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The phosphatidylcholine-transfer protein catalyzed import of phosphatidylcholine into isolated rat liver mitochondria

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In order to study the individual steps involved in the import of phosphatidylcholine (PC) into rat liver mitochondria, a number of PC analogues were introduced into the outer membrane of isolated mitochondria. Two fluorescent PC species, i.e. 1-palmitoyl-2-(16-bimanylthio)hexadecanoyl-PC (bimane-PC) and 1-palmitoyl-2-(10-pyrene)decanoyl-PC (pyrene-PC), and one radiolabeled PC species, i.e. 1-palmitoyl-2-[1-^{14}C]oleoyl-PC (^{14}C-POPC), were studied. The PC analogues were introduced from small unilamellar vesicles with the use of the PC-specific transfer protein. The amount of PC imported was quantified by reisolation of the mitochondria. Import of the fluorescent PC species was monitored by on-line fluorescence spectroscopy. The distribution of the newly inserted PC between the outer and the inner membrane was assessed by separation of the two membranes using digitonin treatment. All analogues tested remained exclusively localized in the outer membrane thereby suggesting that additional (extramitochondrial) factors are required to initiate transfer of PC to the inner membrane.

Introduction

Mitochondria in mammalian cells have a limited capacity to synthesize their membrane phospholipids. This capacity is restricted to the synthesis of the negatively-charged phospholipid cardiolipin and its precursors [1–3]. The other major phospholipids found in the outer and inner mitochondrial membrane (i.e., phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI)) are synthesized in the endoplasmic reticulum (ER) whereafter they are transferred to the mitochondrial outer and inner membrane. Although the exact mechanism of the transport of newly synthesized lipid from ER to mitochondrion has not been established, cytosolic phospholipid transfer proteins have been suggested to play an important role in this process, especially for PC [4,5]. The flow of lipid between the mitochondrial boundary membranes has received much less attention. Since phospholipid transfer activity could not be demonstrated in the intermembrane space [1,6,7], it is considered highly unlikely that soluble lipid carrier proteins participate in this process. Similarly, monomer diffusion through the intermembrane space can be largely ruled out except perhaps for phosphatidic acid [7]. Rather it has been suggested that contact sites between inner and outer membrane may represent a route enabling phospholipid transfer between the inner leaflet of the outer membrane and the outer leaflet of the inner membrane [1,6,8]. However, no direct experimental evidence is available to support this model. It should be stressed that by whatever mechanism lipids are transferred between the two boundary membranes the process must be highly controlled in

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidic acid; ER, endoplasmic reticulum; TNP-PE, trinitrophenyl-PE; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-PE; *N*-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-PE; ^{14}C-POPC, 1-palmitoyl-2-[1-^{14}C]oleoyl-PC; bimane-PC, 1-palmitoyl-2-(16-bimanylthio)hexadecanoyl-PC; pyrene-PC, 1-palmitoyl-2-(10-pyrene)-decanoyl-PC; PC-TP, PC-specific transfer protein; SUV, small unilamellar vesicles; Hepes, 2-[4-(2-hydroxyethyl-1-piperazinyl)]ethanesulfonic acid; TLC, thin-layer chromatography; LSC, liquid scintillation counting; R_f, relative mobility; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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order to guarantee the maintenance of the distinct lipid composition of these two membranes [1].

This paper addresses the question whether transfer of PC from the outer to the inner membrane as demonstrated in vivo (e.g., Ref. 6) can be mimicked in vitro using isolated mitochondria. These experiments require preparations of highly intact mitochondria which show minimal contamination with other organelles. We have recently described an improved procedure to isolate rat liver mitochondrial suspensions which satisfy these criteria [15]. Here we present newly developed methods for the introduction of labeled phospholipids into the outer membrane, for monitoring the import process and for assessing the distribution of labeled lipid between the outer and inner membrane. In the present study, this methodology has been used to study the characteristics of the import of phosphatidylcholine into rat liver mitochondria. Import was from small unilamellar vesicles and was mediated by the PC-specific transfer protein. The effect of mitochondrial respiration on PC import was tested by performing the experiments both in the absence and presence of succinate. The rationale behind this was that respiration induces large changes in mitochondrial conformation [9]. Furthermore, it has been suggested that energization of the inner membrane through respiration is accompanied by a substantial increase in the number of contact sites between outer and inner membrane [10].

Experimental Procedures

Materials. 1-Palmitoyl-2-(16-bimanylthio)hexadecanoyl-PC (bimane-PC) was a gift from Dr. J.R. Silvius (McGill, Montreal). 1-Palmitoyl-2-(10-pyrene)-decanoyl-PC (pyrene-PC) was purchased from Molecular Probes (Eugene, OR). 1-Palmitoyl-2-[1-^{14}C]oleoyl-PC (^{14}C-POPC) (52 Ci/mol) and [1α,2α(n)-^{3}H]cholesteryl oleoyl ether (32 Ci/mmol) were obtained from Amersham. The PC-specific transfer protein (PC-TP) was isolated from bovine liver and stored as described earlier [11]. PC was isolated from egg-yolk according to published procedures [12]. Phosphatidic acid (PA) was prepared from egg-yolk PC as described earlier [13]. Trinitrophenyl-PE was prepared from egg-PE as described by Van Duijn et al. [14]. *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)PE and *N*-(lissamine rhodamine B sulfonyl)-PE were purchased from Avanti Polar-Lipids (Birmingham, AL). The purity of the various lipid samples was regularly checked by TLC in chloroform/methanol/water (65 : 25 : 4, v/v). Digitonin was obtained from Merck and used without further purification. [*phenyl*-3,*benzyl*-^{3}H]Pargyline hydrochloride (22.8 C/mmol) was purchased from New England Nuclear. All other biochemicals used were of the highest purity available and obtained from regular commercial sources.

Isolation and characterization of mitochondria. Mitochondria were isolated from the liver of adult, male Wistar rats, as described elsewhere [15]. The respiratory control ratio and P/O-ratio amounted to 5.4 ± 0.6 (n = 29) and 2.07 ± 0.14 (n = 10), respectively, with succinate (5 mM) as substrate. The integrity of the outer membrane was routinely checked from the degree of latency of the oxidation of exogenously-added reduced cytochrome *c* by cytochrome *c* oxidase [16]. This latency was around 90% (mean: 89.4 ± 2.7 (n = 20)). The purity of the mitochondrial suspensions was determined by marker enzyme analysis and electron microscopy, as detailed elsewhere [15].

Small unilamellar donor vesicles. PC analogues were introduced into the mitochondria from small unilamellar vesicles (SUV). The vesicles were made by hydrating a dry lipid film in 2 ml buffer A (without inorganic phosphate; see below), vortexing and ultrasonication (10 times 30 s, with 2.5 min cooling time) under a N_2 flow and in ice-water at 60 W using a Branson sonifier (medium-size tip). Next, the suspension was centrifuged at 15 000 rpm in a Sorvall SS-34 rotor for 15 min at 4° C. The following vesicle compositions (in molar percentages) were used: (i) pyrene-PC donor vesicles (70 or 10, pyrene-PC; 10 or 70, egg-PC; 5, egg-PA; 10, TNP-PE; 5, *N*-Rh-PE); (ii) bimane-PC donor vesicles (10, bimane-PC; 75, egg-PC; 5, egg-PA; 10, NBD-PE); (iii) ^{14}C-POPC donor vesicles (2.5, ^{14}C-POPC; 92.45, egg-PC; 5.0, egg-PA; 0.05, [1α,2α(n)-^{3}H]cholesteryl oleoyl ether).

Conditions of PC import. All experiments were carried out at 30° C in the following medium (hereafter referred to as Buffer A): 130 mM KCl, 5 mM Hepes, 2 mM potassium phosphate, 1 μM rotenone (pH 7.4). Unless otherwise indicated, PC import was performed under the following standard conditions: 5 μM donor vesicle phospholipid, 5 μg PC-TP/ml, and 125 μg mitochondrial protein/ml which corresponds to a concentration of 11.2 μM total mitochondrial PC [15]. Usually, the process was started by addition of mitochondria from a freshly isolated 25–30 mg/ml suspension on ice.

Percentage of labeled PC in outer monolayer of donor SUV. The percentage of labeled PC which was present in the outer monolayer of the donor SUV was determined as follows. For the two fluorescent species, 2 μg PC-TP was added to 0.5 μM donor SUV in a volume of 2 ml buffer A (see above) in the fluorescence cuvet. Subsequently, acceptor SUV composed of egg-PC/egg-PA (95 : 5, mol/mol) were added from a 2 mM stock solution to obtain molar ratios of acceptor/donor SUV of 20, 30, 40 and 50. At all these ratios, the equilibrium level of transfer of labeled PC from the quenched donor to the unquenched acceptor SUV was determined by fluorimetry. The percentage of fluorophore in the outer monolayer was then calculated by dividing the above

intensity by that obtained upon addition of 25 μl of a 10% (v/v) Triton X-100 solution. The latter intensity was corrected for the direct effect of Triton X-100 by comparing the fluorescence intensity of unquenched SUV of 0.5 mol% fluorescent PC in egg-PC in the absence and presence of Triton. The percentage changes in intensity were +135.3 and −31.4% for pyrene- and bimane-PC, respectively, in the presence vs. the absence of Triton. For donor SUV containing ^{14}C-POPC, rat liver mitochondria (1, 2, 3 and 4 mg protein) were used to deplete the outer monolayer of the donor SUV. After 30 min at 30 °C, mitochondria and donor SUV were separated by centrifugation whereafter ^{14}C and ^{3}H counts were determined in pellet and supernatant, as described below. In all cases, it was found that 70.0 ± 2.1% ($n = 6$) of the labeled PC is present in the outer monolayer of the donor SUV.

Back-exchange experiments. The reversibility of the PC import process was assessed by the following back-exchange experiments. Import of bimane-PC, pyrene-PC or ^{14}C-POPC was performed by incubation at 30 °C for 30 min in the absence or presence of 5 mM succinate, as described above, followed by reisolation and resuspension of the mitochondria. Each preparation was divided in two halves. One half was further incubated in buffer A in the absence of succinate, supplemented with 1 mM KCN. The other half was incubated in the presence of 5 mM succinate. With this set-up we were able to assess simultaneously the effects of succinate respiration on PC import and its subsequent export. Further additions: 11.4 μg/ml PC-TP, and 175 μM egg-PC/egg-PA (95 : 5, mol/mol) SUV acceptor vesicles. Both during PC import and export 1 ml samples were taken at regular intervals, and centrifuged to quantify the mitochondrion-incorporated PC analogue.

Quantification of import. Quantitative data on the extent of import of the labeled PC analogues as a function of time and experimental condition were obtained by separation of donor SUV and rat liver mitochondria via centrifugation of 1 ml samples for 4 min at 14000 rpm in an Eppendorf microcentrifuge (model 5415). When studying ^{14}C-POPC import, mitochondrial pellets were dissolved with 50 μl formic acid whereafter 900 μl buffer A was added. Both the pellet fraction and the supernatant were counted in duplicate by adding 425 μl aliquots to 6 ml Insta-gel (Packard) followed by counting in a Packard 1500 Tri-Carb Liquid Scintillation Analyzer. When fluorescent PC species were used, pellets were dissolved in 1 ml buffer A plus 50 μl 10% (v/v) Triton X-100 after which a 900 μl aliquot was transferred to a fluorescence cuvet, and supplemented with buffer to a final volume of 2 ml. Supernatants (900 μl) were measured after adding 1.1 ml buffer A and 50 μl 10% Triton X-100. The different fluorophores were quantified by calibration of their fluorescence intensities against standards of known concentration.

Fluorescence spectroscopy. Fluorescence spectroscopy was carried out on a SPF-500C spectrofluorimeter (SLM Aminco Instruments), equipped with a thermostatically controlled sample holder and a magnetic stirring device. The bandpasses used were 2.5 and 5.0 nm for excitation and emission, respectively. Unless indicated otherwise, the following excitation (and emission) wavelengths (nm) were used: pyrene-PC, 343 (378); bimane-PC, 390 (468); *N*-Rh-PE, 390 (463); NBD-PE, 473 (535).

Fluorescence microscopy. Phase contrast and fluorescence microscopic images were obtained on a Leitz Orthoplan microscope equipped with epiillumination. Micrographs were taken on Kodak triX-pan film (800 ASA). For bimane-PC specific fluorescence detection, the Leitz filter block D was used having an excitation bandpass filter between 355 and 425 nm, and a low pass 460 nm emission filter.

Evaluation of potential PC metabolism. Mitochondria harbour a number of enzymes which could in principle metabolize the incorporated PC species, e.g., via deacylation/reacylation reactions. Since this would lead to erroneous results, the degree of metabolism was assessed by performing TLC analysis on Bligh and Dyer extracts [17] of mitochondria after import of bimane-PC, pyrene-PC or ^{14}C-POPC in the absence or presence of 5 mM succinate. For the fluorescent species, TLC was performed with $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v) as the eluent. Then the fluorescent spots were scraped from the plate, extracted with 2.5 ml $CHCl_3/CH_3OH/H_2O$ (1 : 2.2 : 1, v/v), the silica pelletted by centrifugation and the amount of fluorescent lipid measured by fluorescence spectroscopy. In some cases, for simplicity donor vesicles were used which contained pyrene- or bimane-PC as the only fluorescent species. Two different solvent systems for TLC were used in the case of ^{14}C-POPC, i.e. the above in which ^{3}H-cholesteryl oleoyl ether has R_f 1.0, and hexane/diethyl ether/acetic acid (85 : 15 : 2, v/v) in which ^{14}C-POPC has R_f 0.0. Spots were visualized by iodine vapour and autoradiography using Kodak Dental film, scraped from the plate and counted by LSC in Insta-Gel (Packard). In all cases, the label was only detectable in the PC spot indicating that the import studies were not complicated by concomitant lipid metabolism.

Selective removal of the outer membrane. Quantitative and selective lysis of the outer membrane from rat liver mitochondria was accomplished with digitonin, according to a slight modification of a method described elsewhere [15]. Briefly, after PC import or prolonged incubation at 30 °C, mitochondria were reisolated by centrifugation for 10 min at 7000 rpm in a SS-34 rotor at 4 °C. The pellet was resuspended in a medium containing 220 mM mannitol, 70 mM sucrose, 2 mM Hepes, and 0.5 g/l bovine serum albumin to obtain a 20 mg/ml suspension. This preparation was then mixed with a 6-fold excess of a non-incubated 20 mg/ml

mitochondrial suspension in the above medium whereafter digitonin treatment was performed as described [15]. By this approach we were able to measure marker enzymes for the various mitochondrial compartments in parallel to the distribution of the PC species over outer and inner membrane.

Control experiments were performed to establish whether the incubated mitochondria displayed a similar sensitivity towards digitonin in terms of marker enzyme and phospholipid profiles as non-incubated mitochondria for which the original procedure had been worked out [15]. To this end, mitochondria were incubated as described for the PC import experiments (except that egg-PC/egg-PA (95 : 5, mol/mol) donor vesicles were used), reisolated, resuspended to 20 mg/ml, treated with digitonin without prior addition of non-incubated mitochondria, and centrifuged for 10 min at 10000 rpm. Marker enzyme activities and phospholipid content of the resulting pellet and supernatant were measured as described previously [15]. It appeared that these incubated mitochondria were as susceptible to digitonin as non-incubated mitochondria [15].

As indicated above, however, digitonin treatment is usually carried out on a mixture of incubated and non-incubated mitochondria. In this mixture, the incubated mitochondria might have a different susceptibility towards digitonin than the non-incubated mitochondria. Further control experiments were conducted to study this possibility. ^{3}H-labeled pargyline was used for this purpose. Pargyline is a specific and covalent inhibitor of monoamine oxidase, as demonstrated by McCauley and co-workers [18], and can therefore serve as a convenient outer membrane marker. First, we have established the feasibility of this method by comparing the extent of labeling with ^{3}H-pargyline to the activity of monoamine oxidase present. Non-incubated, control mitochondria (80 μl 20 mg/ml) were treated with an equal volume of digitonin solution in the range of 0.0 to 0.3 mg digitonin/mg protein and centrifuged whereafter the pellets were resuspended in 150 μl 50 mM potassium phosphate, pH 8.0. Monoamine oxidase activity was assayed on 25 μl of these suspensions according to Ref. 19. The activity decreased gradually from 100% to 12% of the control in the range of 0.1 to 0.2 mg digitonin/mg protein (data not shown). At higher digitonin concentrations no further decrease in activity was observed. To the remaining 125 μl of the above suspensions 2 μl of an ethanolic 0.5 μM solution of ^{3}H-pargyline was added, and the samples incubated at 25 °C for 30 min. Thereafter, a large excess of unlabeled pargyline (1 μl 50 mM) was added to suppress the unspecific incorporation of ^{3}H-pargyline in the mitochondrial membranes. After a further incubation of 5 min, the samples were centrifuged for 10 min at 15000 rpm in a SS-34 rotor and resuspended in 125 μl 50 mM potassium phosphate containing 0.4 mM unlabeled pargyline. After 5 min at 25 °C, the mitochondria were reisolated, resuspended in 100 μl 50 mM potassium phosphate whereafter duplicate samples were taken for LSC. The percentage of ^{3}H counts remaining in the mitochondrial pellet as a function of the digitonin/protein ratio was identical to the residual monoamine oxidase activity (see above). These observations confirm that ^{3}H-pargyline labeling may serve as a marker for monoamine oxidase in digitonin-treated samples.

This procedure was then used to specifically monitor incubated, ^{3}H-pargyline labeled mitochondria with respect to their susceptibility to digitonin in the presence of an excess of non-incubated mitochondria, i.e. according to the procedure used for determining the intermembrane distribution of the labeled PC analogues. The experiment consisted of the following steps. Labeling of mitochondria (6 mg protein) with ^{3}H-pargyline (0.5 μM, 20 μl) was carried out for 5 min at 30 °C in a final volume of 1 ml buffer A. Subsequently, the suspension was diluted 15-fold with buffer A and 10 μl 50 mM unlabeled pargyline was added. After 5 min, mitochondria were reisolated (10 min, 7000 rpm, 4 °C in SS-34 rotor) and resuspended to 10 mg/ml in the medium used for digitonin treatment (see above). One-third of the sample remained on ice. The remaining two-third was used for a further 45 min incubation under conditions of PC import in the absence or presence of 5 mM succinate. After reisolation and resuspension, all three mitochondrial preparations were mixed with excess control mitochondria and treated with digitonin as described above. The pellets were counted in duplicate for ^{3}H-pargyline labeling. The results of this experiment (not shown) indicated that digitonin removed the outer membrane from the fraction of incubated, pargyline-labeled mitochondria with an efficiency identical to that observed for non-incubated mitochondria.

The above experiments demonstrated that, under our experimental conditions, digitonin may indeed be used as a tool to assess the intermembrane distribution of the imported PC analogues.

Miscellaneous procedures. Rat liver microsomes were isolated as described elsewhere [15]. Protein was determined with the BCA protein assay reagent (Pierce) supplemented with 0.1% (v/v) SDS and using bovine serum albumin as the standard. Phospholipid phosphorus was determined by the Fiske-SubbaRow method [20].

Results

Three differently labeled analogues of PC (Fig. 1) were used to study their import into rat liver mitochondria. The general procedure employed consisted of incubating isolated mitochondria in the presence of small unilamellar donor vesicles containing the PC ana-

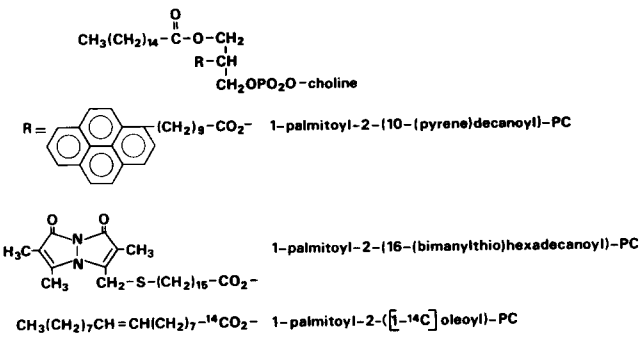

Fig. 1. Structures of the phosphatidylcholines used in this study.

logue and the (bovine liver) PC-specific transfer protein. The latter protein catalyzes a one-to-one PC exchange reaction leading to replacement of endogenous mitochondrial PC with donor vesicle PC, i.e., without modifying the PC content of the mitochondrial membranes [21,22].

Import of bimane-PC

Fig. 2 depicts the results of an import experiment in which the fluorescent PC analogue bimane-PC was used.

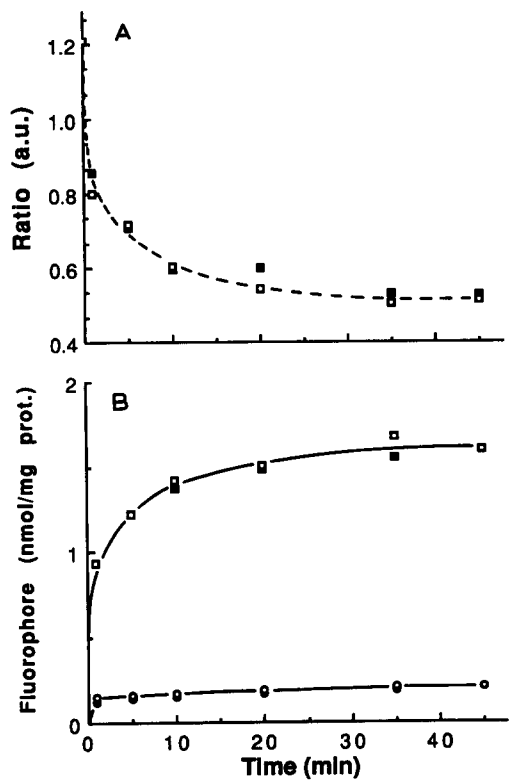

Fig. 2. Import of bimane-PC. Mitochondria were incubated with bimane-PC containing donor vesicles and PC-TP either in the absence (■,●) or presence (□,○) of 5 mM succinate. At the indicated times, samples were taken and processed for quantification of the exchangeable bimane-PC and the non-exchangeable marker NBD-PE by fluorimetry. (A) The ratio of bimane-PC to NBD-PE fluorescence (a.u.) in the supernatant; (B) amount of bimane-PC (□,■) and NBD-PE (○,●) in the mitochondrial pellet.

At the indicated times, mitochondria were reisolated from the import mixture whereafter the supernatant (containing the bulk of the donor vesicles) and the pellet were analyzed for their bimane-PC and NBD-PE content by fluorimetry. The NBD-PE was present in the donor SUV to serve as a non-exchangeable marker allowing correction of bimane-PC incorporation due to aggregation and/or fusion of donor vesicles with the mitochondria. Fig. 2A demonstrates that the donor vesicles became depleted of bimane-PC relative to NBD-PE which was due to PC-transfer protein mediated incorporation of the PC probe into the mitochondria (Fig. 2B). It is interestingly to note that: (i) exchange-equilibrium was reached in approx. 15 min; (ii) both rate (the initial rate amounted to 0.85 ± 0.08 nmol bimane-PC/min/mg protein ($n = 6$)) and extent (1.60 ± 0.04 nmol bimane-PC/mg protein ($n = 6$)) of bimane-PC import were not affected by the addition of succinate to the import buffer.

A crucial prerequisite for the validity of our experimental approach is that all mitochondria participate in the exchange reaction, i.e., receive labeled PC. To test this qualitatively, mitochondrial suspensions were examined by fluorescence microscopy after incubation with donor vesicles containing bimane-PC, in the presence or absence of PC-TP. The bimane-labeled analogue was chosen for this purpose because of its favourable excitation and emission wavelengths, and because of its rapid transfer by PC-TP (see Fig. 2). Mitochondrion-associated fluorescence was only observed when bimane-PC had been incorporated through the action of PC-TP (not shown). As an example, Fig. 3 shows images of a single section of a mitochondrial suspension obtained by phase-contrast (a) and fluorescence microscopy (b), respectively. Comparison of the two images demonstrates a virtually complete overlap of the dark and bright spots indicating that all mitochondria participate in the PC exchange process. Since the mitochondria have a high degree of outer membrane integrity, we presume that the PC-TP mediated incorporation of fluorescent PC occurs predominantly at the level of the outer membrane.

Import of pyrene-PC

The major advantage of employing fluorescent PC species in the present study was that the PC-TP mediated exchange reaction could be monitored on-line in the fluorimeter. By including an efficient quencher of labeled PC fluorescence (i.e., NBD-PE for bimane-PC and TNP-PE for pyrene-PC) in the donor vesicles, only fluorophores incorporated in the mitochondria will give rise to an appreciable fluorescence emission. A typical on-line experiment for pyrene-PC import, as monitored by measuring sequential emission spectra, is shown in Fig. 4. An interesting feature of pyrene fluorescence emission is that it may, in principle, consist of two

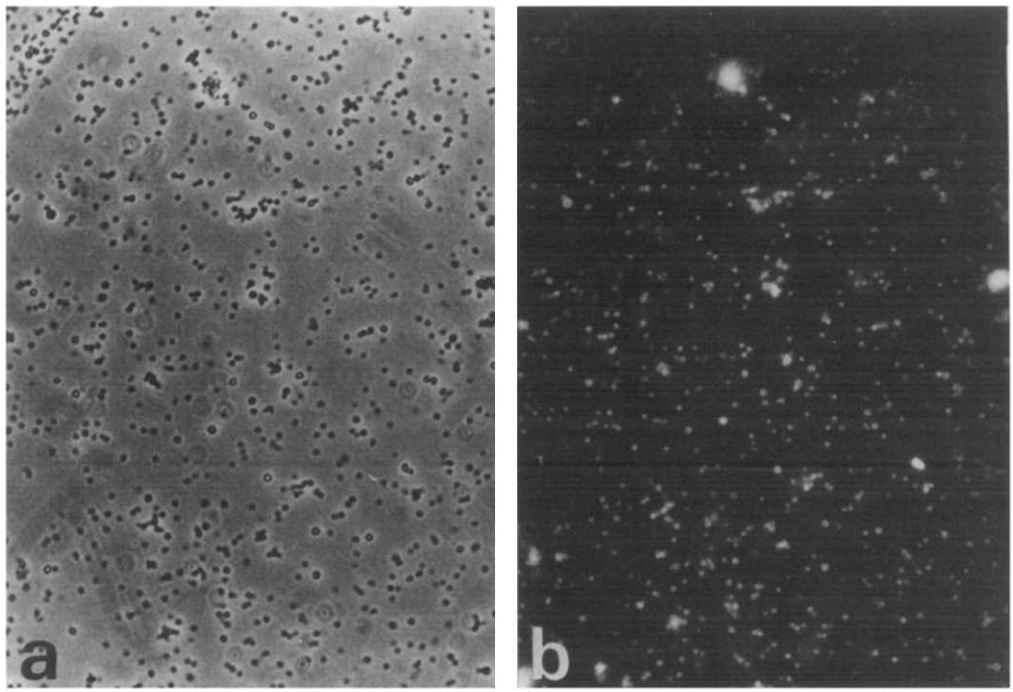

Fig. 3. Phase-contrast (a) and fluorescence microscope (b) images of rat liver mitochondria after import of bimane-PC. Mitochondria were labeled for 15 min at 30 °C whereafter they were reisolated, resuspended to 10 mg/ml and examined by microscopy as described in Methods. Identical fields are displayed in a and b.

spectral components: monomer and excimer fluorescence [23,24]. Monomer emission which has its maximum at 378 nm (see bottom spectra of Fig. 4A and B) is observed at low molar percentages of pyrene-PC. At increasing probe concentrations, the relative contribution of excimer (i.e., excited dimer) fluorescence gradually increases due to an increased probability of complex formation between an excited state and a ground state pyrene monomer [23]. This fluorescence behaviour is evident from the series of emission spectra (Fig. 4) acquired during transfer of pyrene-PC from 70 mol% pyrene-PC SUV to mitochondria in the absence (A) or presence (B) of 5 mM succinate. Addition of PC-TP to the donor SUV results in pure monomer fluorescence (bottom spectra in A and B) due to the fact that part of the protein exchanges its endogenous (non-fluorescent) PC molecule for SUV-associated pyrene-PC (25). Fig. 4 shows that subsequent addition of mitochondria led to spectra containing both monomer and excimer (i.e., the broad peak centered around 480 nm) fluorescence. The contribution to these spectra of monomer fluorescence originating from pyrene-PC associated with PC-TP will gradually decrease as a function of time due to exchange with endogenous, non-fluorescent mitochondrial PC. Clearly, the relative intensity of excimer vs. monomer emission gradually increased as a function of time. The absolute intensity of both monomer and excimer emission were higher in the presence of succinate. Their ratio, however, appeared rather similar in both cases and typically ranged from 0.25 to 0.85. This suggests that the molar percentage of pyrene-PC incorporated into the mitochondria remained the same under both conditions. In the absence of PC-TP, no emission spectra were observed indicating that spontaneous transfer of pyrene-PC to mitochondria was negligible.

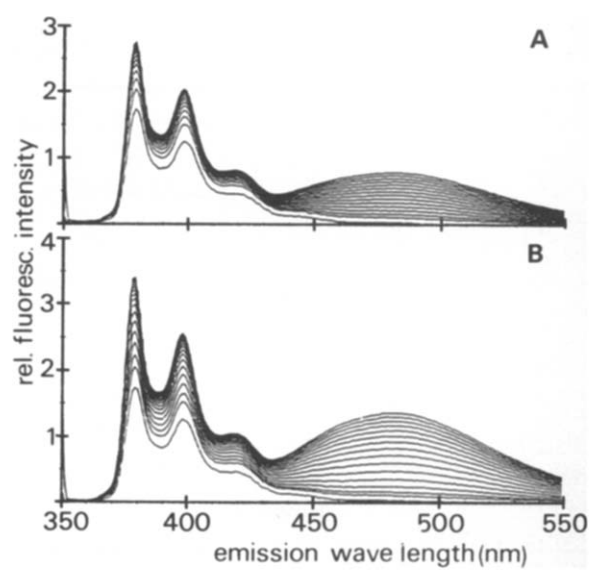

Fig. 4. Fluorescence emission spectra acquired during transfer of pyrene-PC from donor SUV (70 mol% pyrene-PC) to mitochondria mediated by PC-TP in the absence (A) and in the presence (B) of 5 mM succinate. The bottom spectra in A and B are from donor vesicles in the presence of PC-TP. Thereafter, mitochondria were added and spectra taken sequentially every 2 min.

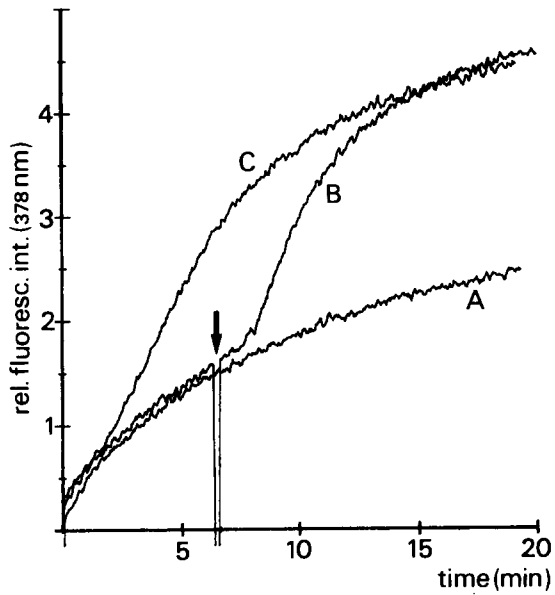

Fig. 5. Import of pyrene-PC as monitored by on-line fluorescence spectroscopy. The following components were sequentially added to 2 ml buffer: (i) donor SUV containing 10 mol% pyrene-PC; (ii) PC-TP; (iii) mitochondria (at $t = 0$). Further additions: (A) no substrate; (B) 5 mM succinate added at time indicated by arrow; (C) 5 mM succinate present throughout. Note that in the absence of mitochondria (not shown) the monomer fluorescence intensity was solely due to PC-TP-associated fluorophore which remained unchanged as a function of time.

Fig. 5 shows an experiment in which the transfer of pyrene-PC from the quenched donor vesicles to the rat liver mitochondria was measured by continuously monitoring the pyrene-PC monomer fluorescence at 378 nm. The transfer resulted in a gradual increase in intensity at this wavelength. Interestingly, the rate and extent of the fluorescence increase were greatly enhanced upon addition of 5 mM succinate. The effect of succinate was fully blocked by the cytochrome bc_1 inhibitor antimycin-A, by KCN and the uncoupler CCCP (not shown). Under these conditions, monomer fluorescence developed according to curve A both in the absence and presence of succinate.

The on-line fluorescence observations could be seriously complicated by changes in the spectroscopic properties of pyrene-PC upon succinate addition. Therefore, the effect of succinate on the import of pyrene-PC was also quantified by reisolating the mitochondria followed by detergent lysis and fluorimetry. The results of an experiment using 70 mol% pyrene-PC donor SUV are shown in Fig. 6. Relative to the import of pyrene-PC in the absence of succinate, the import in the presence of succinate was increased by $61.4 \pm 6.4\%$ ($n = 4$) after 30 min and $53.8 \pm 2.3\%$ ($n = 4$) after 60 min. By measuring the amount of pellet-associated *N*-Rh-PE we could conclude that the spontaneous adsorption of donor vesicles to the mitochondria was very low under both conditions. Essentially identical observations were made when donor SUV containing 10 or 20 mol% pyrene-PC were employed (data not shown) indicating that the effect observed for succinate occurs independently of the amount of pyrene-PC incorporated into the mitochondria.

The following control experiments (not shown) were conducted to assess the specificity of the stimulatory effect of succinate on pyrene-PC incorporation. Firstly, the rate and extent of exchange of pyrene-PC into rat liver microsomes as monitored by on-line fluorimetry remained constant in the range from 0 to 10 mM succinate. Secondly, transfer of pyrene-PC into SUV prepared from the total rat liver mitochondrial lipid extract also showed no succinate dependence. Thirdly, other respiratory substrates (e.g., glutamate, pyruvate + malate, and isocitrate, in the absence of rotenone) could replace succinate in stimulating pyrene-PC import into mitochondria. Fourthly, ATP in the presence of KCN but not in the presence of oligomycin led to a stimulation of pyrene-PC import comparable to that seen with succinate. These data suggest that the electrochemical H^+-gradient across the inner membrane as generated by succinate respiration or by ATP hydrolysis is involved in the stimulation of pyrene-PC import.

Import of ^{14}C-POPC

One of the most abundant molecular species of PC encountered in rat liver mitochondria is 1-palmitoyl-2-oleoyl-PC [26]. For that reason, we have measured the import of ^{14}C-labeled POPC from donor SUV which contained ^{3}H-cholesteryl oleoyl ether as a metabolically-resistant non-exchangeable marker, into isolated mitochondria (Fig. 7). In the presence of PC-TP, there was a rapid incorporation of ^{14}C-POPC. It was consistently found that succinate addition resulted in

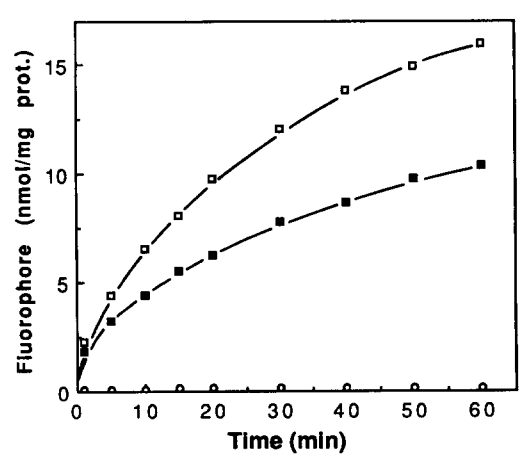

Fig. 6. Quantification of the import of pyrene-PC from 70 mol% pyrene-PC donor SUV in the absence (■,●) or presence (□,○) of 5 mM succinate. At the indicated times, samples were taken and centrifuged to pellet the mitochondria. Pyrene-PC (□,■) and the non-exchangeable marker *N*-Rh-PE (○,●) in the pellet were quantified by fluorimetry in the presence of Triton X-100, as described in Methods.

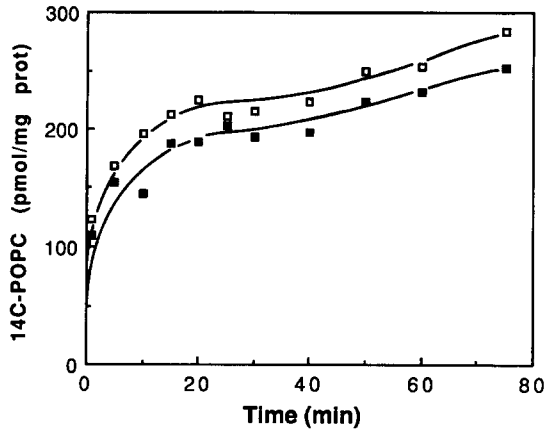

Fig. 7. Import of ^{14}C-POPC from donor SUV containing 2.5 mol% ^{14}C-POPC, and 0.05 mol% ^{3}H-cholesteryl oleoyl ether as a non-exchangeable marker. Conditions: in the absence (■) or presence of 5 mM succinate (□). At the indicated times, samples were taken and centrifuged to pellet the mitochondria.

slightly higher levels of ^{14}C-POPC import than seen in its absence. However, this effect was much less than that observed with pyrene-PC (Fig. 6).

Outer membrane-inner membrane distribution

The central question of the present study is whether the three PC analogues initially incorporated into the outer membrane would be able to flow to the inner membrane. To answer this question we have used digitonin treatment for selective removal of the outer membrane using two different experimental protocols: (i) labeled PC was introduced into the mitochondria by incubation for 60 min in the absence or presence of 5 mM succinate followed by reisolation of the mitochondria and controlled digitonin treatment; (ii) PC import was carried out for 15 min in the absence of succinate whereafter mitochondria were reisolated and further incubated without donor vesicles and PC-TP for 45 min, either in the absence or presence of 5 mM succinate, followed by reisolation and digitonin treatment as above. The results of the latter protocol are summarized in Table I. The data shown were obtained at a digitonin/protein (mg/mg) ratio of 0.3 which had been proven to be optimal [15]. As a control, also the results are shown for mitochondria incubated at 0 °C during the secondary phase of the experiment. Clearly, the major part of the imported PC (approx. 85–90%) was released from the mitochondrial pellet by digitonin treatment irrespective of the type of PC species introduced and the conditions of incubation. Identical results were obtained when the experiment was conducted according to protocol (i) (not shown). Since 85–90% of the outer membrane marker monoamine oxidase was liberated from the mitochondrial pellet (see also Ref. 15) we assume that the 10–15% of labeled PC remaining with the pellet represents outer membrane contamination. We have carefully checked whether digitonin efficacy was identical for the various mitochondrial preparations, i.e., was not affected by pretreatment conditions, both in terms of marker enzyme profiles and phospholipid distribution (see Methods). This being the case, the results in Table I indicate that even upon prolonged incubation the three labeled PC analogues remained essentially restricted to the outer membrane.

Back-exchange of imported PC to excess acceptor vesicles

To gain insight into the reversibility of the PC import process, we have performed back-exchange experiments. After PC-TP mediated import of labeled PC from donor SUV, mitochondria were reisolated and further incubated in the presence of PC – TP and a large excess of (unlabeled) acceptor SUV composed of egg-PC and egg-PA (95 and 5 mol%, respectively). Both during the import and the back-exchange phase samples were withdrawn at regular intervals and centrifuged to separate mitochondria from donor and acceptor SUV, respectively. Import and back-exchange experiments were carried out in the presence and absence of succinate. As an example, Fig. 8A and B depict the results for bimane-PC and pyrene-PC, respectively. In agreement with the data in Figs. 2B and 6, bimane-PC import was unaffected while pyrene-PC import was greatly stimulated by succinate. For the back-exchange phase, only the data obtained in the absence of succinate are shown. Those obtained in the presence of succinate, however,

TABLE I

Digitonin treatment for determining mitochondrial localization of labeled PC

Import of PC was accomplished by incubating mitochondria for 15 min, in the absence of substrate, whereafter mitochondria were reisolated by centrifugation and resuspended for digitonin treatment as described in Methods. One-third of the suspension was left at 0 °C. The rest was further incubated at 30 °C under PC import conditions, except that donor vesicles and PC-TP were omitted. In addition, one-third received 5 mM succinate. After 45 min the latter two mitochondrial samples were centrifuged and the pellets resuspended to 20 mg/ml. Digitonin treatment was performed at digitonin/protein (mg/mg) ratios ranging from 0.0 to 0.3 as detailed in Methods. Results are shown at a ratio of 0.3 only.

PC species	Percentage of labeled PC released by 0.3 mg digitonin/mg protein [a]		
	0 °C [b]	– succ. [b]	+ succ. [b]
Bimane-PC	84.0	89.7	88.0
Pyrene-PC	84.4	91.8	88.8
^{14}C-POPC	84.3	87.3	85.4

[a] The amount of label found in the pellet at 0.0 mg digitonin/mg protein was set at 100%. Values are the average of duplicate samples.

[b] Conditions during secondary incubation were: (i) at 0 °C in medium used for digitonin treatment; (ii) at 30 °C in import buffer either in the absence or presence of 5 mM succinate.

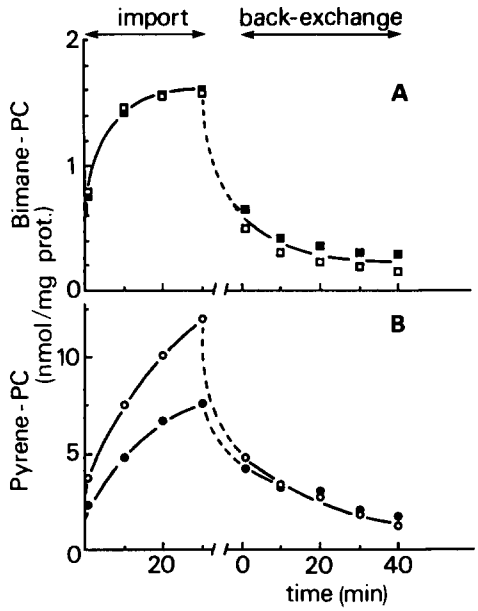

Fig. 8. Import and back-exchange of bimane-PC (A) and pyrene-PC (B). Import was in the absence (closed symbols) or presence (open symbols) of 5 mM succinate. After reisolation and resuspension, mitochondria were further incubated to determine back-exchangeability of PC, as detailed in Methods. Back-exchange was either in the absence or the presence of succinate. For simplicity, only the data on back-exchange in the absence of succinate are shown.

were essentially identical. Irrespective of the absence or presence of succinate during back-exchange, and irrespective of the condition used for import, both bimane-PC and pyrene-PC were readily and essentially completely exchanged to the acceptor SUV. This was also found for similar experiments with ^{14}C-POPC (not shown).

Discussion

This paper describes in vitro import of phosphatidylcholine into rat liver mitochondria. The major goal of these experiments was to establish whether PC newly inserted into the outer leaflet of the outer membrane is able to flow to the inner membrane. The strategy followed was to exchange endogenous mitochondrial PC for a labeled, exogenous species donated from donor SUV through the action of the PC-transfer protein. Inherent to the mode of action of this protein the PC content of the mitochondria should remain unaltered.

Our experimental set-up was aimed at achieving an initial insertion of donor PC into the outer leaflet of the outer membrane. All available evidence seems to indicate that the exchange mediated by PC-TP indeed occurs at this location. The most important arguments in favour of this notion are: (i) the mitochondria have a highly intact outer membrane; (ii) the mitochondrion-associated, bimane-PC specific fluorescence as detected by fluorescence microscopy (Fig. 3) was homogeneously distributed among individual mitochondria; (iii) lysis of the outer membrane by digitonin led to virtually complete removal of incorporated label (Table I).

The distribution of labeled PC between inner and outer membrane was determined using digitonin-induced removal of the outer membrane under conditions where the inner membrane remained largely intact [15]. As detailed in Methods, the validity of using digitonin under the various experimental conditions was carefully verified. It appeared that the PC analogues did not undergo significant transfer to the inner membrane (Table I): only 10–15% of it was retained in the latter fraction. It is important to note that similar levels of the activity of monoamine oxidase [15], and similar amounts of the outer membrane proteins porin and glutathione transferase (as deduced from Western blots employing specific anti-sera (Hovius, R. and Van der Linden, P., unpublished data)) remained associated with the inner membrane fraction as well. This suggests that the major part of the labeled PC present in the pellet at 0.3 mg digitonin/mg protein might be accounted for by remnants of the outer membrane still being attached to the inner membrane. This would imply that, within experimental error, the newly imported PC species tested here were exclusively localized in the outer membrane.

The question then arises whether the virtual absence of transfer of labeled PC to the inner membrane is due to the fact that this process is intrinsically slow for rat liver mitochondria per se or that certain stimulatory elements required to promote it are absent in our in vitro system. The transfer of radiolabeled phospholipids (especially PC and PE) between the endoplasmic reticulum and the mitochondrial membranes of rat liver has been extensively studied both in vivo and in vitro (e.g., using prelabeled microsomes as a donor). From these studies it is evident that the rate of lipid transport from ER to outer membrane in general is considerably faster than that between outer and inner membrane [1,2,6,8,27–33]. Nevertheless, most of the above investigations indicate that there is a significant transport of labeled phospholipid from the outer to the inner membrane within the time frame of our experiments (e.g., Refs. 6,8,27,28,30,32). As an example, Blok et al. [6] showed that 60 min after injection of ^{32}P-phosphate there was essentially isotopic equilibrium for PC between ER and outer mitochondrial membrane of rat liver while the ratio of the specific radioactivity of inner over outer mitochondrial membrane PC amounted to 0.3–0.4. Interestingly, this ratio continued to increase gradually up to a value of 0.7 during prolonged incubation for another 60 min [6]. Similar observations were made by Ruigrok et al. [8] after perfusing rat liver with [*methyl*-^{14}C]choline chloride followed by isolation of subcellular organelles and mitochondrial subfractionation. In addition, these authors observed that perfusion at elevated calcium concentrations led to a greatly in-

creased rate of PC transfer from outer to inner membrane. In conclusion, both in vitro using labeled microsomes in the presence of cytosol and unlabeled mitochondria, and in vivo using pulse labeling a significant intramitochondrial transfer of PC was observed to take place in a 60 min period. The extent of this transfer would have been well above the minimum level of detection of our digitonin method.

The absence of a detectable transfer of labeled PC from the outer to the inner mitochondrial membrane under our experimental conditions suggests that this process requires certain cytosolic components. We have recently obtained evidence which supports this suggestion (Hovius, R., Nicolay, K., unpublished experiments). Rat liver cytosol appeared to stimulate transfer of PC to the inner membrane, in a time- and concentration-dependent manner.

As discussed above, the three labeled PC analogues were restricted to the outer membrane. The question then arises where the labeled PC was localized in the outer membrane, e.g., did it have access to the entire outer membrane or only to part of it. Although no definite conclusion can be drawn as yet, it is valuable to discuss the data in terms of the sizes of the endogenous mitochondrial PC pools available for exchange by exogenous donor PC. Table II is a compilation of the data available thus far. It is of note that in the exchange reaction bimane-PC has completely equilibrated between donor vesicles and mitochondria. As for pyrene-PC and ^{14}C-POPC equilibrium has not been completely attained yet. This implies that the mitochondrial PC pools calculated from the import data with these two probe lipids are a minimal value. The numbers fall in two categories: (i) 17–19 nmol PC/mg protein for bimane-PC and for pyrene-PC in the absence of respiratory substrate; (ii) 32–38 nmol PC/mg protein for ^{14}C-POPC and for pyrene-PC in the presence of 5 mM succinate. As detailed elsewhere [15], mitochondrial PC amounts to 89 nmol/mg total mitochondrial protein and is equally distributed between both boundary membranes. This implies that the numbers in the above first category are approximately 40% of the outer membrane PC pool whereas those in the second category are in the order of 80% of the entire outer membrane pool of PC. The latter finding is in good agreement with our observation that the labeled PC species remained exclusively localized in the outer membrane (Table I).

A straightforward explanation for the differences in pool size available to the three PC analogues is lacking. Firstly, the transbilayer distribution of PC across the outer membrane of rat liver mitochondria is not known. The only relevant report to data on this topic is that from Sperka-Gottlieb et al. [34] on outer membrane vesicles derived from mitochondria of the yeast *Saccharomyces cerevisiae*. Using a bovine heart PC/PI transfer protein preparation, these authors came to the conclusion that 48% of the outer membrane PC was localized in the cytoplasmic leaflet. Secondly, the rate of transbilayer movement of the present PC analogues and, in general, of endogenous mitochondrial PC is unknown.

TABLE II

Mitochondrial PC pools available for exchange by PC-TP

Import of bimane-PC, pyrene-PC and ^{14}C-POPC was carried out for 60 min in the absence or presence of 5 mM succinate, as described in the legends to Figs. 3, 6 and 7, respectively, and in Methods. Thereafter, mitochondria were reisolated and the amount of labeled PC incorporated quantified. The latter was corrected for contamination with donor vesicles using the appropriate non-exchangeable marker to obtain the net amount of label introduced through PC-TP mediated exchange. The size of the mitochondrial PC pool in which the analogue was introduced was calculated as follows. From the net amount of label incorporated into the mitochondria, the amount of labeled PC remaining in the outer leaflet of the donor vesicles was calculated (the percentage label present in the outer leaflet of the donor vesicles initially had been determined as described in Methods). This allowed the molar percentage of labeled PC remaining in this leaflet after exchange to be calculated. Assuming equal molar percentages of labeled PC in the exchangeable fractions of the donor vesicles and mitochondria (i.e., assuming exchange equilibrium) the size of the mitochondrial PC pool available to exchange for the different PC analogues was then calculated.

PC species	Exchangeable PC pool (nmol/mg protein) [a]	
	− succ [b]	+ succ [b]
Bimane-PC [c]	17.5	17.8
Pyrene-PC [d]	19.2	38.2
^{14}C-POPC [d]	32.4	35.3

[a] Values are averages from two independent experiments with duplicate determinations in each experiment.
[b] Import was in the absence or presence of 5 mM succinate.
[c] Equilibrium in the exchange reaction had been reached.
[d] Equilibrium had not been completely reached.

Conclusions

We have shown that three different PC analogues (two fluorescent- and one radio-labeled) were readily imported into the outer membrane of isolated rat liver mitochondria. The absence of the transfer of any of these lipids to the inner membrane suggests that certain additional factors (e.g., cytosolic proteins) are necessary to drive this process for PC.

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