Translocation of 5-Lipoxygenase to the Membrane in Human Leukocytes Challenged with Ionophore A23187*

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Challenge of human peripheral blood leukocytes with ionophore A23187 resulted in leukotriene (LT) synthesis, a decrease in total cellular 5-lipoxygenase activity, and a change in the subcellular localization of the enzyme. In homogenates from control cells, greater than 90% of the 5-lipoxygenase activity and protein was localized in the cytosol (100,000 x g supernatant). Ionophore challenge (2 μM) resulted in a loss of approximately 58% of the enzymatic activity and 35% of the enzyme protein from the cytosol. Concomitantly, there was an accumulation of inactive 5-lipoxygenase in the membrane (100,000 x g pellets) which accounted for at least 45% of the lost cytosolic protein. There was a good correlation between the quantities of LT synthesized and 5-lipoxygenase recovered in the membrane over an ionophore concentration range of 0.1-6 μM. The time course of the membrane association was similar to that of LT synthesis. Furthermore, although the pellet-associated enzyme recovered from ionophore-treated leukocytes was inactive, an irreversible, Ca2+-dependent membrane association of active 5-lipoxygenase could be demonstrated in cell-free systems. To determine whether ionophore treatment induced proteolytic degradation of 5-lipoxygenase, the total activity and protein content of 10,000 x g supernatants from control and ionophore-treated cells were examined. These supernatants, which included both cytosolic and membrane-associated enzyme, showed a 35% loss of 5-lipoxygenase activity but only an 8% loss of enzyme protein as a result of ionophore challenge (2 μM). Therefore, the majority of the loss of 5-lipoxygenase activity was most likely due to suicide inactivation during the LT synthesis, rather than to proteolytic degradation. Together these results are consistent with the hypothesis that ionophore treatment results in a Ca2+-dependent translocation of 5-lipoxygenase from the cytosol to a membrane-bound site, that the membrane-associated enzyme is preferentially utilized for LT synthesis, and that it is consequently inactivated. Thus, membrane translocation of 5-lipoxygenase may be an important initial step in the chain of events leading to full activation of this enzyme in the intact leukocyte.

The enzyme 5-lipoxygenase catalyzes the first two steps in the biosynthesis of the leukotrienes (LT) from arachidonic acid (1-3). These steps include first, the oxidation of arachidonate at carbon 5 to yield 5(S)-hydroperoxy-6,8,11,14-eicosatetraenoic acid (5-HPETE), followed by the subsequent conversion of 5-HPETE to 5,6-oxido-7,9,11,14-eicosatetraenoic acid (LTA4). LTA4 serves as the precursor for the potent chemotactic factor, LTB4, as well as for the smooth muscle contracting agonists LTC4, LTD4, and LTE4. These products are secreted by leukocytes after exposure to various immunologic and inflammatory stimuli, and their biological activities suggest that they may play a role in immediate hypersensitivity reactions and inflammation (4). Therefore, the regulation of the 5-lipoxygenase may be of great importance to the pathophysiology of these processes.

The 5-lipoxygenase has now been purified from human and porcine peripheral blood leukocytes, as well as from rat basophilic leukemia cells (2, 5, 6). In each case, maximal activity of the purified protein required Ca2+ and ATP. In addition, the human enzyme was also stimulated by three distinct protein fractions from the leukocytes, one of which was membrane-associated (5, 7). Although these findings suggested a potentially complex regulatory mechanism for the human leukocyte 5-lipoxygenase, the active components in these protein fractions have not been characterized, and their mode of action remains unknown. Thus, little is actually understood about the regulation of 5-lipoxygenase activity in the intact cell.

When subcellular fractions of human leukocyte homogenates are examined for 5-lipoxygenase activity, greater than 90% of the enzyme is found in the cytosolic fraction (100,000 x g supernatant) (7, 8). However, a number of observations point to a potential role for cellular membrane in the modulation of 5-lipoxygenase activity. First, the substrate arachidonic acid is derived from membrane phospholipids through the action of specific phospholipases. High concentrations of the substrate are therefore likely to occur only near the membrane. Second, 5-lipoxygenase is an extremely hydrophobic enzyme whose primary sequence possesses regions having homology with the lipid interfacial binding sites of pancreatic lipase and lipoprotein lipase (9). Third, 5-lipoxygenase activity in cell-free systems is stimulated by phosphatidylcholine micelles and crude cell membrane preparations (100,000 x g pellets) (6, 7). Finally, the findings that 5-lipoxygenase is a Ca2+-dependent enzyme and that elevations in intracellular Ca2+ are required to activate the 5-lipoxygenase pathway in intact cells suggest the possibility that Ca2+ could induce a translocation of the 5-lipoxygenase from a cytosolic to a membrane site. Similar observations have been made for other Ca2+-dependent enzymes such as protein kinase C and phospholipase C (10, 11). Earlier attempts to demonstrate such a phenomenon in the case of human leukocyte 5-lipoxygenase showed that homogenization of cells in the presence of Ca2+ did in fact result in the recovery of large quantities of 5-lipoxygenase activity in cell membrane fractions (100,000 x g...
pellets) (8). However, this membrane association required unphysiologically high Ca\(^{2+}\) concentrations and was readily reversible upon Ca\(^{2+}\) removal. Thus, the true relevance of this binding remained questionable.

It was the purpose of the present study, therefore, to determine the fate of 5-lipoxygenase in intact leukocytes challenged with the ionophore A23187 as a stimulus of LT synthesis. For this purpose, we have raised an antisemium to human leukocyte 5-lipoxygenase and have developed an immunoblot assay to detect inactive as well as active enzyme protein. This technique was used in conjunction with routine enzyme assay to monitor 5-lipoxygenase activity. Our results suggest that membrane association of 5-lipoxygenase may be an important step in the activation of this enzyme.

**MATERIALS AND METHODS**

**Human Leukocyte Suspensions**—Human leukocyte concentrates (buffy coat) were obtained from local blood collection centers. Contaminating erythrocytes were removed by dextran sedimentation followed by hypotonic lysis in a Trias-buffered ammonium chloride solution as described (5). After the second lysis step, the cells were washed twice in Dulbecco's phosphate-buffered saline (GIBCO) and suspended at a concentration of 4 x 10\(^7\) cells/ml in Dulbecco's phosphate-buffered saline. The resulting suspension consisted of a population of monocytes, lymphocytes, and neutrophils in ratios comparable to those in normal human peripheral blood.

**Incubation Conditions**—For most experiments, 20-ml aliquots of the leukocyte suspensions were warmed to 37°C, and the desired concentrations of ionophore A23187 (Sigma) were added as a stock solution in dimethyl sulfoxide. The dimethyl sulfoxide concentration was maintained at less than 0.1% in all samples in each experiment and never exceeded 0.1%. The cells were incubated for the desired time period and were then placed on ice. To each incubation mixture was added 800 pl of 100 mM EDTA (4 mM final concentration), and 1-ml aliquots were then removed and combined with 1 ml of ethanol containing 1 nmol/ml prostaglandin (PG) B\(_2\) (Sigma), for assay of LT\(_B\) content. The remaining 19.8 ml of the cell suspensions was subjected to centrifugation at 500 x g for 12 min, and the cell pellets were resuspended in 3.5 ml of KPB-1 (50 mM potassium phosphate buffer, 100 mM NaCl, 2 mM EDTA, 1 mM diithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 60 pl/mg soybean trypsin inhibitor, pH 7.1).

For time course studies, 400 ml of cell suspension was warmed to 37°C. Four aliquots (20 ml) were then removed, placed on ice, and combined with 800 pl of 100 mM EDTA, in tubes numbered 1-4. Ionophore A23187 (2 pl final concentration) was then added to tubes 2 and 4 and the samples were then allowed to remain at 37°C for the remaining 30 min of cell suspension. Then, at 30, 60, 90, 120, 180, 240, 360, and 600 s, duplicate 20-ml aliquots were removed and combined with 800 pl of 100 mM EDTA on ice. At the same time, 1-ml aliquots were also removed and combined with 1 ml of 1 nmol/ml PGB\(_2\) in ethanol for LT\(_B\) assay. At the end of the incubation period the cells were collected by centrifugation and resuspended in KPB-1 as described above.

**Cell Homogenation and Preparation of Subcellular Fractions**—Leukocytes were homogenized by sonication at 4°C using a Cole-Parmer 4710 series ultrasonic homogenizer (Cole-Parmer Instrument Co., Chicago, IL). The samples received three pulsatile bursts of 20 s each. The homogenate was subjected to centrifugation at 100,000 g supernatants at 60-90% saturation of ammonium sulfate. This preparation contained a 5-lipoxygenase stimulatory factor that has been described previously (5, 8, 12). Samples were warmed to 37°C, and reactions were initiated by the addition of 5 pl of a solution of 20 mM arachidonic acid (100 pl final concentration) and 0.4 mM 15-hydroperoxy-11,13-eicosadienoic acid (2 pl final concentration, see below). After a 10-min incubation period, samples were combined with 1 ml of ethanol containing 2 nmol of 13-hydroxytetraenes (5, 6, 8, 11, 14-eicosatetraenoic acid) and extracted with chloroform as described above. The samples were then analyzed by high-performance liquid chromatography on Nova-Pak C18 eluted at 1 ml/min with methanol:water:triethanolamine (75:25:0.1, pH 2.0, 1:1 v:v:v).

**Immunoblot Analysis of 5-Lipoxygenase**—5-Lipoxygenase was purified to near homogeneity as described (5). For immunization of rabbits, suspensions were prepared by combining 2 ml of a solution of 5-lipoxygenase (150 pg/ml in the final chromatography buffer) with Ribi adjuvant (Ribi Immunochem Research Inc., Hamilton, MT) according to manufacturer's directions. Rabbits received two subcutaneous injections of 1 ml each followed in 2 weeks by 20 intradermal injections of 0.1 ml each. One week later, the rabbits again received 2 subcutaneous 1-ml injections. The serum was tested for immuno-reactivity using 500 ng of purified 5-lipoxygenase spotted onto nitrocellulose paper as the antigen. Assays were performed with a Bio-Rad Immunoblot Assay Kit (Bio-Rad) employing horseradish peroxidase-labeled goat anti-rabbit IgG according to manufacturer's instructions. One week after the final injection, the rabbit antisera gave a positive reaction in this assay at dilutions of 1:5000. Preimmune serum was negative in this assay.

For immunoblot analysis of the 5-lipoxygenase content of leukocyte 100,000 x g supernatants and pellets, 100-pl samples were combined with 50 pl electrophoresis sample buffer and boiled immediately to prevent proteolytic degradation (see "Results"). Aliquots (20 pl) of the resulting solutions were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 15 x 12-cm, 10% acrylamide gels overlaid with a 4% acrylamide stacking gel by the method of Laemmli (14). The proteins in the gels were then transferred to nitrocellulose paper using a Bio-Rad Trans-Blot Cell according to manufacturer's directions. Transfers were carried out overnight at 100 V in a buffer containing 30% current for the column format. Immediately after protein transfer, nitrocellulose blots were blocked in a solution of 3% (w/v) gelatin in Tris-1 (20 mM Tris-Cl) contain-
ing 0.5 M NaCl, pH 7.5). After washing twice with TriS-2 (Tris-1 containing 0.05% (v/v) Tween 20), the blots were incubated for 3 h at room temperature with a 1:150 dilution of antisera in Tris-2 containing 1% (w/v) gelatin. The blots were then washed twice again with TriS-2 and finally incubated with a solution of TriS-2 containing 1% (w/v) gelatin, 6 μCi/ml [3H]-labeled goat anti-rabbit IgG (Du Pont-Nen, Boston, MA), and a 1:5,000 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG (Bio-Rad Immoblot Assay Kit). The blots were subjected to five washes in a solution of 0.5% (v/v) Nonidet P-40 in Dulbecco’s phosphate-buffered saline, dried, and exposed to x-ray film for 24 h. Autoradiograms were scanned with a LKB 2202 Ultrascan laser densitometer (LKB Produktor, Bromma, Sweden).

**Protein Assay**—Protein concentrations were determined by the method of Bradford (15) using a Bio-Rad Protein Assay Kit with bovine γ-globulin as the standard. The protein contents of 100,000 x g supernatants and pellets were 7–8 and 5–6 mg/ml, respectively.

**RESULTS**

**Effect of Ionophore Stimulation on LT Synthesis, and the Subcellular Localization of 5-Lipoxygenase Activity**—Leukocyte suspensions were challenged for 10 min at 37 °C with 2 μM ionophore. At the end of this time period the cells had produced LTB4 (2.97 ± 1.10 nmol/106 cells), 20-carboxy-LTB4 (1.13 ± 0.49 nmol/106 cells), and 20-hydroxy-LTB4 (1.38 ± 0.66 nmol/106 cells, mean ± S.D., n = 7). In addition, minor quantities of 5-HETE and the nonenzymatic hydrolysis products of LTA4 (16) were also detected, although these together comprised less than 10% of the total 5-lipoxygenase products. Cells incubated without ionophore synthesized no 5-lipoxygenase metabolites.

To determine the effects of ionophore challenge on 5-lipoxygenase activity, cells were homogenized, 100,000 x g supernatants and pellets were prepared, and these were subjected to enzyme assay. In contrast to the findings with intact cells, the major 5-lipoxygenase product of cell-free preparations was 5-HETE/5-HETE, as has been reported previously (1–3). Since LT consistently comprised less than 15% of the total lipoxygenase products under these conditions, quantitation of 5-lipoxygenase activity was based on the amount of 5-HETE/5-HETE synthesized during a standard 10-min incubation period. Fig. 1 shows the total amount of 5-lipoxygenase activity recovered in supernatants and pellets prepared from cells incubated in the presence and absence of ionophore. Consistent with previously published data (8) the majority of the enzymatic activity (>90%) was recovered in the supernatant fraction regardless of ionophore exposure. The specific activity of supernatant samples was 6–8-fold higher than that of pellet samples, suggesting that 5-lipoxygenase was predominantly a soluble enzyme. Of interest, however, was the finding that ionophore challenge caused a 55% loss of enzymatic activity from the supernatant fraction. This loss was statistically significant as determined by the paired Student’s t test (p < 0.01). A decrease in 5-lipoxygenase activity in the 100,000 x g pellets was also observed, although this difference was not statistically significant.

There are a number of possible explanations for the observed loss of 5-lipoxygenase activity from the cytosol of ionophore-treated cells. First, 5-lipoxygenase in cell-free systems is irreversibly inactivated during its reaction with arachidonic acid (12, 17–18), and it is quite possible that this also occurs when the enzyme is utilized for LT synthesis in intact cells as well. Second, activation of leukocytes by ionophore may cause a release of proteases that degrade the enzyme. Third, there may be a movement of the enzyme from the cytosol to a membrane-associated site. In this case one might expect to see increases in enzyme activity in the 100,000 x g pellets; however, if the enzyme is also inactivated as a result of LT synthesis, such increases would not be detectable.

**Immunoblot Analysis of 5-Lipoxygenase Protein**—To determine the fate of 5-lipoxygenase in ionophore-activated leukocytes, we required a technique that would detect the enzyme whether or not it was catalytically active. We therefore developed an immunoblot assay technique utilizing a polyclonal antiserum raised against highly purified enzyme. Fig. 2 shows the results obtained when immunoblot analysis was performed on 100,000 x g supernatants and pellets prepared from leukocytes incubated in the presence and absence of iono-
Three protein bands were specifically detected by this technique. The band at 80,000 daltons corresponded to the 5-lipoxygenase, and co-migrated with purified enzyme (see Fig. 3). The recognition of the 63,000-dalton band was best explained by the fact that the enzyme preparation used to immunize the rabbit was contaminated by a 63,000-dalton protein that eluted approximately 1 min after the 5-lipoxygenase. This impurity possessed no 5-lipoxygenase activity, and we have never observed degradation of the enzyme to a 63,000-dalton form. We therefore believe that this protein is not related to the 5-lipoxygenase itself, although we have not absolutely discounted this possibility. The origin of the 50,000-dalton species is less clear. It may be an intracellular proteolytic degradation product of 5-lipoxygenase, although we have never directly observed conversion of the 80,000-dalton protein to a 50,000-dalton species under any of our experimental conditions.

Inspection of the data shown in Fig. 2 indicated that the 80,000-dalton 5-lipoxygenase band was present in all of the leukocyte samples. However, the intensity of the band in the supernatant from the ionophore-treated cells was visibly reduced when compared with the sample from control cells. Also notable was the marked increase in the intensity of the 5-lipoxygenase band in the membranes of ionophore-treated cells as compared to controls. These results suggested that ionophore activation had resulted in an association of the 5-lipoxygenase with cellular membranes and that this could account at least in part for the loss of enzyme protein and activity from the cytosol.

To quantitate the extent of the membrane association of the 5-lipoxygenase, we prepared 100,000 X g supernatants and pellets and added to them known quantities (0-120 ng) of purified enzyme. We then performed immunoblot analysis and measured the intensity of the 5-lipoxygenase band by laser densitometry. The results (Fig. 3) indicated that there was a linear relationship between the increase in the 80,000-dalton band intensity and the quantity of 5-lipoxygenase in the sample. This allowed us to determine the absolute quantities of enzyme in ionophore-treated and control supernatant and pellet samples. From these data we could show that ionophore treatment resulted in a loss of approximately 5.3 μg of 5-lipoxygenase protein from the cell supernatant samples (Fig. 4). The 5-lipoxygenase content of the pellet samples increased by 2.4 μg. Thus, approximately 45% of the enzyme lost from the supernatants had been recovered in the pellets of ionophore-treated cells.

**Figure 3. Quantitation of 5-lipoxygenase (5-LO) in supernatants and pellets by immunoblot analysis.** The intensity of 5-lipoxygenase labeling was different for supernatant and pellet samples. Therefore, quantitation required that separate standard curves be constructed for known amounts of enzyme analyzed in the presence of both supernatant and pellet preparations. For this purpose, it was desirable to minimize the background due to endogenous 5-lipoxygenase in the supernatant and pellet samples. Thus, the calibration curves were constructed by adding known quantities of purified 5-lipoxygenase (5) to 100,000 x g pellets from control cells and to 100,000 x g supernatants from cells incubated for 10 min at 37°C with 10 μM ionophore to cause a maximal depolarization of the enzyme. These standard samples were subjected to SDS gel electrophoresis and immunoblot analysis along with the 100,000 x g supernatants from control and ionophore-treated leukocytes for which the quantities of 5-lipoxygenase were to be determined. The resulting autoradiograms were scanned by laser densitometry. Inset, 80,000-dalton region of the autoradiogram obtained from a typical immunoblot analysis. Lane identities are as follows: lanes 1-6, a 100,000 x g pellet from control leukocytes to which had been added 120, 67, 45, 22, 11, and 0 ng of purified 5-lipoxygenase, respectively; lanes 7 and 8, duplicate 100,000 x g pellets from control cells; lanes 9 and 10, duplicate 100,000 x g pellets from ionophore-treated cells (2 μM ionophore); lanes 11 and 12, duplicate 100,000 x g supernatants from control cells; lanes 13 and 14, duplicate 100,000 x g supernatants from ionophore-treated cells (2 μM ionophore); lanes 15-20, a 100,000 x g supernatant from leukocytes treated with 10 μM ionophore to which had been added 0, 11, 22, 45, 67, and 129 ng of 5-lipoxygenase, respectively. Main figure, calibration curves generated from the autoradiogram in the inset. The area of the densitometric peak corresponding to the 5-lipoxygenase band from lane 6 was subtracted from the corresponding peak areas from lanes 1-5. Similarly the area of the 5-lipoxygenase peak from lane 15 was subtracted from the corresponding peak areas from lanes 16-20. This provided the peak areas attributable only to the added purified enzyme. These values are plotted against the quantity of added enzyme for pellet (O) and supernatant (C) samples. The lines drawn through the points were obtained by linear regression analysis (r = 0.96 for supernatant samples, and r = 0.99 for pellet samples). These curves were then used to determine the quantity of 5-lipoxygenase present in the samples analyzed in lanes 7-14.

**Figure 4. Relationship between Membrane Association of 5-Lipoxygenase, LT Synthesis, and Ionophore Concentration.** The data in Figs. 1 and 4 indicated that ionophore treatment resulted in loss of 5-lipoxygenase protein from the cytosol and its accumulation in the membrane fraction. This result supported the hypothesis that activation of leukocytes with ionophore led to a translocation of the cytosolic enzyme to a membrane site and that it was the membrane-associated enzyme that was utilized for LT synthesis. Consequently, this 5-lipoxygenase has been inactivated, explaining the failure to observe increased enzymatic activity in the pellet samples. If membrane translocation is an early step in 5-lipoxygenase activation, then one would expect a good correlation between the quantity of translocated enzyme and the amount of LT synthesized by the cells at various ionophore concentrations. That this was true is illustrated by the data in Fig. 5 which show the total amount of LTB₄ synthesized by leukocytes at ionophore concentrations of 0-6 μM. This is compared to the amount of 5-lipoxygenase activity disappearing from the supernatant and enzyme protein appearing in the pellet fractions from the cells. Note that the concentration dependence curves are steep for all three parameters at ionophore concentrations of 0-1 μM. Increasing concentrations above 1 μM ionophore caused relatively small increases in LT synthesis or membrane association of the enzyme.

**Time Course of LT Synthesis and Membrane Association of 5-Lipoxygenase.**—If membrane association of 5-lipoxygenase
we have investigated the time course of LT synthesis by
pared. The quantity of 5-lipoxygenase present in duplicate samples
was determined by immunoblot analysis as shown in Fig.

is an activation step for the enzyme, then it must occur prior
to LT synthesis. We therefore needed an alternate way to stop
the translocation process. Because we could show that little
to no translocation or LT synthesis occurred in the absence
of free extracellular Ca++, we postulated that addition of excess
EDTA after ionophore challenge might stop the ongoing
translocation process. This proved to be the case as is shown
in Fig. 6. In this experiment, we challenged leukocytes with 2
µM ionophore at 37 °C. At the desired times, aliquots were
removed and combined with EDTA to measure translocation,
or ethanol to measure LT synthesis. That EDTA effectively
stopped the translocation process was indicated by the fact
that less enzyme had disappeared from the cytosol or had
appeared in the membrane in samples to which EDTA was
added soon after ionophore as opposed to later. The resulting
time-course data shown in Fig. 6 suggested that translocation
began soon after ionophore addition and actually preceded
LT synthesis.

Some care must be taken in interpreting the data in Fig. 6,
however, because if translocation did not stop immediately
after the EDTA addition, then the process would appear to
be occurring more rapidly than it actually did. There was no
way to know exactly how quickly translocation stopped after
the addition of EDTA; however, we could determine how long
was required for LT synthesis to cease. Fig. 7 shows the
results of an experiment in which duplicate leukocyte suspen-
sions were incubated for 10 min with 2 µM ionophore. One
suspension was maintained at 37 °C throughout the incuba-
tion period. The other suspension was placed on ice 2 min
after ionophore challenge, and EDTA (4 mM final concentra-
tion) was added. Aliquots were removed from both samples at
the indicated times and combined with ethanolic PGBz solu-
tion for assay of total LTB2 production. The results indicated
that LT synthesis ceased within 90 s of EDTA addition. This
was not observed in the absence of EDTA. Because 90 s was required for EDTA addition to affect 5-lipoxygenase activity, it is likely that a similar time may be required for translocation to be affected as well. Therefore, we can conclude from Fig. 6 that the time course of translocation is similar to the time course of LT synthesis, but it is impossible to know from available techniques which process precedes the other.

Association of Active 5-Lipoxygenase with Cellular Membrane—Although the idea of membrane translocation of 5-lipoxygenase as an early activation step is an attractive hypothesis, the data presented thus far are also consistent with the idea that the membrane is simply a repository for inactivated enzyme. For example, cytosolic 5-lipoxygenase may be activated, synthesize LT, become inactivated, and subsequently become associated with the membrane, possibly as an initial step toward degradation. If this were true, then one would not expect active enzyme to become membrane-associated. We therefore sought to determine whether 5-lipoxygenase could bind to membrane in its active form. To test this hypothesis, it was necessary to perform the experiment in a cell-free system, because in the intact cell Ca\(^{2+}\) activation was always accompanied by LT synthesis and, consequently, enzyme inactivation. Prior experiments (8) had demonstrated a Ca\(^{2+}\)-dependent membrane association of active 5-lipoxygenase in such systems. However, excessively high Ca\(^{2+}\) concentrations had been required to demonstrate the association, and the enzyme was readily released from the membrane upon removal of Ca\(^{2+}\). This was not characteristic of the membrane association observed in ionophore-treated leukocytes. In the earlier studies, membrane association was demonstrated in 100,000 x g pellets prepared from cells homogenized at 4 °C in the presence of Ca\(^{2+}\). We therefore questioned whether temperature might be an important factor in the translocation process. To test this hypothesis, we prepared 10,000 x g supernatants from homogenates of leukocytes never exposed to ionophore. These supernatants were warmed to 37 °C and incubated for 1 min in the presence or absence of 2 mM Ca\(^{2+}\) (added in the presence of 2 mM EDTA). The samples were then placed on ice, and EDTA and/or Ca\(^{2+}\) was added to each so that the final concentrations were 4 mM EDTA and 2 mM Ca\(^{2+}\) in both. The samples were then subjected to centrifugation for the preparation of 100,000 x g supernatants and pellets. The results (Fig. 8) showed that 5-lipoxygenase activity in the 100,000 x g supernatants had decreased as a result of exposure to free Ca\(^{2+}\) at 37 °C and that there was an accumulation of enzymatic activity in the membrane fraction. Similar results were obtained over a range of added Ca\(^{2+}\) of from 2 to 3 mM (in the presence of 2 mM EDTA). The membrane association was also very rapid, being complete in less than 15 s. These results indicated that at 37 °C active 5-lipoxygenase could associate with membrane in the presence of very low free Ca\(^{2+}\) concentrations, because the effect was observed when only enough Ca\(^{2+}\) was added to equal the concentration of EDTA. Furthermore, this association was not reversible as indicated by the fact that excess EDTA was added to tubes prior to centrifugation. Thus we could demonstrate an irreversible, Ca\(^{2+}\)-dependent membrane association of active 5-lipoxygenase exposed to relevant Ca\(^{2+}\) concentrations at 37 °C.

Stability of 5-Lipoxygenase to Proteolytic Degradation—The data in Fig. 4 indicated that only 45% of 5-lipoxygenase protein lost from the cytosol was recovered in the pellet. Therefore, the question arose as to the fate of the remaining enzyme. It is likely that the amount of enzyme in the pellets was underestimated due simply to physical losses in resuspending the membranes after centrifugation. However, it was impossible to know whether incomplete recovery could account for all of the apparent enzyme loss. Therefore, we considered the possibility that some of the 5-lipoxygenase
may have been destroyed by proteases that had been activated as a result of ionophore treatment. This possibility was investigated by examining 5-lipoxygenase activity and protein content of 10,000 × g supernatants from leukocytes incubated in the presence and absence of ionophore. Because 10,000 × g supernatants included enzyme from both the cytosol and membrane fractions, significant losses due to proteolysis should be detectable regardless of the subcellular localization of the enzyme. To accentuate the effects of any endogenous protease activity, we also prepared supernatants from which our routine protease inhibitors were omitted. The results are summarized in Table I. When protease inhibitors were omitted from the homogenization buffer in the absence of ionophore treatment, there was a 22% reduction in enzymatic activity accompanied by a comparable (23%) loss of enzyme protein as determined by immunoblot analysis. Under these conditions (no protease inhibitors), ionophore treatment resulted in an additional 40% loss in enzymatic activity and 33% loss of 5-lipoxygenase protein. These results indicated that, without protease inhibitors, degradation of 5-lipoxygenase protein did occur and was augmented by exposure of the cells to ionophore. However, these results are in marked contrast to those obtained in the presence of protease inhibitors. In this case, ionophore treatment effected a 34% loss of 5-lipoxygenase activity, but only an 8% loss of enzyme protein. Thus, when protease inhibitors were included during homogenization, the majority of the loss of enzymatic activity could not be attributed to proteolysis.

This result suggested that, in the presence of the protease inhibitors phenylmethylsulfonyl fluoride and soybean trypsin inhibitor, the 5-lipoxygenase was relatively resistant to proteolysis. We decided to test this conclusion further in experiments in which 10,000 × g supernatants containing the protease inhibitors were incubated at 37 °C in the presence and absence of Ca2+, to activate potential Ca2+-dependent proteases. The results (Fig. 9A) showed that 5-lipoxygenase activity was extremely stable in the absence of Ca2+ but that Ca2+ addition (0.2 μM in excess of EDTA) caused a marked loss of enzymatic activity. The instability of enzymatic activity was less severe in supernatants from ionophore-treated cells, although the mechanism for this difference is not known. Despite the apparent instability of 5-lipoxygenase activity in the presence of Ca2+, Fig. 9B shows that there was no significant loss of 5-lipoxygenase protein under any of the incubation conditions. Thus, the stability of the enzyme to proteolysis in the presence of protease inhibitors was confirmed.

One additional discovery was made, however, during the course of these studies concerning the susceptibility of 5-lipoxygenase to proteolysis. For the time-course study shown in Fig. 5B, supernatants were incubated at 37 °C, and aliquots were removed at the indicated times and combined with electrophoresis sample buffer containing SDS. We observed
that it was imperative to boil these samples immediately, or extensive degradation of the 5-lipoxygenase would ensue. This is illustrated in Fig. 10 which shows the stability of the enzyme in 10,000 x g supernatants incubated at 37 °C for a period of up to 60 min, as compared to the total break down of the protein after incubation for 60 min at 25 °C in the presence of the electrophoresis sample buffer. This proteolysis occurred in both ionophore-treated and control supernatants regardless of the presence or absence of Ca2+ or protease inhibitors. Thus, it is clear that a protease exists in 10,000 x g supernatants that is not inhibited by phenylmethylsulfonyl fluoride or soybean trypsin inhibitor, is not Ca2+-dependent, and is active in the presence of 0.5% SDS. This protease is capable of degrading 5-lipoxygenase, but only after it has been denatured.

**DISCUSSION**

In the present studies we have attempted to ascertain the fate of 5-lipoxygenase in leukocytes challenged with ionophore A23187. Our findings may be summarized as follows. Challenge with 2 μM ionophore resulted in a loss of 30–50% of the 5-lipoxygenase activity from leukocyte 10,000 x g supernatants. However, immunoblot analysis revealed that there was only an approximately 10% decrease in total 5-lipoxygenase protein. Therefore, the loss of enzymatic activity was due primarily to suicide inactivation as opposed to proteolytic degradation. When 10,000 x g supernatants were subjected to centrifugation at 100,000 x g, greater than 90% of the 5-lipoxygenase activity was recovered in the supernatants, regardless of ionophore treatment. However, supernatants from ionophore-treated cells showed a 36% reduction in total 5-lipoxygenase protein, and there was a 3-fold increase in inactive enzyme in the 100,000 x g pellet. A close inspection of the data in Fig. 3 and Table 1 indicated that, of the approximately 5.3 μg of 5-lipoxygenase protein lost from the cytosol as a result of the ionophore challenge, approximately 1.5 μg (10% of the total cytosolic enzyme) may have been proteolytically degraded, and 2.4 μg was recovered in the pellet. This accounts for 74% of the lost enzyme. The remaining 26% was most likely attributable to physical loss in manipulating the pellets; however, this cannot be quantitated with certainty. Thus, we can conclude that ionophore challenge resulted in suicide inactivation of 5-lipoxygenase, a translocation of the enzyme from the cytosol to the membrane, and a small amount of proteolytic degradation.

The most important question arising from these data regards the physiological significance of the membrane association of the enzyme. Relevant to this question is the fact that the enzyme accumulating in the membrane was not active, suggesting that it had undergone suicide inactivation and had been utilized for LT synthesis. This finding, in conjunction with the fact that there was a good quantitative and temporal correlation between LT synthesis and membrane association of the enzyme is consistent with the idea that Ca2+-dependent membrane translocation is an early activation step for the enzyme. Also consistent with this idea are the results from experiments in cell-free systems that showed that 5-lipoxygenase activity was stimulated by membranes (7) and that the active enzyme could bind to membrane in the presence of Ca2+. However, it is also true that the loss of enzymatic activity from 100,000 x g supernatants (55%, Fig. 1) exceeded the loss of enzyme protein (36%, Fig. 3). Thus the cytosol also contained enzyme that may have been utilized for LT synthesis and undergone suicide inactivation. Therefore, we cannot rule out the possibility that LT synthesis occurred in the cytosol and that the membrane simply served as a repository for inactivated enzyme. In addition, we must acknowledge that ionophore is not a physiological stimulus, and the translocation phenomenon may be an artifact of excessive intracellular Ca2+ concentrations. Unfortunately, attempts to utilize more appropriate stimuli, such as fMet-Leu-Phe or serum-coated zymosan, resulted in levels of LT synthesis that were very low in comparison with those elicited by ionophore, and our presently available techniques are not sensitive enough to detect the low levels of membrane translocation that may have occurred.

Clearly much remains to be learned concerning the regulation of 5-lipoxygenase in the intact leukocyte. In cell-free systems, 5-lipoxygenase can be activated by Ca2+ in the absence of membranes, indicating that, under these circumstances, membrane association is not required for activity. Furthermore, Ca2+ must be able to directly affect enzyme activity by a mechanism not involving membrane translocation. However, in cell-free assays, free arachidonic acid, Ca2+, and ATP are usually provided to maximize enzymatic activity. It is quite possible that, in the intact cell, the enzyme may be membrane-associated in order to obtain arachidonic acid that is being released from phospholipid stores. This is a plausible hypothesis due to the fact that cytosolic concentrations of arachidonate are not likely to be high. It is also intriguing to postulate that membrane translocation in the intact cell may effect a functional change in the enzyme itself. 5-Lipoxygenase catalyzes both the 5-lipoxygenase and the LTA synthase reactions (1–3). However, in cell-free systems, the coupling between the two reactions is very poor so that 5-HPETE is the major product. This is in contrast to the case in intact cells in which LT synthesis is highly efficient. Whether membrane association or some other aspect of the intracellular environment is responsible for this difference remains an interesting question for future research.

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