Selective Uptake of $^3$H]Arachidonic Acid Into the Dense Tubular System of Human Platelets

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We have used quantitative electron microscopic autoradiography to characterize the subcellular distribution of arachidonoyl phospholipids following brief (5 minutes) exposure of unstimulated human platelets to $^3$H]arachidonic acid. Labeled arachidonate was taken up rapidly and incorporated into phospholipids. Phospholipid radioactivity was preserved and spatially fixed during tissue processing for electron microscopy. Analysis of autoradiographs showed that following a brief exposure to 750 nmol/L $^3$H]arachidonate, there is selective labeling of an internal membrane compartment composed of the dense tubular system and the open canalicualar system. The plasma membrane, platelet granules, and nonmembranous cytoplasm were not labeled. Since the open canalicualar system is continuous with the plasma membrane and since phospholipids in continuous membranes are freely diffusible, our observations indicate that $^3$H]arachidonate was incorporated into phospholipids within the dense tubular system and not the open canalicualar system. Thus, the dense tubular system, known to contain cyclooxygenase activity, incorporates arachidonate selectively following brief exposure to this fatty acid, presumably to concentrate it in proximity to enzymes for icosanoid synthesis.

**THE PRODUCTION of icosanoids, oxygenated metabolites of arachidonic acid, requires cellular mechanisms for uptake and release of arachidonate from phospholipids. It has been demonstrated recently that icosanoid production can be impaired despite normal cyclooxygenase activity, an abundant supply of cellular arachidonate, and release of arachidonate from phospholipids. In this situation, compromised production of icosanoids is due to markedly decreased activity of arachidonoyl-CoA synthetase, an enzyme that appears to function as an icosanoid precursor fatty acid uptake system in cells. Similar results have been obtained using a mutant cell line devoid of arachidonoyl-CoA synthetase activity. Thus, the process of arachidonate incorporation into the cell, the earliest step in the pathway from arachidonate to icosanoids, has an effect on the production of icosanoids following stimulation by agonists.

In this study, we investigated the incorporation of a five-minute pulse of 750 nmol/L $^3$H]arachidonate into subcellular membrane compartments in the platelet. Arachidonate metabolism and icosanoid production have been characterized extensively in blood platelets. Platelets contain a high affinity arachidonoyl-CoA synthetase, which permits selective accumulation of icosanoid precursor fatty acids that are rapidly esterified into phospholipids.

We have identified the subcellular sites of arachidonoyl phospholipids following brief exposure of unstimulated human platelets to $^3$H]arachidonate. Our findings indicate that after five minutes of exposure to arachidonate, virtually all of the incorporated $^3$H]arachidonate is found in the dense tubular system, suggesting an important role for this internal membrane system in arachidonate metabolism in the platelet.

**MATERIALS AND METHODS**

**Isolation of platelets and labeling with $^3$H]arachidonate.** Platelets were isolated from human blood according to the method of Baenziger et al. and resuspended at a concentration of 5 x 10$^8$/mL in platelet wash buffer (26.2 mmol/L sodium phosphate and 6.8 mmol/L potassium phosphate, pH 6.5, 118 mmol/L sodium chloride, 5.6 mmol/L glucose). Four aliquots (0.2 mL) were incubated with 750 nmol/L [5,6,8,9,11,12,14,15-$^3$H]arachidonate (135 Ci/mmol, Amersham, Arlington Hts, IL) to label the platelets to a high specific radioactivity and allow development of an adequate number of grains for quantitative autoradiography; four aliquots were incubated with 3.7 nmol/L [3H]arachidonate and used in assays to determine the amount of [3H]arachidonate removed from platelets during processing of the samples for electron microscopy; four aliquots were incubated in the absence of radiolabeled arachidonate and used to determine background grain density. Following incubation for 5 minutes at 37°C to allow incorporation of exogenous [3H]arachidonate, 0.8 mL of platelet wash buffer was added to each tube and the cells centrifuged at 12,000 x g for ten seconds. The platelets were resuspended in 1 mL of platelet wash buffer containing 1 mg/mL fatty acid free bovine serum albumin (Sigma, St Louis), centrifuged again at 12,000 x g for ten seconds, and resuspended in 0.2 mL of platelet wash buffer without bovine serum albumin. The platelets were incubated for an additional 5 minutes at 37°C and then fixed by the addition of 1 mL of tannic acid-glutaraldehyde (3% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.4, containing 1.5% tannic acid and 2 mmol/L CaCl$_2$). The fixed platelets were pelleted at 12,000 x g for one minute and resuspended in tannic acid-glutaraldehyde for additional fixation for two hours at room temperature.

**Preparation and analysis of autoradiographs.** Platelets were processed for electron microscopic autoradiography according to methods developed in our laboratory. Briefly, the fixed platelets were rinsed with 0.1 mol/L sodium cacodylate buffer, pH 7.4, containing 2 mmol/L CaCl$_2$, post-fixed with 2% osmium tetroxide in 0.1 mol/L sodium cacodylate buffer for one hour at 4°C, rinsed in 0.8% sodium acetate buffer, pH 7.2, and stained en bloc with 0.5% uranyl magnesium acetate in 0.8% sodium acetate buffer overnight at 4°C. The stained platelets were rinsed with acetate buffer, dehydrated rapidly at 4°C with increasing concentrations of acetone, and infiltrated with Spurr’s low viscosity epoxy resin.

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Platelets incubated with 750 nmol/L \([^{3}H]arachidonate\) were embedded in Spurr's resin by pelleting the cells in polypropylene capsules containing resin. These platelets were sectioned for analysis with electron microscopy and autoradiography. Platelets incubated with 3.7 nmol/L \([^{3}H]arachidonate\) were used to assess preservation of radioactivity during tissue processing. Following each step, platelets were pelleted, and the processing reagent was transferred to a vial. Radioactivity removed from the platelets during each step was quantified with liquid scintillation spectrometry. At the completion of processing, the platelets were dissolved in NCS (Amersham), and radioactivity retained in the cells was determined with liquid scintillation spectrometry.

The chemical composition of radiolabeled material removed during acetone dehydration was determined by thin layer chromatography. The combined acetone washes of platelets incubated with 750 nmol/L \([^{3}H]arachidonate\) were concentrated into a small volume by evaporation under nitrogen and analyzed by thin layer chromatography in a phospholipid separation system containing CHCl₃:CH₃OH:glacial acetic acid:H₂O (60:30:16:3.2).

 Autoradiographs were prepared according to a modification of the flat substrate method of Salpeter and Bachmann, as previously described. Pale gold sections, approximately 100 nm thick, were mounted on collodion-coated slides, shadowed with a thin (5 to 6 nm) layer of carbon, and coated with monolayers of Ilford L-4 nuclear tract emulsion. The slide-mounted sections were stored in light-tight boxes at 4°C for 96 hours. The emulsion was developed in 1.1% p-phenylenediamine and 12.6% sodium sulfite, and fixed in 30% sodium thiosulfate, as previously described. Sections were collected on 200-mesh copper grids and examined in a Phillips 200 EM electron microscope at 60 KeV. Sections prepared from unlabeled platelets for assessment of background grain density were placed on the same collodion-coated slides as the radiolabeled tissue sections. Previous studies from our laboratory have demonstrated no significant artifacts due to positive or negative chemography or latent image fading in the autoradiography procedures used in these studies.

 Autoradiographs were analyzed according to the mask overlay method of Salpeter et al using the computer program of Land and Salpeter. This method accounts for the problem of image spread in which a grain is formed overlying one compartment when the radiolabeled probe is located in a different compartment. This method uses a mathematical model describing the spread of grains around point sources of radiation and estimates of the probabilities that a point source of radioactivity located in one structure will give rise to grains overlying any other structure. The half-distance (radius of a circle around a point source in which 50% of grains will be contained) for the autoradiographic system used is approximately 150 nm measured in a system with tritium, monolayers of Ilford L-4 emulsion, 100 nm thick sections, and p-phenylenediamine developer. Although p-phenylenediamine is an inefficient developer (approximately 1 grain per 15 disintegrations), it produces small compact grains that minimize obstruction of underlying fine structure.

A total of 150 randomly selected photographs containing 410 platelets and 741 grains was analyzed at a final print magnification of ×48,000. All structures appearing in the electron micrographs were assigned to individual source compartments defined as potentially labeled structures. Source compartments, encompassing the entire photographic area, included specific membraneous organelles of platelets, all remaining platelet cytoplasm, and extracellular space between the platelets. The dense tubular system and the open canicular system of membranes could be readily distinguished by their ultrastructure, but because of their small size and intimate anatomic proximity, the spread of grains resulting from radiolabeled sources in either of the two compartments overlapped to such an extent that they could not be resolved autoradiographically. Accordingly, these two internal membrane compartments were combined to form a single source compartment referred to as DTS/OCS. Additional source compartments analyzed were the plasma membrane, mitochondria, all platelet granules including dense bodies, all remaining nonmembranous platelet structures, and the extracellular space between platelets.

Results

\([^{3}H]arachidonate\) was taken up rapidly by platelets and incorporated into phospholipids. In separate experiments, we determined that over 90% of esterified radiolabeled arachidonate was present in phospholipids. Platelets incubated with 3.7 nmol/L \([^{3}H]arachidonate\) for five minutes at 37°C incorporated approximately 37% of the exogenous labeled arachidonate. Loss of cell radioactivity during tissue processing for electron microscopy was minimal. As shown in Table 1, 86% of incorporated radioactivity was retained by platelets following tissue processing. Approximately one third of the radioactivity removed during processing was recovered in the initial aqueous fixation steps and presumably reflects removal of radioactivity loosely associated with the platelets rather than extraction of incorporated cellular lipids. Most of the remaining radioactivity removed during processing was recovered during the dehydration and resin infiltration steps involving organic solvents. Thin layer chromatographic analysis of material extracted during dehydration in acetone showed that virtually all radioactivity was present in neutral lipid (data not shown). Thus, the modest amount of radioactivity removed during tissue processing for electron microscopy was predominantly unincorporated radioactivity or radioactivity in neutral lipid. Labeled phospholipids, nearly quantitatively preserved, were spatially fixed during tissue processing. Thin layer chromatographic analysis of the small amount of labeled phospholipid removed during dehydration with acetone revealed that no single phospholipid species was extracted selectively. Because there is little conversion of arachidonate to other fatty acids and the bulk of oxygenated arachidonate metabolites exit the cell, radioactivity retained in platelets was assumed to be \([^{3}H]arachidonate\). In this regard, Banerjee and Rosenthal showed in human skin fibroblasts incubated 5 minutes with submicromolar concentrations of arachidonate that a maximum of 10% of incorpor-

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Table 1. Loss of Radioactivity During Tissue Processing

<table>
<thead>
<tr>
<th>Step</th>
<th>Percent of Total Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tannic acid—glutaraldehyde + rinse</td>
<td>4.41</td>
</tr>
<tr>
<td>Osmium tetroxide + rinse</td>
<td>0.91</td>
</tr>
<tr>
<td>Uranyl magnesium acetate + rinse</td>
<td>1.91</td>
</tr>
<tr>
<td>Dehydration</td>
<td></td>
</tr>
<tr>
<td>70% acetone</td>
<td>0.47</td>
</tr>
<tr>
<td>95% acetone</td>
<td>0.26</td>
</tr>
<tr>
<td>100% acetone</td>
<td>3.35</td>
</tr>
<tr>
<td>Resin infiltration</td>
<td></td>
</tr>
<tr>
<td>Resin:acetone, 1:1</td>
<td>1.32</td>
</tr>
<tr>
<td>100% resin</td>
<td>0.75</td>
</tr>
</tbody>
</table>

Total percent removed, 13.40; Percent retained in tissue, 86.60. Total dpm retained in tissue, 7.41 × 10⁶ dpm. Total platelet number in sample, 1 × 10⁶.
rated arachidonate is elongated to 22:4n-6. Furthermore, at the concentration of exogenous arachidonate we used, arachidonate is rapidly esterified into phospholipids with minimal conversion to iocosanoids.

Fig 1 shows the results of analysis of electron microscopic autoradiographs. It shows the size of each source compartment expressed as percent of total photographic area (Fig 1A), the specific activity of each source compartment expressed as grains per square micrometer of structure area (Fig 1B), and the percent of total radioactivity contained in each compartment (Fig 1C). Only two structures were labeled. The combined internal membrane compartment composed of the dense tubular system and open canalicular system had a grain density of approximately 26 grains per square micrometer and contained approximately 90% of total platelet radioactivity. Remaining radioactivity was associated with the mitochondrial compartment. Background grain density, determined from sections of unlabeled platelets, was negligible (approximately 0.1 grain/100 μm²).

Figs 2 and 3 show representative low and high magnification electron microscopic autoradiographs of human platelets labeled with [³H]arachidonate. Platelets exhibited typical discoid morphology and contained numerous granules, indicating they were in the unstimulated condition.

DISCUSSION

Quantitative electron microscopic autoradiography is particularly well suited for subcellular localization of arachidonoyl phospholipids. Radiolabeled arachidonate is available with high specific activity, thus minimizing emulsion exposure intervals, and the polyunsaturated acyl moieties are extensively cross-linked by osmium tetroxide, resulting in spatial fixation of arachidonoyl phospholipids, precluding their extraction or intracellular translocation during tissue processing. This provides a significant advantage over cellular fractionation methods in which hours may elapse between the end of an interval of arachidonate incorporation and the collection of fractions for analysis of arachidonate content. With electron microscopic autoradiography, there is no such period for redistribution of arachidonate among subcellular membranes, because fixation preserves the distribution of

![Fig 1](image1.png)

![Fig 2](image2.png)
Fig 3. A high magnification autoradiograph showing the relative size of grains (dense black structures) and the membranous components of the dense tubular system and open canalicular system (arrows). Original magnification \( \times 35,000 \); current magnification \( \times 23,800 \). Bar = 1 \( \mu m \).

arachidonate immediately following the interval of incorporation.

The results of the present study demonstrate that brief (five minutes) exposure of human platelets to physiologic concentrations of arachidonate in vitro results in rapid and selective labeling of an intracellular membrane compartment that includes the dense tubular system and open canalicular system. This combined membrane compartment comprised only 1.5% of total photographic area but contained approximately 90% of labeled arachidonate. Remaining arachidonate radioactivity was localized to mitochondria. The significance of the low level of incorporation of \([\text{H}]\)arachidonate in mitochondria in our experiments is unknown. The plasma membrane was not labeled. Because arachidonate has been shown to rapidly translocate between subcellular membranes, the subcellular distribution of arachidonate is likely to be different following longer incubations of platelets with arachidonate. For example, in a study involving a one-hour exposure of human platelets to \( ^{14}\text{C}\)-arachidonate (0.4 nmol/L), Lagarde et al separated surface and intracellular membranes by high voltage free flow electrophoresis and found significant amounts of \( ^{14}\text{C}\)-arachidonate in both surface and intracellular membranes.\(^\text{18} \) Also, both Lagarde et al\(^\text{18} \) and Perret et al\(^\text{19} \) found endogenous arachidonate in isolated plasma membranes from human platelets. In light of our findings, it appears that arachidonate is initially incorporated in the dense tubular system with subsequent translocation of an arachidonate pool to the plasma membrane. In an electron microscope autoradiography study of mouse fibrosarcoma cells labeled with \([\text{H}]\)arachidonate, it was shown that the amount of arachidonate in the plasma membrane increased four- to eightfold as the incubation time with \([\text{H}]\)arachidonate was extended from fifteen minutes to two hours, indicating arachidonate movement into the plasma membrane.\(^\text{1} \) Thus, the difference in incubation time with radiolabeled arachidonate may explain the presence of arachidonate in the plasma membrane in Lagarde's studies with a one-hour arachidonate exposure and the absence in our studies (five-minute incubation). This would also explain the presence of endogeneous arachidonate in isolated plasma membranes. With regard to arachidonate compartmentation within phospholipids following brief exposure to arachidonate, it has been shown that after a fifteen-minute incubation, greater than 60% of incorporated arachidonate is esterified into phosphatidylycholine.\(^\text{20} \)

Although the tubulovesicular membranes of the dense tubular system and open canalicular system could be distinguished by their different ultrastructures, their intimate anatomic proximity and the spread of grains precluded autoradiographic resolution of the two compartments. However, the open canalicular system is continuous with the plasma membrane, which we have shown was not labeled. Furthermore, studies by Pagano et al\(^\text{23-25} \) using fluorescent phospholipids demonstrate that phospholipids within continuous membrane systems are freely diffusible. Based on their data on the rate of phospholipid diffusion within membranes, grains associated with the plasma membrane would be present if the open canalicular system were highly labeled.

Our data indicate that arachidonate was incorporated into phospholipids in the dense tubular system rather than in the open canalicular system. The dense tubular system is considered to be the endoplasmic reticulum of mature platelets. This internal membrane system contains peroxidase and glucose-6-phosphatase, enzymes present in the rough endoplasmic reticulum of megakaryocytes.\(^\text{26-28} \) We have recently shown that the endoplasmic reticulum is a major storage site for arachidonoyl phospholipids in a mouse fibrosarcoma line.\(^\text{3} \) Moreover, analysis of major membrane pools of rat liver has shown that the endoplasmic reticulum is enriched in arachidonate relative to the plasma membrane.\(^\text{29} \) These findings are compatible with our results, which suggest that arachidonate is rapidly and selectively incorporated into phospholipid in the dense tubular system of the platelet. In nucleated cells, the nuclear membrane appears to be a kinetically preferred site for the incorporation of arachidonate into phospholipids and may also serve as a reservoir for arachidonate released for icosanoid production.\(^\text{3} \) Using a mutant cell line devoid of arachidonoyl-CoA synthetase activity, we have demonstrated that incorporation of arachidonate into the nuclear membrane is impaired.\(^\text{3} \) Despite normal cyclooxygenase and phospholipase activities and abundant intracellular arachidonate, mutant cells synthesize significantly smaller amounts of icosanoids than control cells.\(^\text{13} \) Thus, agonist-mediated icosanoid production may depend on appropriate subcellular compartmentalization of arachidonoyl phospholipids, presumably controlled by the high affinity arachidonoyl-CoA synthetase. This compartmentation concept is supported by observations that enzymes of icosanoid synthesis such as cyclooxygenase and prostaglandin I\(_2\) synthetase reside in the nuclear membrane and endoplasmic reticulum.\(^\text{30-32} \) Our findings that the dense tubular system becomes rapidly and selectively labeled with newly incorporated arachidonate suggest this membrane
system may function in a manner analogous to the nuclear membrane and endoplasmic reticulum of nucleated cells. In fact, Carey et al.\(^1\) have shown, using platelet membrane fractions isolated by high voltage flow electrophoresis, that platelet cyclooxygenase and thromboxane synthetase activities are found predominantly in an intracellular membrane fraction with little of either activity in surface membrane fractions, granules, or cytosol. The localization of arachidonate metabolizing enzymes in their studies resembles the localization of incorporated arachidonate in our experiments. In an earlier study, Gerrard et al. demonstrated that of several subcellular platelet fractions, the one most enriched in elements of the DTS was the most efficient in converting \(^{14}C\)-arachidonate to eicosanoids.\(^2\) Thus, present evidence indicates that in platelets arachidonate is incorporated into phospholipids in the dense tubular system and, when liberated upon platelet activation, the free arachidonate is converted to thromboxane \(A_2\) by enzymes in the same membrane compartment.

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Selective uptake of [3H]arachidonic acid into the dense tubular system of human platelets

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