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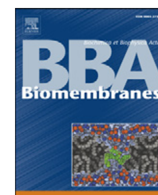

Mitochondria isolated in nearly isotonic KCl buffer: Focus on cardiolipin and organelle morphology

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ABSTRACT

Rat liver mitochondria were isolated in parallel in two different isolation buffers: a standard buffer containing mannitol/sucrose and a nearly physiological KCl based solution. The two different organelle preparations were comparatively characterized by respiratory activity, heme content, microsomal and Golgi contamination, electron microscopy and lipid analyses. The substitution of saccharides with KCl in the isolation buffer does not induce the formation of mitoplasts or disruption of mitochondria. Mitochondria isolated in KCl buffer are coupled and able to maintain a stable transmembrane charge separation. A number of biochemical and functional differences between the two organelle preparations are described; in particular KCl mitochondria exhibit lower cardiolipin content and smaller intracristal compartments in comparison with the standard mitochondrial preparation.

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1. Introduction

The isolation method for mitochondria was developed for the first time in 1948 by Palade et al., who were able to isolate functionally active mitochondria by using a medium not containing electrolytes. In particular hypertonic solutions containing sucrose were found to be more protective for mitochondria, which after isolation could be stained with Janus green B, but not with vital red, as well as mitochondria in intact cells [1].

In addition, Palade observed that in homogenates prepared in nearly isotonic 0.85% NaCl or KCl containing medium mitochondria very quickly became swollen and spherical. It is still generally believed that the presence of KCl in the isolation buffer does not allow the isolation of intact mitochondria and results in the formation of mitoplasts. Nowadays the isolation medium of mitochondria from different tissues consists of a buffered solution containing nearly iso-osmotic sucrose or mannitol or a combination of both sugars, since the use of monosaccharides such as mannitol results in better coupled isolated mitochondria [2]. However, it should be considered that iso-osmotic sucrose and/or mannitol solutions are neither isotonic nor physiological. Indeed, the presence of an impermeable solute in the isolation buffer could induce abnormal ions and water fluxes across the membranes of mitochondria altering the composition of matrix.

The present study was undertaken to examine the possibility of isolating coupled and functionally active mitochondria from rat liver by using a nearly physiological buffer containing isotonic KCl instead of sugars. The biochemical and morphological features of mitochondria isolated in KCl buffer are here described and compared with those of mitochondria isolated using the standard method.

2. Materials and methods

2.1. Materials

Phenylmethanesulfonylfluoride (PMSF), oligomycin, carbonylcyanide-3 chlorophenylhydrazone (CCCP), acetyl-coenzyme A, 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) safranin O and 58 K Golgi protein antibody were all purchased from Sigma (St. Louis, MO).

All organic solvents used were commercially distilled and of the highest available purity (Sigma-Aldrich). Plates for thin layer chromatography (TLC) (Silica gel 60A), obtained from Merck, were washed twice with chloroform/methanol (1:1, v/v) and activated at 120 °C before use.

2.2. Isolation of rat liver mitochondria and microsomes

Animal maintenance, handling and sacrifice were conducted as recommended by the institutional laboratory animal committee of the University of Bari.

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Rat liver mitochondria were isolated by differential centrifugation following a previously described protocol [3]. Two different isolation buffers were used: a) mannitol buffer, containing 0.22 M mannitol, 0.075 M sucrose, 1 mM EDTA, 10 mM Hepes, pH 7.4, and 0.25 mM PMFS or b) KCl buffer containing 0.18 M KCl, 1 mM EDTA, 5 mM MOPS, pH 7.25, and 0.25 mM PMFS. The latter has been previously used to isolate mitochondria from rat heart [4]. The 4000 g supernatant from the mitochondrial preparation was further centrifuged at 100,000*g* for 1 h to obtain a microsomal pellet also containing Golgi complex membranes.

The mitochondrial and microsomial fractions were suspended in mannitol or KCl isolation buffer at a protein concentration of 50–60 mg/ml, as determined by the Biuret method.

2.3. Measurement of oxygen consumption rate

The respiratory activity of liver mitochondria was measured polarographically with a Clark-type electrode, in an all-glass reaction chamber magnetically stirred, at 25 °C. Mitochondrial proteins were suspended at a final concentration of 0.5 mg/ml in a medium containing 75 mM sucrose, 50 mM KCl, 2 mM KH_2PO_4, 2 mM $MgCl_2$, 10 μM EGTA, 30 mM Tris–Cl, pH 7.4. State 4 respiration was started by the addition of succinate (10 mM) in the presence of 2 μg/ml rotenone.

2.4. Measurement of membrane potential

The membrane potential in intact mitochondria was measured as previously described [5] following safranin fluorescence quenching at 525 nm (excitation) and 575 nm (emission) with a Jasco FP 6200 spectrofluorimeter. Mitochondria (0.25 mg/ml) were suspended in 1 ml of the same medium described for respiratory activity measurements, supplemented with 5 μM safranin, at 25 °C. The transmembrane potential was generated by adding 10 mM succinate in the presence of 1 μg/ml rotenone.

2.5. Analysis of cytochrome content

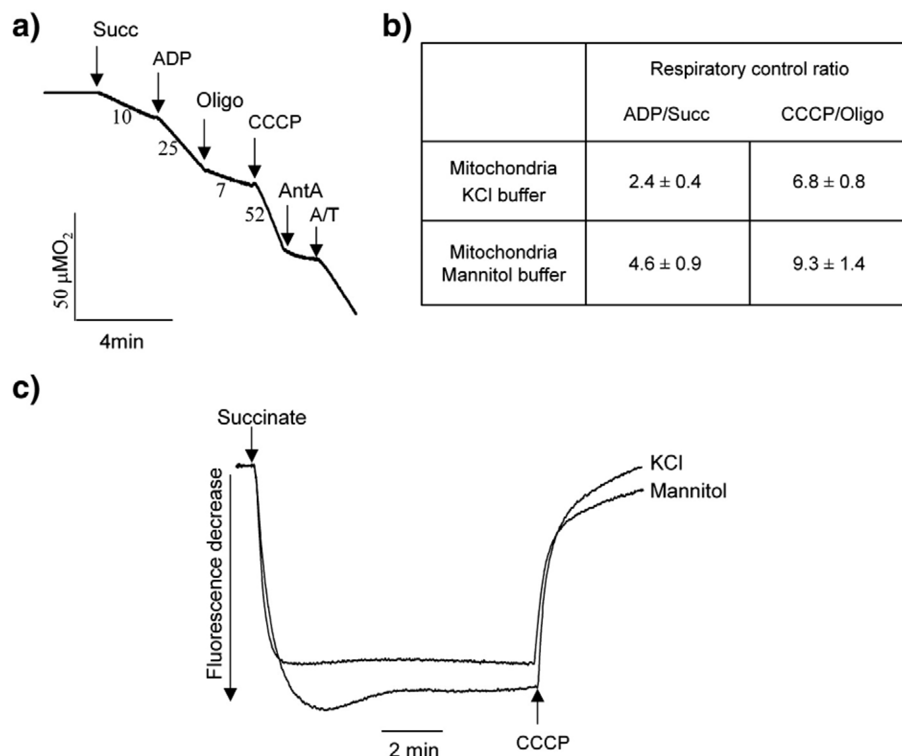
Mitochondria (2.5 mg) were added to 1 ml of 5 mM Hepes (pH 7) and 1% Triton. The cytochrome content was calculated from differential spectra (dithionite reduced minus ferrycianide-oxidized) by using millimolar extinction coefficients of 19.1 and 24 for the c-type (550–540 nm) cytochromes and cytochrome aa_3 (605–630 nm), respectively.

2.6. Assay of NADPH–cytochrome c reductase activity

The determination of NADPH–cytochrome *c* reductase was carried out according to Pederson et al. [6]. Fifty micrograms of proteins of mitochondrial or microsomal fraction was added to 0.3 M phosphate buffer (pH 7.5) containing 75 μM cytochrome *c*, 0.1 mM NADPH and 1 μg/ml rotenone. The reduction of cytochrome *c* was measured spectrophotometrically by following the increase in absorbance at 550 nm.

2.7. Western blotting analysis of 58 K Golgi protein

The mitochondrial and microsomal fractions were subjected to SDS/PAGE for the detection of 58 K protein, a marker of the Golgi complex [7]. Ten micrograms of proteins of each fraction was loaded on the gel. The gel was blotted onto a nitrocellulose membrane and probed by a mouse monoclonal anti-58 K Golgi protein antibody. Immunoblot was performed with HRP-conjugated anti-mouse antibody using the chemiluminescence ECL kit. Relative optical densities of bands were quantified by densitometric analysis (Quantity One-Biorad; BioRad Italy, Segrate, Italy).



	Respiratory control ratio	
	ADP/Succ	CCCP/Oligo
Mitochondria KCl buffer	2.4 ± 0.4	6.8 ± 0.8
Mitochondria Mannitol buffer	4.6 ± 0.9	9.3 ± 1.4

Fig. 1. a) Oxygen consumption measurement in rat liver mitochondria prepared in KCl based buffer. Where indicated ADP (0.5 mM), oligomycin (2 μg/ml), CCCP (0.5 μM), antimycin A (2 μg/ml) and ascorbate/TMPD (10 mM/0.2 mM) were added. Numbers on the trace refer to the rate of oxygen consumption as nmol $O_2\,min^{-1}\,mg\,protein^{-1}$. b) RCR values of mannitol/sucrose and KCl mitochondria. Values are reported as ADP stimulated vs. succinate supported respiration and as CCCP stimulated vs. oligomycin inhibited respiration. c) Transmembrane potential measurements in respiring mitochondria. Transmembrane potential was set up by the addition of 10 mM succinate (plus rotenone) to mitochondria suspended at 0.25 mg protein/ml in the reaction mixture described for oxygen consumption experiments. Where indicated 0.5 μM CCCP was added. KCl and mannitol refer to mitochondria prepared in KCl and mannitol based buffer, respectively.

2.8. Lipid extraction and analyses

Total lipids were extracted using the Bligh and Dyer method [8]; the extracts were carefully dried under N_2 before weighing and then dissolved in chloroform. Total lipid extracts were analysed by TLC on silica gel 60A plates (Merck, 20×10 cm, layer thickness of 0.2 mm). Lipids were eluted with chloroform/methanol/acetic acid/water 85:15:10:3.5, (v/v) or exane/diethyl ether/acetic acid 35:15:0.5, (v/v) and detected by spraying with 5% sulphuric acid, followed by charring at 120 °C. The quantitative analyses of CL content were performed by video densitometry, using the software ImageJ (http://rsb.info.nih.gov/ij). The lipid standard curve was linear in the concentration range of 1–10 µg. For the isolation of each component in the lipid extract, preparative TLC of the total lipid extract was carried out on silica gel 60A plates (Merck, 20×20 cm, 0.5 mm thick layer) in the solvent constituted by chloroform/methanol/acetic acid/water 85:15:10:3.5, (v/v). The silica in each band was scraped from the plate and the lipids were extracted five times with chloroform/methanol (1:1, v/v). The combined supernatants were brought to dryness under a stream of nitrogen. The total lipid extracts and the lipids extracted from each TLC band were analysed by electrospray ionization mass spectrometry (ESI-MS). For negative ion mass spectrometry, lipids were dissolved in chloroform/methanol (1:1, v/v) except for total lipid extracts and cardiolipin, which were converted into the ammonium salt form as described by Kates [9]. Typically, 4.5 ml of 0.2 N HCl was added to a solution containing about 10 mg of lipids in 5 ml of chloroform/methanol (1:1, v/v). The biphasic system was mixed and centrifuged. After removal of upper phase, the lower chloroform phase was washed twice with 4 ml of methanol/water (10:9 v/v). The chloroform phase (containing the free acid form of lipids) was immediately neutralized by the addition of ammonium hydroxide to pH 7–8 and brought to dryness. The residue was dissolved in chloroform/methanol (1:1, v/v).

For positive ion mass spectrometry, lipids were dissolved in chloroform/ammonium acetate 1 mM in methanol (1:3, v/v). ESI-MS spectra were obtained with an API QSTAR mass spectrometer (Applied Biosystem/MSD Sciex, Concord, Ontario, Canada) equipped with a Turbo ion spray interface. MS–MS measurements were carried out by fragmenting the target ions at the proper collision energy (usually−80 eV).

2.9. Electron microscopy

Samples of mitochondrial pellets were fixed in a mixture of 3% paraformaldehyde and 1% glutaraldehyde in 0.1 mol/l PBS at pH 7.4 for 4 h at 4 °C. Some specimens were postfixed in 1% OsO_4 in PBS for 30 min at 4 °C. Fixed specimens were dehydrated in ethanol and then embedded in Epon (TAAB, Reading, England). Ultrathin sections were mounted on nickel mesh grids and stained routinely with uranyl acetate and lead citrate [10].

The grids were observed under a Morgagni 268 electron microscope (FEI; Hillsboro, OR).

Table 1
Heme content, NADPH–cytochrome *c* reductase activity and Golgi contamination in mitochondria prepared in mannitol/sucrose or KCl based buffer. Mean ± standard deviation of triplicates is reported.

	Mitochondria mannitol buffer	Mitochondria KCl buffer
Heme content (nmol/mg protein)		
aa_3	0.21 ± 0.04	0.14 ± 0.02
cytochrome *c*	0.20 ± 0.08	0.13 ± 0.04
NADPH–cytochrome *c* reductase (%)	12.3 ± 6.8	30.4 ± 5.6
Golgi contamination (%)	15 ± 2.5	22 ± 4.1

3. Results

3.1. Respiratory activity and biochemical analyses

The oxygen consumption activity of rat liver mitochondria prepared in a KCl based medium was examined. The polarographic trace reported in Fig. 1a shows coupled succinate supported electron transfer activity, as indicated by the increase in the rate on addition of ADP as well as after the addition of the uncoupler CCCP to oligomycin inhibited respiration. Respiratory Control Ratio (RCR) values are reported in Fig. 1b, where a comparison is made between KCl and mannitol/sucrose prepared mitochondria. The RCR value measured in KCl mitochondria as ADP vs. succinate supported respiration was about half of that exhibited by mannitol mitochondria, whereas the RCR values measured in both preparations as CCCP vs. oligomycin inhibited respiration were definitely closer each other. The RCR differences between the two mitochondrial preparations were almost stable over a period of several hours after the completion of the isolation procedure; on average, RCR values declined by 2 to 5% for both preparations after 3 h. Although KCl mitochondria show a different coupling efficiency in comparison with mannitol mitochondria,

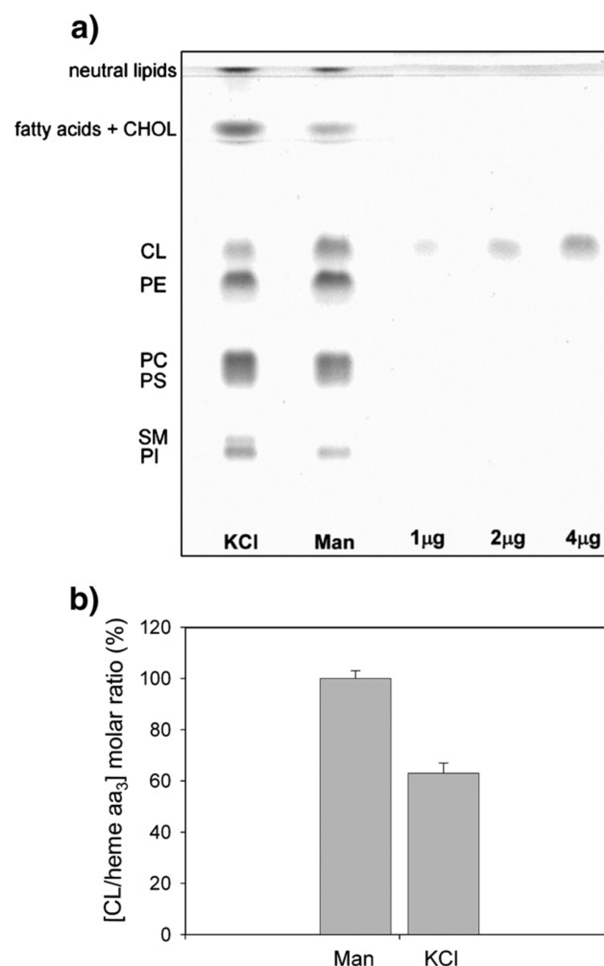


Fig. 2. Thin layer chromatography (TLC) of total lipid extracts of rat liver mitochondria. a) Lipid profile of mitochondria isolated in KCl buffer (KCl) and in mannitol/sucrose buffer (Man). Seventy micrograms of each total lipid extract and 1, 2 and 4 µg of cardiolipin standard were loaded on the plate. b) The bar graph shows quantitative determination of CL in the two different TLC lipid profiles; data are means of three experiments. Lipids were eluted with chloroform/methanol/acetic acid/water 85:15:10:3.5, (v/v) and detected by spraying with 5% sulphuric acid, followed by charring at 120 °C. Lipid bands were assigned by comparison with authentic standards: phosphatidylinositol (PI), sphingomyelin (SM), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE), cardiolipin (CL), and cholesterol (CHOL).

it appears that the substitution of saccharides with KCl in the isolation buffer does not compromise the membrane tightness. This aspect was further substantiated by the observation that KCl mitochondria were able to develop and maintain an uncoupler-sensitive membrane potential to an extent comparable with that measured in mitochondria prepared in mannitol based medium (Fig. 1c). The transmembrane potential of both the mitochondrial preparations was similarly stable over time.

Spectroscopic determinations of heme content are also reported in Table 1. It can be seen that the heme aa_3 and cytochrome *c* content of KCl mitochondria amounted to about 2/3 of that found in mannitol/sucrose mitochondria.

The extent of contamination by the microsomal fraction as well as by the Golgi membranes was estimated by measuring the activity of NADPH–cytochrome *c* reductase and the presence of the 58 K Golgi marker protein (typically associated with the cytoplasmic surface of Golgi apparatus in rat liver [7]), respectively. The data are reported in Table 1, and the values of microsomal and Golgi membrane contamination in the mannitol/sucrose preparation are also reported for comparison. Analyses of microsomal contamination indicate that 85% and 70% of final pellet is represented by mitochondria in mannitol/sucrose and KCl preparations, respectively; the contamination by Golgi membranes is also slightly higher in KCl mitochondria.

3.2. Lipid analyses

Lipids were extracted from the two different mitochondrial preparations by the Bligh and Dyer method [8]. The lipid/protein ratio was found to be 0.24 ± 0.02 and 0.3 ± 0.04 in the mannitol and KCl mitochondria, respectively.

Lipid extracts of mitochondria isolated in mannitol and KCl buffers were analysed by TLC and by ESI-MS. The combination of these two techniques is particularly powerful for the analysis of mitochondrial lipid extracts. Fig. 2a shows the TLC lipid profiles of the two different mitochondria preparations. The Rf of various lipid bands were compared with those of authentic standards. The polar lipids in the extracts were identified in Rf order as phosphatidylinositol (PI), sphingomyelin (SM), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and cardiolipin (CL). The neutral lipids were close to the solvent front.

It can be seen that cardiolipin content was lower in KCl mitochondria than in mitochondria prepared in mannitol/sucrose buffer. Quantitative lipid determination by videodensitometric analyses of TLC plate showed that the CL content of mitochondria isolated in KCl buffer was 1/3 that of standard mitochondria. The low level of CL in KCl mitochondria could be only due to “dilution” of the mitochondrial lipid marker cardiolipin by lipids of extra-mitochondrial membranes

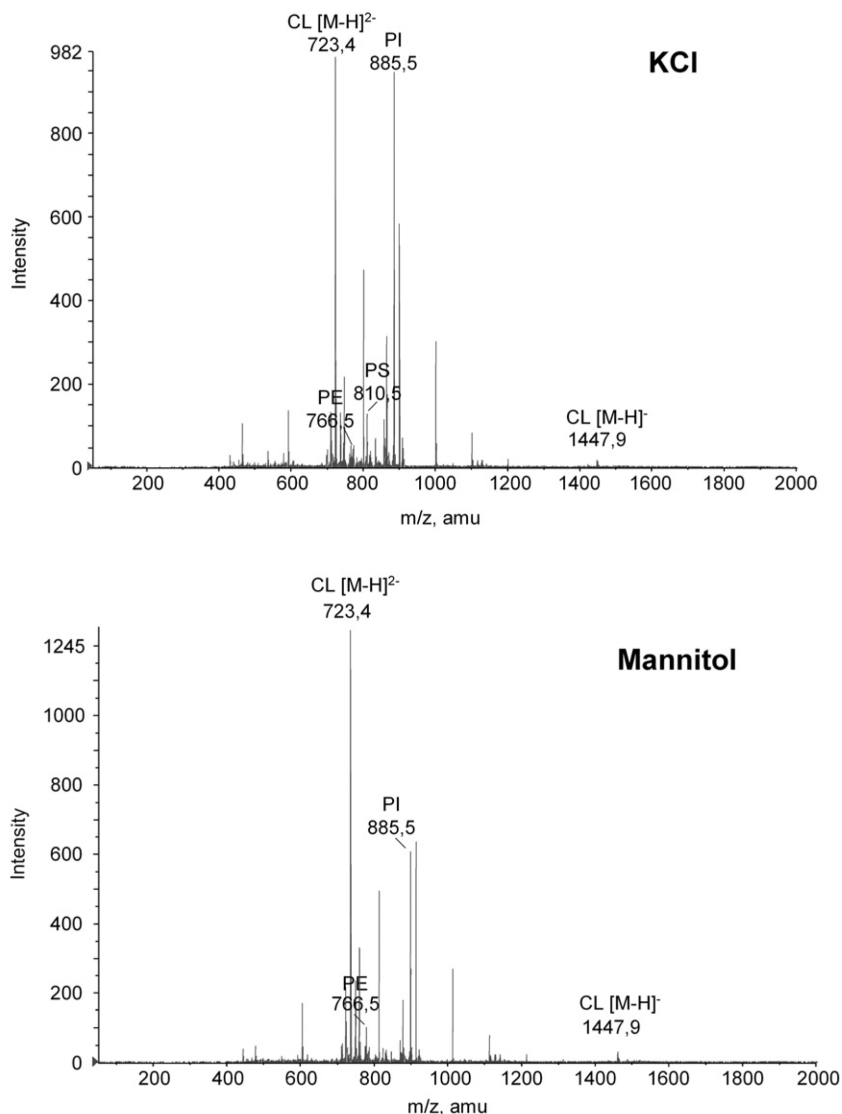


Fig. 3. Negative ion ESI-MS spectra of total lipid extracts of rat liver mitochondria isolated in KCl buffer (a) and in mannitol buffer (b).

(see data in Table 1). However, by expressing the CL content as CL/(heme aa3) molar ratio it is possible to obtain a reliable estimation of cardiolipin levels of the organelles and compare the two preparations, regardless of the accompanying contaminations. From CL/(heme aa3) values of the two preparations (reported in Fig. 2b), it can be concluded that KCl mitochondria typically show a significant decrease in cardiolipin (about 40% less).

As regards the other phospholipids, it can be seen that the bands assigned to PI and PS are more intense in the lipid extract of mitochondria isolated in KCl buffer and in addition that the band of SM is only clearly visible in the KCl profile. The SM associated to mitochondria isolated in mannitol buffer does not typically exceed 1% of total lipid extract [11], thus the presence of higher amounts of SM in KCl mitochondria can be explained by the presence of membrane fragments arising from the trans Golgi network (TGN) and/or endosomes [12]. As far as cholesterol and fatty acids are concerned, it can be seen that these two components comigrate and were enriched in KCl mitochondria compared to the standard preparation; by analysing the lipid extracts of the two different preparations of mitochondria with an appropriate TLC solvent allowing the separation of cholesterol, fatty acids and other neutral lipids, we found that cholesterol, diglycerides, triglycerides and cholesteryl esters, but not fatty acids, were enriched in the lipid extract of mitochondria isolated in KCl buffer (data not shown).

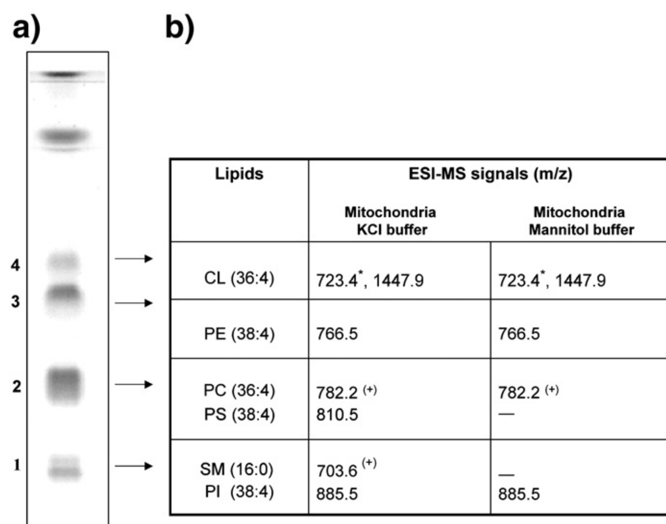
Further insights into the lipid composition of the two different mitochondria preparations were obtained by ESI-MS analyses, described in detail in Fig. 3.

The same main peaks are present in the ESI-MS (−) spectra of the two lipid extracts but with different intensities; the peak at 766 m/z indicates the presence of phosphatidylethanolamine (PE 38:4), the peak at 885 m/z represents phosphatidylinositol (PI 38:4) and the peak at 810 m/z represents phosphatidylserine (PS 38:4).

Since CL is a unique phospholipid having four chains and potentially carrying two negative charges, the cluster centred at 723.4 m/z can be attributed to bicharged CL having four C18 chains with eight total unsaturations. In order to gain direct information about the CL chains, daughter-fragment spectra (MS–MS analysis) of this molecular species were obtained (not shown). The MS–MS spectrum of the molecular species corresponding to 723.47 m/z, in both ESI-MS lipid profiles, shows only one negative ion at 279.2 m/z, which represents a C18:2 chain, thus confirming the previous assertion that the ion at 723.47 m/z has eight total unsaturations. In addition, four main bands were isolated by preparative TLC from each lipid extract. The ESI-MS analyses of the lipids extracted from silica of each TLC band confirmed assignments in Fig. 2. Fig. 4 reports the main peaks in the ESI-MS analyses (either in the negative or positive mode) of various TLC bands. The ESI-MS data here presented are in agreement with those reported in a recent study of identification of lipids tightly bound to isolated cytochrome *c* oxidase [13].

3.3. Morphology

Finally, comparative electron microscopy analyses were performed (Fig. 5). Mitochondria isolated in mannitol/sucrose buffer showed predominantly condensed profiles. In agreement with previous literature reports [2], intracristal spaces (corresponding to the light areas inside the condensed or dark organelles) were altered and more expanded than intracristal spaces of orthodox mitochondria (in situ); the outer membrane was generally seen to be in close apposition to the inner boundary membrane. One hundred twenty-five condensed mitochondria per 500 μm^2 were present in the mannitol preparation; about 10% of those was swollen and contained fragmented cristae. Mitochondria isolated in KCl buffer amounted to 75 per 500 μm^2 and were nearly all in the condensed state also. Interestingly, in this case the intracristal spaces (light areas) appear less expanded or in other words the matrix volume appears larger compared to mannitol



Lipids	ESI-MS signals (m/z)	
	Mitochondria KCl buffer	Mitochondria Mannitol buffer
CL (36:4)	723.4*, 1447.9	723.4*, 1447.9
PE (38:4)	766.5	766.5
PC (36:4)	782.2 (+)	782.2 (+)
PS (38:4)	810.5	—
SM (16:0)	703.6 (+)	—
PI (38:4)	885.5	885.5

Fig. 4. Isolation and characterization of individual polar lipids present in the total lipid extract of rat liver mitochondria isolated with mannitol/sucrose or KCl buffer. TLC lipid profile in (a) refers to the lipid extract of mitochondria isolated in KCl buffer. Lipid bands of both Mann and KCl TLC lanes were scraped from the silica and analyzed by ESI-MS. In the table (b) the molecular ions (m/z) obtained by ESI-MS analyses in negative (−) or in positive (+) mode of each band are reported. (*) Bicharged molecular ion $[M\text{-}2H]^{2-}$.

mitochondria; furthermore in some mitochondria (10% of the condensed forms) the space between the inner and outer membranes appears enlarged. This feature is also present, although to a lesser extent (5%), in our mannitol preparations (not shown) and even in images shown by others [2]. In addition, altered (i.e. swollen) mitochondria (about 10%) together with a considerable presence of membrane debris and glycogen were observed in KCl mitochondria. It is concluded that mitochondria isolated in mannitol/sucrose or KCl buffer are in two different condensed states. The presence of different condensed states of mitochondria has been previously described in the classical studies of Hackenbrock [14,15].

4. Discussion

Although liver mitochondria prepared in KCl medium appear to be less pure than those obtained by using the mannitol/sucrose isolation buffer, they are bio-energetically active and able to maintain a stable transmembrane charge separation (Fig. 1). Accordingly, functionally active mitochondria were isolated from rat heart in KCl containing buffer [4].

Compared to standard mitochondrial preparations, KCl prepared mitochondria exhibit, among other things, a lower content of cardiolipin, which correlates only in part with the higher presence of microsomal and Golgi membranes. In other words the reduction of cardiolipin content of KCl mitochondria is more pronounced than we would expect on the basis of extra-mitochondrial membrane presence and/or heme content. This aspect is quite evident when the CL content is referred to the amount of heme aa3 in each preparation (Fig. 2b).

Other literature studies have pointed out that the amount of cardiolipin in biomembranes is not constant and can be modulated by different factors. In yeast, for example, it has been shown that pH difference across the inner membrane as well as osmotic factors may affect mitochondrial cardiolipin level [16]. Furthermore, several evidences indicate that osmotic stress is an important factor regulating the levels and distribution of cardiolipin in bacteria [17–19].

Also it cannot be excluded that the reduced levels of CL, together with the increase in PI, PS and cholesterol in the lipid extract from KCl prepared mitochondria, could be, at least in part, a consequence of different alterations of phospholipid and cholesterol metabolism and

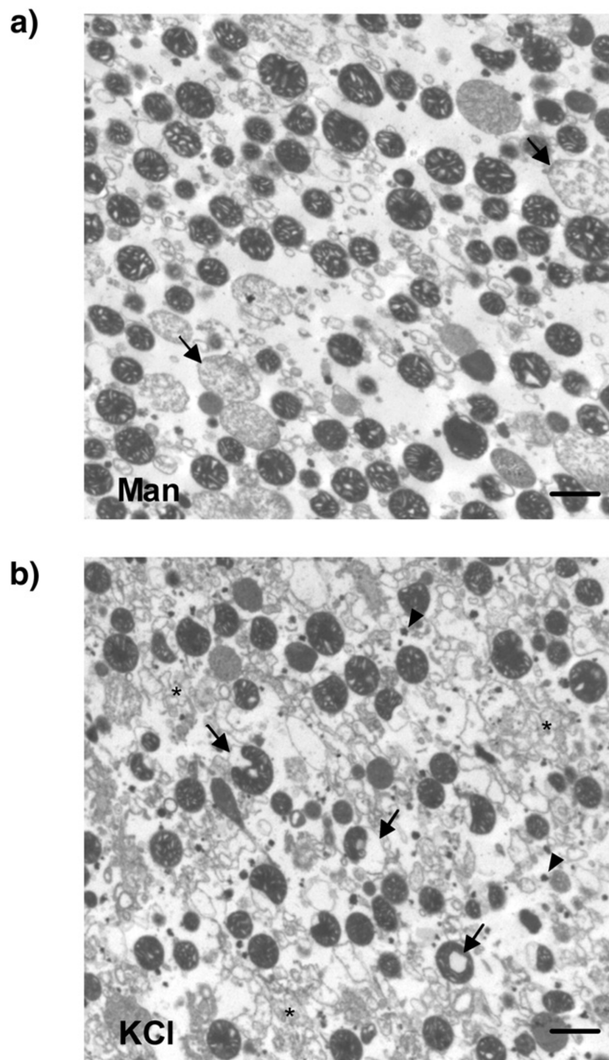


Fig. 5. Ultrastructure of mitochondria isolated from rat liver by using mannitol/sucrose buffer and KCl buffer. a) Mitochondria isolated in mannitol buffer (Man) display predominantly condensed profiles. Some of them show swollen and fragmented cristae (arrows). Mitochondria isolated by using KCl buffer (KCl) are numerically inferior of mitochondria in mannitol buffer. In some mitochondria the space between the membranes is very extended (arrows). Membrane debris (asterisks) and glycogen stores (arrowheads) can also be observed. Scale bar 1 μm. Mitochondria in a) and b) are in two different condensed states; the area of intracristal spaces appears to be larger in a) than in b).

trafficking in the two preparations. During the isolation procedure of cell organelles most interrelationships between cellular subcompartments are altered and/or destroyed. In the case of mitochondria most of the natural contacts with other intracellular membrane systems are probably lost. The traffic of lipids from and to mitochondria implies transfers between i) the inner and outer membranes through the contact sites and ii) the outer mitochondrial membrane and other cellular membranes (ER). The contacts and lipid exchanges between different membranes, as well as the activity of enzymes of the lipid metabolism, might be differently altered during the isolation procedures using KCl or mannitol/sucrose buffers.

Since of the well known cardiolipin–protein interaction with the ADP-ATP carrier as well as with the ATP-synthase (complex V) [20], the low level of cardiolipin in KCl mitochondria might be responsible, at least in part, for the observed lower RCR value (ADP vs. succinate supported respiration) as compared to that of standard mitochondria (see Fig. 1b).

The greater presence of extra-mitochondrial membranes in KCl mitochondria compared to mannitol/sucrose mitochondria could simply be due to differences in sedimentation of intracellular membrane fragments in KCl buffer.

However, in line with the above discussion on lipid trafficking, we favor the hypothesis that the use of KCl buffer during the isolation of mitochondria might preserve part of the structural link between the mitochondrial network and other intracellular membrane systems. For example, it is well known that the zones of close contact between ER and mitochondria, which are called MAM (mitochondria associated membranes) [21], support communication between the two organelles including bioenergetics and cell survival [22].

The nearly physiological saline buffer could also reduce alterations of ionic and water fluxes across the inner and outer membranes of mitochondria, which could even alter the morphology of isolated organelles. It has recently been proposed that the topology of the mitochondrial inner membrane is a regulatory property of mitochondria, under the control of proteins and lipids that affect inner membrane curvature and fusion [23]; interestingly, there is a correlation between cardiolipin content and curvature of biomembranes [24,25].

The novel mitochondrial preparation herein characterized might be useful to further investigate: a) the structural transitions that occur in the cristae during matrix expansion and contraction; b) the influence of inner membrane shape in the diffusion of ions and metabolites between the intracristal and intermembrane compartments, and c) the possible relationship between cardiolipin content and curvature of the inner membrane.

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