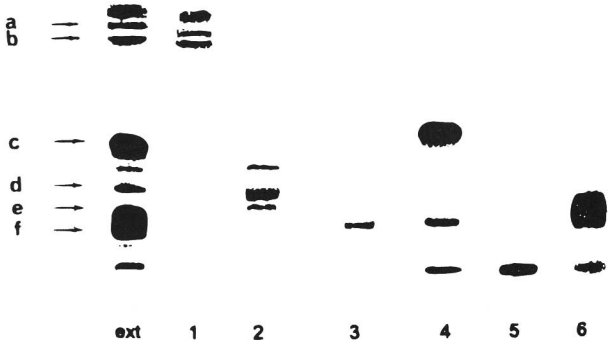

Fig. 5. TLC of fractions from silicic acid column chromatography of lipid extract of mitoplasts. The lipids were stained with anisaldehyde. For experimental details, see Step 1 in Results. Lanes 1–6 correspond to the fractions in Table II. a, cholesterol; b, fatty acids; c, phosphatidylethanolamine; d, cardiolipin; e, phosphatidylcholine; f, phosphatidylserine.

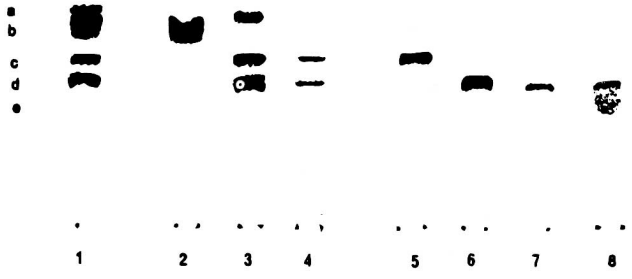

Fig. 6. TLC of active fractions in silicic acid chromatography. Lanes 1–7 were stained with copper acetate, Lane 8 is the fraction of Lane 7 after staining with iodine vapor. Lane 1, fraction 1, see Step 1; Lanes 2–4, fractions after second silicic acid column chromatography, see Step 2; Lanes 5–8 are the fractions after the third silicic acid column chromatography, see Step 3.

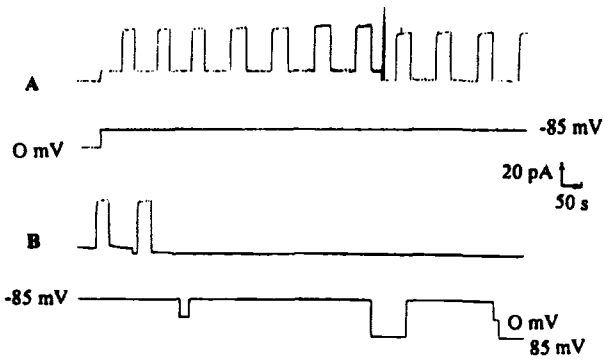

Fig. 7. Oscillations of channel activity. A Ca^{2+} gradient of 4 : 1 was used with 10 mM as the lower concentration. Other conditions correspond to those in Fig. 1. In A, the activity in the beginning of the experiment is shown; in B, when the channel closed permanently after 2 h. The lower traces show the applied voltage.

ence of fairly high concentrations of Ca^{2+} (10 and 40 mM) and Ca^{2+} gradients of 1:4 over the membrane, strikingly regular, clock-like opening and closing of the channel with a conductance of 500 pS were observed (Fig. 7A). In this experiment the oscillations lasted for 2 h, when the channel suddenly remained permanently closed (Fig. 7B), even when changing the applied potential.

Figure 8 shows the same oscillating 500-pS channel reconstituted in another membrane under the same conditions. However, there was a regular spiking-like channel opening superimposed upon the regularly opening and closing channel. In Fig. 8A the channel under the potential of −100 mV initially was in the open state when spiking commenced. Then the regular oscillatory closing and opening of the channel occurred for more than 3 h (Fig. 8A–D). Reversing the potential did not change the general behavior of the system (Fig. 8A). It is noteworthy that during this experiment one channel only was oscillating while several channels remained open (Fig. 8B). Note also one instant (Fig. 8C), at +100 mV, when all channels were closed for the time between two spikes. This suggests that there is a connection between spiking and the channel state. With time there was a gradual decrease in spike amplitude (Fig. 8D). During the 3.5 h duration of this experiment, the time between spikes was slightly prolonged (Table III). The table also shows that the duration the channel stayed open decreased with time while the duration it remained closed was marginally affected.

The lowest concentration of Ca^{2+} at which an oscillating channel was observed was 0.5 mM (Fig. 9). At this concentration of the cation, the conductance of the channel was 20 pS.

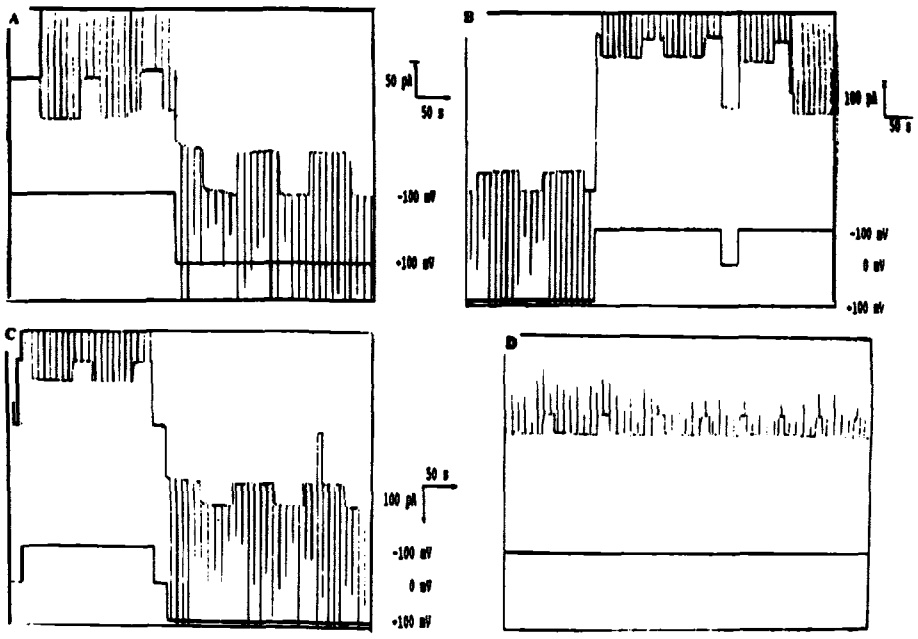

Fig. 8. Oscillation and spiking of channel activity. The experimental conditions were as in Fig. 7. In A, the channel activity in the beginning of the experiment is shown, in B, after 0.5 h; in C, after 1 h, and in D, after 3.5 h.

Table III. Changes in Channel Open/Closed Times and in Spiking Frequency with Time

	Duration of experiment		
	10 min	1 h	3.5 h
Duration of open state (s)	40	16	8.6
Duration of closed state (s)	57	56	43
Time between spikes (s)	7.8	9.7	11

DISCUSSION

This study presents evidence showing that mitochondria contain a component capable of inducing oscillating channel activity in a lipid bilayer in the presence of Ca^{2+}. Under appropriate conditions this activity was continuous for 2–3 h with at most small changes. The channel-forming component is probably localized in the inner mitochondrial membrane though the outer membrane was not excluded; in mitoplasts the outer membrane may still stick to the inner membrane at adhesion sites. The fractionation of organelles yielded highly purified mitochondria with negligible contamination by microsomal membranes as judged by membrane marker enzyme activities (Table I) and by electron microscopy (Fig. 3). The extraction procedure mainly extracts lipids but may contain also other hydrophobic substances.

The channel properties. The studied channel could be activated only in the presence of Ca^{2+}. The concentration of Ca^{2+} influenced the cation–anion selectivity of this channel. At lower concentrations of Ca^{2+} it behaved as a Ca^{2+} channel with low specificity over Cl^-; at higher concentrations the channel behaved rather as a Cl^- channel. This may be due to a change in the charges of the channel gate due to binding of Ca^{2+}. A change in the specificity from a cation to a Cl^- channel has recently been reported for a preparation that may represent the mitochondrial calcium uniporter (Gainutdinov *et al.*, 1994).

Fig. 9. Oscillation of channel activity at low concentration of Ca^{2+}. Experimental conditions correspond to those in Fig. 1, but the concentration of Ca^{2+} was 0.5 mM and the applied voltage 50 mV.

Oscillations in channel activity for extended periods were obtained in the presence of a Ca^{2+} gradient both at high, 10–40 mM (Figs. 7 and 8), and at lower Ca^{2+} concentrations, 0.5 mM (Fig. 9). In both cases the oscillations continued even when the voltage was reversed (Fig. 8), without changes in the properties of the channel.

Oscillations in channel activity have been studied in artificial lipid bilayers (Toko *et al.*, 1991; Yagisawa *et al.*, 1993) and membrane-like monolayers formed from dioleylphosphate or triolein (Urabe and Sakaguchi, 1993) absorbed on a porous filter (Toko *et al.*, 1986, 1991) and in monolayers of oleate at interfaces (Kawakubo and Fukunaga, 1990). One plausible model is that the oscillatory behavior is due to phase transitions between the liquid crystalline state and the gel phase brought about by adsorption and resorption of protons by the membrane surface. This is influenced by changes in cation permeabilities (Yagisawa *et al.*, 1993). One may modify this model by assuming that cations passing through the channel become bound to charged groups in the membrane causing phase transition and closing of the channel. When the cations are lost from the sites, the phase transition reverses and the channel is reopened. The constant open and closed times would then be dependent upon the rates of penetrating the channel, followed by binding to negatively-charged sites, and the rates of diffusion and phase transition. This model is supported by the studies of the effect of Ca^{2+} at 1–10 mM concentrations on phase transitions in bilayers (Antonov *et al.*, 1992). In rats on a diet devoid of essential fatty acids, mitochondrial cardiolipin and phosphatidylcholine contained less of these which could be expected to influence their phase transition behavior, and under oscillatory conditions the oscillatory behavior was indeed changed (Stancliff *et al.*, 1969; Williams *et al.*, 1972).

The spiking may well be related to the phenomenon. The spikes represent very shorts burst of high permeability. It has been found that cardiolipin may form reversed intramembrane vesicles in lipid bilayers

in the presence of Ca^{2+} and also very short-lived inverted micellar intermediates (Siegel, 1984). If such vesicles were momentarily open, they could mediate a fast flux of ions.

An alternative model is that Ca^{2+} influences the properties directly by binding to a regulatory site. This was suggested for an insulin-activated cation channel in skeletal muscle, which in patch-clamp studies exhibited insulin-dependent oscillatory opening and closing in the presence of 1 mM Ca^{2+} (McGeoch and Guidotti, 1992). This channel is mainly a Na^+ channel but allows some penetration of Ca^{2+}. This cation would then slowly penetrate the channel and become bound on the inside of the patch-clamp thereby inhibiting the channel which would be reactivated when the cation had diffused away from its binding site.

The nature of the channel-forming component. Our interest was focused on the hydrophobic Ca^{2+}-binding components present in the mitochondrial extract. During the purification the active fraction was found to have components exhibiting carbohydrate staining and binding lectin. It appeared at this point likely that we were dealing with a glycolipid or glycopeptide. On further purification the channel-forming component was separated from the glycocomponent (Fig. 6, Lane 8). Then, however, the channel activity no longer exhibited oscillatory behavior (not shown). It therefore seems likely that components present in the less extensively purified fraction (Fig. 5, Lane 1), i.e., glycocomponents, fatty acids, and/or cholesterol, may modulate the channel in such a way that it becomes oscillatory. It is of interest that insulin may induce oscillations in a Na^+ channel (McGeoch and Guidotti, 1992), that oxidized cholesterol may change the channel activity of porin (Gallucci *et al.*, 1996), and that arachidonic acid modulates agonist-activated Ca^{2+} entry in glandular cells (Shuttleworth, 1996).

The ^{1}H-NMR spectra indicated that the component contains aliphatic chains with carbonyls. There was some free carboxyl, probably due to contamination from free fatty acids. The component forms oligomers with M_R 750 or 1500. The elucidation of the structure of this lipid compound is in progress.

Relation to mitochondrial channels. Using the patch-clamp technique, a number of channels have been demonstrated in the mitochondrial inner membrane (Kinnally *et al.*, 1992). One slightly anion-selective one has a conductance of 107 pS in 150 mM KCl which is in the range of the presently studied one. Of special interest are Ca^{2+} channels and Ca^{2+}-activated channels. The putative channel component of the mitochondrial calcium uniporter has been purified and its properties described (Mironova *et al.*, 1994), but it is not extracted into the lipid extract and is Ruthenium red-sensitive in contrast to the presently studied channel. In mitochondria there is also a Ca^{2+}-activated, Cyclosporin A-sensitive megachannel; however, its conductance is much higher (Szabó *et al.*, 1992). Of interest is calciphorin, a Ca^{2+}-ionophoretic, 3-kDa protein found in rat liver mitochondria (Ambudkar *et al.*, 1984), but its activity is also inhibited by Ruthenium red and its M_R is higher. A ionophore similar to Gramicidin D was extracted from beef heart mitochondria using Folch extraction and silicagel chromatography (Blondin *et al.*, 1971) as in this study. However, the above-mentioned channels are peptides and there was no indication in the IR spectra of such.

Phospholipids that may mediate increased transport of Ca^{2+} have been extracted from mitochondrial inner membrane proteins (Medvedev *et al.*, 1982; Sokolove and Brenza, 1983). More specifically, oxidized unsaturated fatty acids (Kim and LaBella, 1985; Serhan *et al.*, 1981) may also act as Ca^{2+} ionophores (Serhan *et al.*, 1981) as has been reported also for phosphatidic acid (Serhan *et al.*, 1981) and cardiolipin (Kim and LaBella, 1985); on the other hand such findings have also been disputed (Holmes and Yoss, 1983). Of special interest are lysophospholipids and free fatty acids produced by phospholipase A_2 that may increase Ca^{2+} and H^+ permeabilities in phospholipid bilayers when a membrane potential is induced (Saris, 1972). However, the phospholipids were well separated from the presently studied component by the chromatographic techniques used, and oxidized fatty acids could be expected to induce ion leaks rather than channel activities with well-defined conductances. The Ca^{2+}-binding was not associated with the fatty acid fraction (Fig. 4 and Table II).

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