Intracellular Transport of Phosphatidylcholine to the Plasma Membrane

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ABSTRACT We have used pulse-chase labeling of Chinese hamster ovary cells with choline followed by plasma membrane isolation on cationic beads to study the transport of phosphatidylcholine from the endoplasmic reticulum to the plasma membrane. We have found that the process is rapid (t½ [25°C] = 2 min) and not affected by energy poisons or by cytochalasin B, colchicine, monensin, or carbonyl cyanide p-chlorophenylhydrazone. Cooling cells to 0°C effectively stops the transport process. The intracellular transport of phosphatidylcholine is distinct in several ways from the intracellular transport of cholesterol (Kaplan, M. R., and R. D. Simoni, 1985, J. Cell. Biol., 101:446-453).

In the study reported here, we have used a rapid plasma membrane isolation procedure developed previously (15) to study the translocation of phosphatidylcholine (PC) from the endoplasmic reticulum to the plasma membrane of Chinese hamster ovary cells. We have characterized the process of PC transport to the plasma membrane and compared the properties of this process with those for cholesterol transport.

MATERIALS AND METHODS

Materials: [methyl-3H]Choline chloride (80 Ci/mmol), [methyl-14C]choline chloride (50.5 mCi/mmol), 1, 2-14C-acetate (55.0 mCi/mmol), and [2-3H]-glycerol (500 mCi/mmol) were from New England Nuclear (Boston, MA). Choline chloride (crystallized three times), cytochalasin B, colchicine, and carbonyl cyanide p-chlorophenylhydrazone (CCCP) were from Sigma Chemical Co. (St. Louis, MO). Monensin was from Calbiochem-Behring Corp. (La Jolla, CA). Silica gel G plates (250 µm) were from Analtech, Inc. (Newark, DE), and tissue culture media and serum from Gibco Laboratories (Grand Island, NY).

Cell and Culture Conditions: Chinese hamster ovary cells were grown in suspension culture in a moist 5% CO2 environment at a density of 0.4-1 x 10⁶ cells/ml in alpha minimal Eagle's medium supplemented with 10% lipoprotein-depleted fetal calf serum (16), 0.1 mg/ml streptomycin, and 100 IU/ml of penicillin.

Prelabeling and Pulse-Chase Radiolabeling of Cells: 16-24 h before the experiment, cells were suspended in alpha minimal Eagle's medium as above except it was pyruvate-free and choline-free. At this time, 14C-acetate was added (1 µCi/100 ml suspension), and cells were allowed to grow for 16-24 h. Cells were then harvested and resuspended in choline-free alpha minimal Eagle's medium as above, which has been additionally buffered with 15 mM HEPES, pH 7.0 (10⁶ cells/ml). Cells were equilibrated to 25°C, unless otherwise stated, and the radioactive precursor added. The precursor was made up of [3H]choline (25 µCi per 1.5 x 10⁶ cells) diluted with cold choline chloride (62.5 nM final concentration in the incubation mixture). After labeling for 2 min, 1 mM choline chloride chase was added at 25°C (unless otherwise stated).

Metabolic Energy Depletion: Energy poisons were added from a
neutral solution of 1 M KF and 0.1 M KCN, diluted to 310 mM. The inhibitors mixture was added to the cell suspension to a final concentration of 20 mM KF and 2 mM KCN.

**Lipid Analysis:** Cells were extracted (17), then the lipids separated on silica gel G plates that had been preactivated for 1 h at 110°C. Plates were developed in chloroform/methanol/acetic acid/water at 25:15:4:2 (vol/vol). Spots were identified by running lipid standards and visualized by iodine vapor. The following Rf values have been observed in this system: sphingomyelin, 0.23; PC, 0.4; PE, 0.72. The PC spots were scraped and the radioactivity measured in a toluene/Triton scintillation mixture after adding 1 ml of water to the silica.

**Determination of Lipid Phosphorus:** Lipid was extracted from the silica by the following sequence of solvents: twice with 3 ml of chloroform/methanol/acetic acid/water at 25:15:4:2 (vol/vol), once with 2 ml methanol, then 2 ml methanol/acetic acid/water at 94:1:5 (vol/vol). Quantitative elution was verified by counting the silica. Solvents were then blown down and phosphate determined by the method of Bartlett (18). As a control, an area of the silica where no sample had been applied was assayed.

**[3H]Glycerol [4C]Choline Double Labeling:** Cells were grown for 16–24 h in a choline-free medium as described for prelabeling except the label was omitted. Cells were then resuspended in fresh choline-free medium buffered with 15 mM HEPES at 107 cells/ml. [3H]Glycerol (1 mCi per 8 × 107 cells) and [4C]choline chloride (1 μCi per 8 × 107 cells) were added, and cells were incubated for 2 h at 25°C. Plasma membranes were isolated and the lipid analyzed for 4C and 3H counts.

**Plasma Membrane Isolation on Beads:** Plasma membranes were isolated on DEAE-Sephadex beads exactly as we described previously (15), namely, pulse-chase labeling followed by rapid isolation of the plasma membrane on DEAE-Sephadex beads. We chose [3H]choline as a precursor, which, under our conditions, is efficiently incorporated into PC, yielding a high constant for all samples within an experiment (Table I). It is important that the chase of [3H]choline with choline be effective. That is verified in the inset to Fig. 2, indicating that the addition of 1 mM choline to the cells quickly chases the [3H]choline (62.5 nM).

**RESULTS**

**Incorporation of [3H]Choline into PC**

To follow the rate of transport of newly synthesized PC to the plasma membrane, we used the methodology developed previously (15), namely, pulse–chase labeling followed by rapid isolation of the plasma membrane on DEAE-Sephadex beads. We chose [3H]choline as a precursor, which, under our conditions, is efficiently incorporated into PC, yielding a high enough specific activity during a 2-min labeling. We have determined that >85% of the [3H]choline that was incorporated into lipid in 2 min is associated with PC (not shown).

Since lipid analysis is multistep and recoveries vary between samples, it is essential to have an internal standard. We thus prelabeled cells with [4C]acetate for 16–24 h before the experiment and have established that the 4C/mass for PC is constant for all samples within an experiment (Table I). It is important to verify that during the chase, PC synthesis continues at a constant rate, and that kinetics of transport is not affected by changes in the level of synthesis. The linear incorporation of [3H]choline into PC under continuous labeling at 25° and 37°C is shown in Fig. 1. It is also important that the chase of [3H]choline with choline be effective. That is verified in the inset to Fig. 2, indicating that the addition of 1 mM choline to the cells quickly chases the [3H]choline (62.5 nM).

**Kinetics of PC Transport and the Effect of Energy Poisons**

Initial attempts to follow the kinetics of PC transport to the plasma membrane and is calculated as [3H]/[4C] PC in plasma membrane/ [3H]/[4C] PC in cells × 100.

![Figure 1](https://example.com/figure1.jpg)

**FIGURE 1** Incorporation of [3H]choline into PC. Cells were prelabeled with [4C]-acetate (Materials and Methods), then resuspended in choline-free medium at 107 cells/ml. [3H]choline was added (1.7 μCi/ml) in a final concentration of 1 mM choline. Samples were withdrawn at the indicated times, dripped into 10 vol of ice-cold PBS, and washed twice with the buffer. Lipid was then extracted and analyzed. The labeling was carried out at 37°C and 25°C.

<table>
<thead>
<tr>
<th>MINUTES</th>
<th>[3H]/[4C]PC in plasma membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.25</td>
</tr>
<tr>
<td>60</td>
<td>0.50</td>
</tr>
<tr>
<td>120</td>
<td>0.75</td>
</tr>
</tbody>
</table>

**FIGURE 2** Transport of PC to the plasma membrane. The protocol of pulse–chase radiolabeling has been carried out as described in Materials and Methods. At various times during the chase, aliquots of cells are removed and plasma membranes isolated on beads, and a sample of whole cells removed and washed. Lipid has been extracted and [3H]/[4C]PC in plasma membranes and cells determined and the [3H]PC in the plasma membrane calculated as in Materials and Methods. In a separate experiment, the same protocol was followed except that energy poisons were added together with the chase (Materials and Methods). The inset shows the efficiency of a 1 mM choline chase. The values given are [3H]/[4C]PC in cells.

![Figure 2](https://example.com/figure2.jpg)

**TABLE I. Correlation between [4C]PC and PC Mass**

<table>
<thead>
<tr>
<th>PC phosphate</th>
<th>[4C]PC</th>
<th>[4C]PC P/PC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole cells</td>
<td>39.6</td>
<td>2353</td>
</tr>
<tr>
<td>Plasma membrane</td>
<td>10.4</td>
<td>598</td>
</tr>
</tbody>
</table>

Cells were prelabeled with [4C]-acetate. Plasma membranes were isolated from 1.5 × 107 cells and whole cells washed as in Materials and Methods (2.5 × 108 cells per sample). Lipid was extracted from cells and plasma membranes, and PC isolated. [4C] and PC phosphates were determined (Materials and Methods). Each value is an average of three experiments.
plasma membrane at 37°C indicated that the process is extremely rapid. Thus, all experiments were done at 25°C. A typical pulse-chase experiment is presented in Fig. 2. There appears to be a major, very fast component of the process (t1/2 = 2 min) followed by slow arrival of PC which levels off after 2½ h. About 4% of the total PC in the cell is consistently recovered in the plasma membrane fraction when transport is complete. Taken that the plasma membrane yield in this procedure is ~10%, this means that ~40% of the cellular PC is in the plasma membrane.

To find out whether the process is dependent on metabolic energy, a combination of KF and KCN was used. We have previously shown that these poisons stop lipid synthesis in <3 min (19). As indicated in Fig. 2, PC transport does not seem to be affected by depletion of metabolic energy. The apparent difference at the longer time points is not reproducible.

Temperature Dependence of PC Transport

To use the bead/plasma membrane method for measuring PC transport, it is essential that cooling the cells to 0°C effectively arrests the transport such that there is no additional phospholipid redistribution during plasma membrane isolation. To demonstrate this, cells were pulse-labeled with [3H]-choline for 2 min at 25°C, then rapidly cooled to 0°C and held at that temperature for 30 min. They were then rapidly warmed to 25°C, and transport of [3H]PC to the plasma membrane was determined. Little or no transport occurred at 0°C, and when cells were warmed to 25°C, transport was resumed (data not shown).

We have attempted to examine the effect of temperature on the transport process more carefully even though the transport of PC is quite rapid and makes the precise determination of rates difficult. In these experiments, the pulse and the chase were done at the same temperature, since it is difficult to change the temperature between the synthesis phase and the transport phase. The data presented in Fig. 3 shows the effect of temperature between 15°–37°C on the transport process. The half times for transport over this temperature range vary between 1 and 4 min.

![Figure 3](https://example.com/figure3.png)

**Figure 3** Temperature dependence of PC transport. [3H]Choline labeling for 2 min and choline chase were as in Materials and Methods, except that the pulse and the chase were carried out at the indicated temperature. For each curve, cells were pre-equilibrated at the indicated temperature before the pulse and the same temperature kept throughout the experiment: ●, 15°C; ○, 20°C; △, 25°C; □, 37°C.

Effect of Cytochalasin, Colchicine, Monensin, and CCCP on PC Transport

In an attempt to gain some insight into the mechanism of PC transport, several drugs have been screened for their effect on the transport process. For these experiments, it is especially important to preincubate the cells with the drugs in order that they have an opportunity to exert their effects, since the transport process is so rapid. Drugs were added 30 min before the pulse whenever possible, i.e., whenever they did not stop PC synthesis. The percentage of [3H]PC in the plasma membrane at 1 and 60 min after the pulse are presented in Table II. There is considerable variability in the values at 1 min since it is on the steep part of the rate curve. The values at 60 min, however, are all in close agreement, clearly indicating that cytoskeleton-disrupting agents, monensin, or CCCP do not affect the transport of PC to the plasma membrane.

Ruling Out Base Exchange

It is essential to establish that the increase in [3H]PC in the plasma membrane fraction reflects transport from the endoplasmic reticulum rather than direct incorporation of [3H]-choline into the plasma membrane by some lipase-mediated base exchange reaction. One indication that base exchange is not a problem is indicated in the pulse-chase experiments (Figs. 2 and 4) in which the [3H]/[14C]PC in the plasma membrane at the end of the pulse is always much lower than that of the whole cells. This indicates that during the 2-min pulse, [3H]choline is incorporated mainly (or only) into a non-plasma membrane compartment, presumably the endoplasmic reticulum. However, it still remained to be shown that the process measured during the chase is, in fact, transport. To establish this more carefully, cells were double labeled with [3H]glycerol and [14C]choline as described in Materials and Methods and the [3H]/[14C]PC in cells and plasma membranes determined. The values obtained were 0.39 ± 0.03 for plasma membrane and 0.43 ± 0.03 for whole cells (four experiments each). Thus, it appears that both precursors have been incorporated via de novo synthesis.

DISCUSSION

Using choline as a specific precursor of PC, coupled with a

**Table II. Effect of Drugs on PC Transport**

<table>
<thead>
<tr>
<th>Percentage [3H]PC in plasma membrane</th>
<th>Chase time</th>
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<tbody>
<tr>
<td></td>
<td>1 min</td>
</tr>
<tr>
<td>No additions</td>
<td>23.3</td>
</tr>
<tr>
<td>Cytochalasin B (10 μM)</td>
<td>36.7</td>
</tr>
<tr>
<td>Colchicine (100 μM)</td>
<td>21.7 (25.0)</td>
</tr>
<tr>
<td>Monensin (10 μM)</td>
<td>33.3 (26.7)</td>
</tr>
<tr>
<td>CCCP (1 μM)</td>
<td>35.0 (26.7)</td>
</tr>
<tr>
<td>CCCP (25 μM)</td>
<td>33.3</td>
</tr>
</tbody>
</table>

Prelabeling and pulse-chase at 25°C were carried out, with drugs added either with the chase or 30 min before the pulse (results in parentheses). Samples were withdrawn at 1 and 60 min, and the percentage of [3H]PC in the plasma membrane was determined. The control sample contained the same amount of solvent used to apply the drugs (0.25% dimethyl sulfoxide, 0.25% ethanol).
rapid plasma membrane isolation technique, we have determined the rate of transport of PC from its site of synthesis in the endoplasmic reticulum to the plasma membrane. The process is rapid with an apparent half time of transport of ~2 min at 25°C. The transport kinetic pattern may be biphasic, with ~90% of the transported PC being translocated in the rapid first phase followed by a rather slow movement of the remaining 10% over 2 h. Whether the slow rate represents a separate process or is of any significance is not clear.

Choline can be incorporated into PC either via the cytidine diphosphate-choline pathway occurring in the endoplasmic reticulum or by a base-exchange reaction with pre-existing glycerophospholipids (20). Contradicting results can be found in the literature regarding the relative rate and extent of these two pathways (21–23). The fact that we obtained the same ratio of glycerol/choline incorporated into whole cells and plasma membrane means that they got to the plasma membrane by the same pathway, namely, from de novo synthesis in the endoplasmic reticulum. This rules out the possibility that direct choline incorporation into the plasma membrane contributes significantly to the transport process being measured.

It is interesting to compare the rates of phospholipid transport observed in these experiments with those obtained by others. Yaffe and Kennedy (9) have determined that the half time for PC transfer from the endoplasmic reticulum to the mitochondria in baby hamster kidney cells is a few minutes. In the same study, they also demonstrated that the transport of PE was 20–80-fold slower than was the transport of PC. Sleight and Pagano (14) have measured the rate of transport of PE to the plasma membrane in Chinese hamster ovary cells, using a trinitrobenzenesulfonate labeling procedure. They report a biphasic process with a t½ of the rapid phase of 22 min. Mills et al. (13) report that newly synthesized phospholipids appear in the plasma membrane of Acanthamoeba castellani after a lag of 30 min, with the same kinetics as they found for cholesterol movement.

We have also determined that the transport of PC does not appear to be energy dependent (Fig. 2). These are technically difficult experiments because the energy poisons must be added after synthesis and before transport to see an effect. Since the transport process measured here is so rapid, it is possible that the poisons do not act fast enough. We know from previous experiments that KF and KCN together deplete the cells' energy supply in <3 min (19), so we feel that the lack of effect does indeed indicate that the transport process is energy independent. We have also shown that the transport process is not affected by the protonophore CCCP (Table II). With this inhibitor, we were able to preincubate the cells for 30 min before the pulse labeling without abolishing PC synthesis. Sleight and Pagano (14) have similarly reported that the transport of PE in Chinese hamster ovary cells is energy independent. The apparent lack of an energy requirement for PC transport is in contrast to our finding that cholesterol transport from the endoplasmic reticulum to the plasma membrane is energy dependent (15, 19). It should be appreciated that cholesterol is present in the plasma membrane against a large concentration gradient so it is not surprising that transport be energy dependent. PC, however, is probably not preferentially localized in the plasma membrane (4).

The temperature dependence of PC transport also differs strikingly from that observed for cholesterol transport. Cholesterol transport is virtually halted below 15°C, while PC transport continues. Arrest of vesicular transport processes at 15°–20°C has been indicated in different systems (24–26) and may indicate a vesicle-mediated mechanism for cholesterol transport.

It seems clear from our current results that the transport of PC and cholesterol from the endoplasmic reticulum to the plasma membrane are different. The half time for cholesterol transport is ~10 min at 37°C, whereas the half time for PC transport is ~1 min. Cholesterol transport is energy dependent, and PC transport is not. Cholesterol transport ceases below 15°C, while PC transport continues. Furthermore, it is also clear that the transport of both these lipids are kinetically distinct from protein transport from the endoplasmic reticulum to the plasma membrane (15). Whether the transport of the three classes of molecules essential for plasma membrane biogenesis is mechanistically distinct remains to be determined. If they share a single mechanism, then the kinetic differences must be explained.

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REFERENCES


