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## Interactions of 5-Lipoxygenase with Membranes: Studies on the Association of Soluble Enzyme with Membranes and Alterations in Enzyme Activity

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**ABSTRACT:** Treatment of rat basophilic leukemia cells (RBL-1) with the calcium ionophore A23187 resulted in activation of 5-lipoxygenase, as indicated by an induction of leukotriene release [Orning, L., Hammarström, S., & Samuelsson, B. (1980) *Proc. Natl. Acad. Sci. U.S.A.* 77, 2017]. The enzyme activation was accompanied by a time-dependent association of 5-lipoxygenase to the particulate fraction. When cells were lysed in the presence of 0.05-10  $\mu$ M  $\text{CaCl}_2$ , the soluble 5-lipoxygenase became associated with the particulate fraction. This was demonstrated by a decrease in immunoreactivities and enzymatic activities in the soluble fraction and a parallel increase in particulate-associated immunoreactivities. The particulate-bound enzyme was not active.  $\text{Ca}^{2+}$  induced the membrane association of 5-lipoxygenase when added into the incubation mixtures containing the membrane fraction with either the cytosolic fraction or the purified enzyme. 5-Lipoxygenase also bound to the microsomal-enriched fraction in the presence of  $\text{Ca}^{2+}$ . Maximal membrane binding was obtained after a 1-min incubation at 4 °C. When a fixed amount of isolated membranes (0.2 mg of protein) and increasing cytosolic protein (0.5-4 mg) were used, a linear increase in enzyme binding was observed. The binding became saturated at 3 mg of cytosolic protein/mg of membrane protein. 5-Lipoxygenase binding to the membrane fraction was unaffected by pretreatment of the membranes with trypsin but was inhibited by treating with phospholipase  $\text{A}_2$ , suggesting that phospholipids are involved. The membrane-bound enzyme was not extracted by treatment with a chelator (10 mM EDTA) or high salt (2 M NaCl) but was dissociated from membranes with detergents (0.5% sodium dodecyl sulfate, 10 mM Chaps, 0.5% digitonin, 0.5% Triton X-100, or 1% Brij 35). The membrane fraction when added to the cytosol, followed by subsequent addition of  $\text{Ca}^{2+}$  and substrate, stimulated 5-lipoxygenase activity. There was an increase in both the extent of product formation and the pseudo-steady-state velocity. The maximal stimulation was produced by 0.2 mg/mL membranes. In summary, these data suggest that  $\text{Ca}^{2+}$  induces a shift of 5-lipoxygenase from a soluble to a membrane-associated form which is accompanied by activation of the enzyme. Membrane phospholipids may be the elements in the membranes that bind to 5-lipoxygenase.

5-Lipoxygenases are enzymes in leukocytes and mast cells that oxidize arachidonic acid to 5-hydroperoxyeicosatetraenoic acid (5HPETE)<sup>1</sup> and leukotriene  $\text{A}_4$  ( $\text{LTA}_4$ ), which in turn are converted to a variety of products including leukotriene  $\text{B}_4$  and the peptidoleukotrienes ( $\text{LTC}_4$ ,  $\text{LTD}_4$ , and  $\text{LTE}_4$ ) (Borgeat & Samuelsson, 1979a,b; Borgeat et al., 1976; Jakschik & Lee, 1980; Radmick et al., 1980a,b; Panossian et al., 1982; Maas et al., 1982; Maycock et al., 1982). These substances exhibit a broad range of biological actions including effects on cell migration, enzyme secretion, smooth muscle contraction in the respiratory and gastrointestinal tracts, and vascular permeability. Considerable evidence now implicates these products in a variety of acute and chronic inflammatory responses in vivo as well as in acute allergic reactions affecting the airway and skin (Samuelsson, 1983).

5-Lipoxygenase is activated by calcium and ATP (Jakschik & Lee, 1980; Jakschik et al., 1980; Ochi et al., 1983; Hogaboom et al., 1986). It has also been found that  $\text{Ca}^{2+}$  may induce the binding of 5-lipoxygenase to the membranes

(Rouzer & Samuelsson, 1987). This suggests that activation of the enzyme is accompanied by a shift of the enzyme from the cytosol to the membrane within the cell. Considering the tendency of the enzyme to associate with membranes upon stimulation by  $\text{Ca}^{2+}$  and the abundance of its substrate in the plasma membrane, the translocation-activation may be a plausible regulatory mechanism. In the present studies, we demonstrate that treatment of RBL-1 cells with the calcium ionophore A23187 induces a shift of 5-lipoxygenase from the soluble to the particulate form. This is accompanied by an activation of the enzyme, as indicated by the production and release of  $\text{LTC}_4$ . The movement of the soluble enzyme to the particulate fraction occurs in physiological concentrations of  $\text{Ca}^{2+}$ . We have examined the interactions of 5-lipoxygenase

<sup>1</sup> Abbreviations: Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)- $N,N,N',N'$ -tetraacetic acid; Hepes,  $N$ -(2-hydroxyethyl)-piperazine- $N'$ -2-ethanesulfonic acid; 5HPETE, 5-hydroperoxyeicosatetraenoic acid; LT, leukotriene; RBL cell, rat basophilic leukemia cell; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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with membranous components and have attempted to correlate these interactions with the regulation of the enzyme activities. Results indicate that the membrane fraction stimulates the activity of the soluble 5-lipoxygenase, presumably through interacting with the soluble enzyme.

## EXPERIMENTAL PROCEDURES

### Materials

RBL-1 cells were obtained from American Type Culture Collection, Rockville, MD. Fetal calf serum was purchased from Hazelton Labs, Dutchland, PA.  $[1-^{14}\text{C}]$ Arachidonic acid (59.6 mCi/mmol) was obtained from Amersham, Arlington Heights, IL. Arachidonic acid was purchased from NuChek Prep, Inc., Elysian, MN. Goat serum was obtained from GIBCO Labs, Grand Island, NY. Alkaline phosphatase conjugated anti-rabbit IgG (Fc) was purchased from Promega, Madison, WI. Trypsin, phospholipase A<sub>2</sub> (porcine pancreas, 10 mg/mL), CaCl<sub>2</sub>, MgCl<sub>2</sub>, EDTA, EGTA, ATP, Hepes, NaCl, Na<sub>2</sub>PO<sub>4</sub>, sucrose, SDS, Chaps, digitonin, Triton X-100, Brij 35, Tween 20, 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt, and nitro blue tetrazolium were obtained from Sigma, St. Louis, MO. All solvents were HPLC grade and were purchased from Beckman, Fullerton, CA.

### Methods

**Cell Culture and Preparations of Membrane and Cytosolic Fractions.** RBL-1 cells were grown in spinner culture in Eagle's essential medium supplemented with 10% fetal calf serum. Cells were lysed by nitrogen cavitation (800 psi, 10 min) in 50 mM sodium phosphate (pH 7.0) and 1 mM EDTA at  $(6.5\text{--}7.5) \times 10^7$  cells/mL. The cell lysate was centrifuged at 35000g for 20 min. The 35000g supernatant obtained was recentrifuged at 100000g for 1 h, and the soluble fraction and pellet obtained were described as the cytosolic and microsomal enriched fraction, respectively. The 35000g pellet described as the particulate fraction was resuspended in 20 mL of cold 10 mM Hepes buffer, pH 7.3, layered over 40% sucrose containing 10 mM Hepes, pH 7.3, and centrifuged at 100000g for 1 h. The pellet and the pure white material at the sucrose-buffer interface were described as the mitochondrial and membrane-enriched fractions, respectively. The membranes were harvested, resuspended in 20 mL of cold 10 mM Hepes buffer, pH 7.3, and centrifuged at 100000g for 1 h. The resulting pellet was resuspended in the same buffer to give a final protein concentration of 10 mg/mL which was used in all studies.

**Determination of 5-Lipoxygenase Activity.** The standard 5-lipoxygenase assay mixture contained 10 mM Hepes, pH 7.4, 150 mM NaCl, 1 mM EDTA (buffer A), 2 mM CaCl<sub>2</sub>, 2 mM ATP, 10  $\mu\text{M}$   $[1-^{14}\text{C}]$ arachidonic acid (100 000 dpm), and enzyme (cytosolic fraction) in a total volume of 200  $\mu\text{L}$ . The sequence of addition was as follows: buffer A, ATP, cytosol fraction, CaCl<sub>2</sub>, and arachidonic acid. Ca<sup>2+</sup> was added 0.1 min before the addition of arachidonic acid to initiate the enzyme reaction. In some studies, membrane fraction (0.025–0.4 mg/mL) was added to the buffer A and ATP before the addition of enzyme, CaCl<sub>2</sub>, and  $[1-^{14}\text{C}]$ arachidonic acid. The reaction was performed at 20 °C for various times and terminated by the addition of 400  $\mu\text{L}$  of ice-cold acetone. The reaction products were extracted into ethyl acetate (once with 0.8 mL and once with 0.4 mL). A 1.5-mL aliquot of the upper ethyl acetate layer was removed and evaporated to dryness under argon. The evaporated material was resuspended into 70  $\mu\text{L}$  of methanol for reverse-phase high-performance liquid chromatographic analyses as described (Hogboom et al., 1986).

To examine the effects of Ca<sup>2+</sup> on inactivating 5-lipoxygenase, the cytosolic fraction (0.5 mg/mL) was incubated at room temperature, either alone or in the presence of 1 mM free Ca<sup>2+</sup>. In some experiments, 0.2 mg/mL isolated membranes were included in the incubation mixtures whereas ATP was not present. Aliquots of 200  $\mu\text{L}$  were removed at various times for the assays of enzyme activity. The enzyme assays were initiated by the addition of ATP (2 mM) and  $[1-^{14}\text{C}]$ -arachidonic acid (10  $\mu\text{M}$ , 100 000 dpm).

**Association of 5-Lipoxygenase with the Particulate Fraction in Cells Stimulated with the Calcium Ionophore A23187 or Lysed in Ca<sup>2+</sup>-Containing Media.** RBL-1 cells obtained from spinner culture were suspended ( $2 \times 10^7$  cells/mL) in 5 mM Hepes, pH 7.4, 140 mM NaCl, 5 mM KCl, 0.6 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, and 5 mM glucose (buffer B). Cells were treated with 10  $\mu\text{M}$  A23187 for various times (0, 2, 5, and 20 min) at 37 °C. The cells were then centrifuged (200g, 10 min), and supernatants were removed and set aside for assaying the release of lactate dehydrogenase. The cells were washed once with 5 mL of 10 mM Hepes, pH 7.4, and 150 mM NaCl and resuspended in 2–3 mL of 5 mM Hepes, pH 7.4, and 1 mM EDTA. The cells were immediately lysed by nitrogen cavitation (800 psi, 10 min). The soluble (35000g supernatant) and the particulate fractions (35000g pellet) were obtained as described above. The 5-lipoxygenase present in both fractions was determined by enzyme activity assays and immunoblots. In some experiments, cells were suspended ( $2 \times 10^7$  cells/mL) in 5 mM Hepes buffer, pH 7.6, containing various concentrations of free Ca<sup>2+</sup> (0.05–10  $\mu\text{M}$ ). The apparent free Ca<sup>2+</sup> concentrations in the solutions containing EGTA were calculated as described by Bartfai (1979), using a  $K_a$  value of  $8.04 \times 10^7 \text{ M}^{-1}$  for Ca-EGTA at pH 7.6. The cells were immediately lysed by nitrogen cavitation (800 psi, 10 min) in a total volume of 2–3 mL. In other experiments, cells were either lysed in a 100  $\mu\text{M}$  EGTA-containing medium followed by the addition of Ca<sup>2+</sup> (final free  $[\text{Ca}^{2+}] = 10 \mu\text{M}$ ) into the lysate or lysed in 10  $\mu\text{M}$  Ca<sup>2+</sup> followed by the addition of EGTA (1 mM). The soluble (35000g supernatant) and the particulate fractions (35000g pellet) were obtained and assayed for the presence of 5-lipoxygenase.

**Binding of Active or Inactive Cytosolic 5-Lipoxygenase or the Purified Enzyme to the Membrane Fraction.** Membrane fraction (0.2 mg of protein/mL) was incubated with the cytosolic fraction (approximately 0.5–1 mg of protein/mL) at 4 °C for 1 h in buffer A alone, or in the presence of 2 mM Ca<sup>2+</sup> or 2 mM Mg<sup>2+</sup>, in a total volume of 2 mL. In some experiments, the cytosolic fractions (0.5 mg/mL) were preincubated with 2 mM Ca<sup>2+</sup>, 2 mM ATP, and 10  $\mu\text{M}$  arachidonic acid at room temperature for 30 min followed by the addition of the membrane fraction. The incubation was continued at 4 °C for 1 h. At the end of the incubation, the mixture was centrifuged at 35000g for 20 min to separate the membranes from the soluble fraction. The membrane pellet was washed with 5 mL of buffer A. The 5-lipoxygenase activity and immunoreactivity present in the membrane or the soluble fractions were determined.

**Reversibility of the Binding of 5-Lipoxygenase to Membranes.** To examine whether the association of the 5-lipoxygenase with the membrane fraction is reversible, the membrane fraction was preincubated with the cytosolic fraction in the presence of 1 mM free Ca<sup>2+</sup> (4 °C, 1 h) to allow maximal membrane binding. The sample was centrifuged (35000g, 20 min) to precipitate the membranes which are then resuspended in 1 mL of either of the following solutions: 10 mM EDTA, 2 M NaCl, 0.5% SDS, 10 mM Chaps, 0.5%

digitonin, 0.5% Triton X-100, 1% Brij 35. The samples were incubated at 20 °C for 30 min and were centrifuged at 35000g for 20 min. The 5-lipoxygenase extracted into the soluble fraction was determined by immunoblot.

**Modification of the Membrane Fraction by Trypsin and Phospholipase.** The membrane fraction (10 mg/mL protein) was diluted with buffer A to a final concentration of 0.4 mg/mL. The sample was warmed to 25 °C and then treated with either trypsin (50 µg/mL) or phospholipase A<sub>2</sub> (10 µg/mL). Calcium chloride (2 mM) was included in the phospholipase A<sub>2</sub> treatment. After incubation of the preparations for 1 h at 25 °C, membranes from the samples were obtained by centrifugation (35000g, 20 min), were washed twice with buffer A, and were then used in the Ca<sup>2+</sup>-induced membrane binding of 5-lipoxygenase.

**Preparation of Antibodies.** RBL-1 cell 5-lipoxygenase was purified according to Hogaboom et al. (1986). It was electrophoretically pure as determined by silver staining. The enzyme had a specific activity of 2–5 µmol of products mg<sup>-1</sup> (3 min)<sup>-1</sup>. A female New Zealand White rabbit was immunized with 150 µg of 5-lipoxygenase emulsified with an equal volume of Freund's complete adjuvant. At 3 weeks after the primary immunization, the rabbit was boosted with 200 µg of 5-lipoxygenase which was obtained by excision from the SDS-PAGE gel. The gel slices were homogenized in 10 mM Tris-HCl, pH 7.4, and 150 mM NaCl and emulsified with an equal volume of Freund's complete adjuvant. Bleeding was performed 5 weeks after the primary immunization. Diluted sera were used in the immunoblots.

**Immunoblots.** Proteins were separated by SDS-PAGE (Laemmli, 1970) and transferred to nitrocellulose paper (0.35 A for 4 h). The nitrocellulose sheets were blocked with a solution containing 10% goat serum, 3% bovine serum albumin, 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl (TBS) for at least 1 h at room temperature. The nitrocellulose sheets were incubated with antiserum against 5-lipoxygenase diluted in blocking solution (1:500) for 1 h at room temperature. Unbound antibody was washed from nitrocellulose sheets by three changes of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20 (TBST), 5 min each. The nitrocellulose sheets were incubated with alkaline phosphatase conjugated antibody (1:7500 dilution) for 45 min and then washed 3 times in TBST, 15 min each. Immunoreaction products were detected with the addition of 0.2 mg/mL 5-bromo-4-chloro-3-indolyl phosphate and 0.4 mg/mL nitro blue tetrazolium in 100 mM Tris, pH 9.0, 100 mM NaCl, and 5 mM MgCl<sub>2</sub>. The immunoblots were photographed when they were wet, by using a Polaroid CU-5 Land camera, equipped with a no. 8 Kodak Wratten gelatin filter (Eastman Kodak Co., Rochester, NY) and type 665 Land film. The negative films of immunoblots were used for densitometric scannings. The densitometric scannings were performed with a Beckman DU-8 UV/VIS spectrophotometer (Beckman Instruments, Fullerton, CA). In all experiments, samples were electrophoresed on SDS-PAGE paralleled with a standard curve, which was generated by using various amounts of RBL-1 cytosolic fraction. The relative amount of 5-lipoxygenase was obtained on a linear portion of the standard curve. The details of the quantitation of 5-lipoxygenase will be reported elsewhere.

**Protein Concentrations.** These were determined according to Bradford (1976), using bovine serum albumin as the standard.

**Cell Viability.** Release of the cytoplasmic enzyme lactate dehydrogenase was assayed spectrophotometrically (Amador et al., 1963). Trypan blue exclusion was also used to monitor

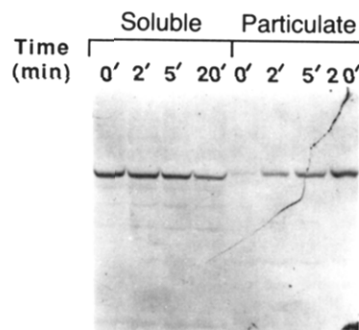


FIGURE 1: Time course of the translocation of 5-lipoxygenase during treatment of RBL-1 cells with the calcium ionophore A23187. Cells ( $20 \times 10^6$  cells/mL) were incubated at 37 °C with 10 µM A23187 for various times (0, 2, 5, and 20 min). Control (0 min) was incubated in the absence of extracellular Ca<sup>2+</sup>. 5-Lipoxygenase present in the soluble (35000g supernatant) and the particulate fractions (35000g pellet) was determined by immunoblot. The results shown are representative of at least three experiments.

cell viability. Cell viability was always greater than 95% when cells were treated with 10 µM A23187 for up to 30 min.

**Preparation of the Calcium Ionophore A23187.** The calcium ionophore A23187 was prepared as a stock solution at 10 mM in dimethyl sulfoxide (DMSO), which was then diluted to the appropriate concentrations with DMSO immediately before use. Each addition was as a 1-µL aliquot. In all experiments, controls and treatments were performed with DMSO in final concentrations equaled to 0.05%.

## RESULTS

**Translocation of 5-Lipoxygenase Induced by the Calcium Ionophore A23187.** The activation of RBL-1 cells by 10 µM calcium ionophore A23187 led to the noncytolytic release of LTC<sub>4</sub> (Orning et al., 1980; Parker et al., 1980). In unstimulated RBL-1 cells, 5-lipoxygenase is cytosolic (Hogaboom et al., 1986). However, upon stimulation by A23187, a portion of the enzyme becomes associated with the particulate fraction. This was demonstrated by examining the relative distribution of 5-lipoxygenase by immunoblot. A polyclonal antibody was raised against the purified RBL-enzyme. Immunoblot analysis of the purified 5-lipoxygenase or the cytosolic fraction revealed a major band of approximately 72 kDa (Figure 1). When cells were stimulated with 10 µM A23187, there was a gradual decrease in the cytosolic 5-lipoxygenase with increasing time of treatment (Figure 1, soluble 0-, 2-, 5-, and 20-min lanes), which was paralleled by an increase in particulate-associated enzyme (particulate 0-, 2-, 5-, and 20-min lanes).

**Association of 5-Lipoxygenase with the Particulate Fraction When Cells Were Lysed in Ca<sup>2+</sup>-Containing Media.** When cells were lysed in an EGTA-containing buffer with no Ca<sup>2+</sup> added, all of the 5-lipoxygenase was recovered in the 35000g soluble fraction, and none could be detected in the particulate fraction (Figure 2A). However, when cells were lysed in buffers containing increasing concentrations of free Ca<sup>2+</sup> (0.05–10 µM), there was a gradual decrease in soluble 5-lipoxygenase (Figure 2A) and enzyme activities (Figure 2B). These changes were paralleled with an increase in the amount of particulate-associated enzyme (Figure 2A). At 10 µM Ca<sup>2+</sup>, only 20% of soluble enzyme activity was recovered, and this is consistent with the results obtained from immunoblotting showing that approximately 20% of enzyme immunoreactivity remained in the soluble fraction (Figure 2B). However, it is difficult to estimate the amount of enzyme in the particulate fraction since the binding of 5-lipoxygenase to the particulate fraction may affect its interactions with the primary or the

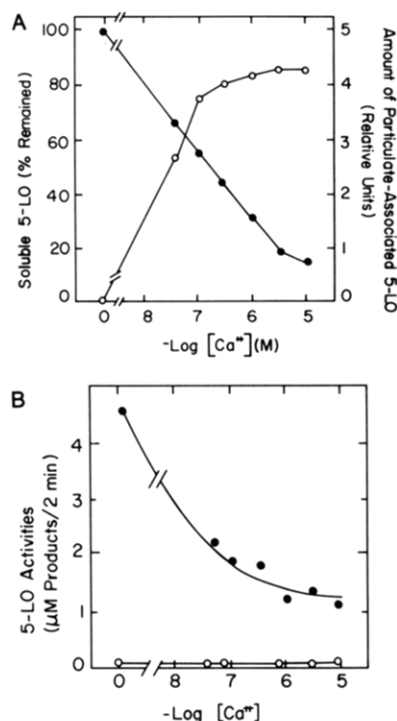


FIGURE 2: Effects of the  $\text{Ca}^{2+}$  concentration on the translocation of 5-lipoxygenase. Cells ( $20 \times 10^6$  cells/mL) were lysed in buffer A containing the indicated concentrations of free  $\text{Ca}^{2+}$ . (a) 5-Lipoxygenase was determined in the resulting soluble (35000g supernatant) (●) and particulate fractions (35000g pellet) (○) by immunoblot. The blot was photographed with a Polaroid CU-5 Land camera, and the relative quantities of the immunoreactive bands were determined by spectrophotometric scanning of the negative film. (b) 5-Lipoxygenase activity was determined in the soluble (●) and the particulate (○) fractions as described under Experimental Procedures.

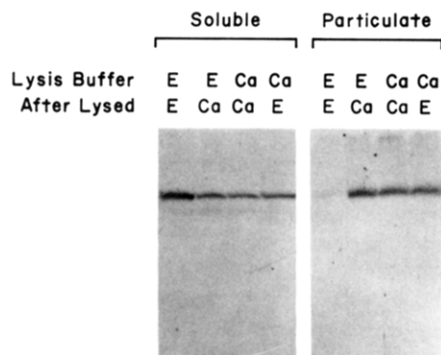


FIGURE 3: Relative distribution of 5-lipoxygenase when cells were lysed and processed under various conditions. Lanes E/E, cells were lysed in the presence of 100  $\mu\text{M}$  EGTA, and the cell lysate was centrifuged in the same medium; lanes E/Ca, cells were lysed at 100  $\mu\text{M}$  EGTA, and 10  $\mu\text{M}$   $\text{Ca}^{2+}$  (free concentration) was then added to the lysate; lanes Ca/Ca, cells were lysed at 10  $\mu\text{M}$   $\text{Ca}^{2+}$ , and the lysate was centrifuged in the same medium; lanes Ca/E, cells were lysed in 10  $\mu\text{M}$  free  $\text{Ca}^{2+}$ , and 1 mM EGTA was then added to the lysate. The soluble and particulate fractions from the individual experiments were obtained and processed in the same way as described under Experimental Procedures.

secondary antibody (alkaline phosphatase conjugated antibody). No enzyme activity was detected in the particulate-associated enzyme.

Figure 3 shows the relative distribution of 5-lipoxygenase between the soluble and the particulate fractions when cells were lysed and then processed under various conditions. When cells were lysed in the presence of EGTA (no free  $\text{Ca}^{2+}$ ), followed by the addition of 10  $\mu\text{M}$   $\text{Ca}^{2+}$  to the cell lysate, there was approximately an 80% decrease in the soluble (E/Ca lane)

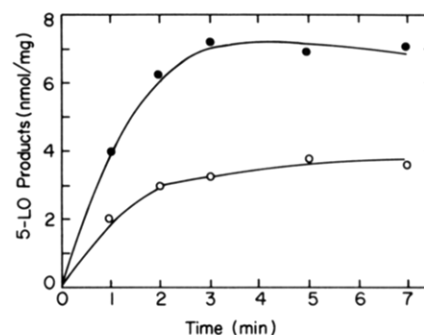


FIGURE 4: Stimulation of 5-lipoxygenase activity by the addition of membranes. Isolated membranes (0.2 mg/mL) were added to the enzyme assay mixture containing buffer A, ATP, and cytosolic fraction (0.5 mg of protein/mL).  $\text{Ca}^{2+}$  was added 0.1 min before the addition of [ $^{14}\text{C}$ ]arachidonic acid to initiate the enzyme reaction. The results shown are representative of at least three experiments. (○) Cytosolic fraction; (●) cytosolic fraction with isolated membranes.

enzyme which was recovered in the particulate fraction (E/Ca lane). Similar distribution of the enzyme was obtained when cells were lysed and then centrifuged in a 10  $\mu\text{M}$   $\text{Ca}^{2+}$ -containing buffer (Ca/Ca, soluble and particulate lanes). This indicates that cell integrity is not required for the  $\text{Ca}^{2+}$ -mediated membrane binding of the enzyme.

The addition of EGTA to the homogenate following lysis in the presence of  $\text{Ca}^{2+}$  shows no change in the distribution of 5-lipoxygenase (Figure 3, Ca/E lane). This suggests that although  $\text{Ca}^{2+}$  is required to initiate the enzyme-membrane association, once the enzyme is bound, the continued presence of  $\text{Ca}^{2+}$  is not required to maintain this association.

**Interaction of the Cytosolic 5-Lipoxygenase with the Membrane Fraction and Its Effects on Enzyme Activation.** Membrane fraction stimulates 5-lipoxygenase activity. Not only is there an overall increase in the extent of product formation but also there is an enhancement of the pseudo-steady-state velocity (Figure 4). The activation by membrane fraction was concentration dependent. Membrane fraction at 0.2 mg/mL produced near-maximal stimulation.

To examine whether the membrane fraction activates the 5-lipoxygenase by association with the enzyme, we studied their possible interactions in the same incubation mixtures used for enzyme assays (0.5 mg/mL cytosolic protein, 0.2 mg/mL membrane protein, 1 mM EDTA, and 2 mM  $\text{Ca}^{2+}$ ). The membrane fraction added showed no detectable 5-lipoxygenase. After incubation in the presence of  $\text{Ca}^{2+}$ , the soluble 5-lipoxygenase was membrane-associated, which parallels a decrease in the amount of soluble enzyme (data not shown). No enzyme activity could be detected for the membrane-bound 5-lipoxygenase. When incubation was performed in the absence of  $\text{Ca}^{2+}$ , or when  $\text{Mg}^{2+}$  was used at the same concentration (2 mM) as  $\text{Ca}^{2+}$ , no enzyme was obtained in the membrane fraction. Therefore,  $\text{Ca}^{2+}$  is required for the binding of enzyme to the membrane fraction. The  $\text{Ca}^{2+}$ -mediated membrane binding of 5-lipoxygenase is rapid, and maximal binding was obtained after 1 min at 4  $^{\circ}\text{C}$  and did not change for an hour.

The cytosolic enzyme which had been inactivated by incubation with  $\text{Ca}^{2+}$ , ATP, and arachidonic acid displayed  $\text{Ca}^{2+}$ -dependent binding to the membrane fraction that was comparable to the active enzyme. However, when free  $\text{Ca}^{2+}$  was removed from the incubation mixture by adding 3 mM EDTA before the addition of membrane fraction, no membrane association of the inactive enzyme could be detected. These results suggest (1) that there is no difference in the extent of membrane binding between the active and inactive 5-lipoxygenase and (2) that the binding of both requires  $\text{Ca}^{2+}$ .

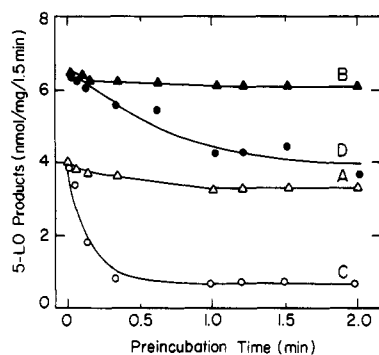


FIGURE 5: Inactivation of 5-lipoxygenase by incubation with  $\text{Ca}^{2+}$ . Incubation mixtures contained 0.5 mg/mL cytosolic fraction, 2 mM ATP, and buffer A, together with the following additions: aliquots of 200  $\mu\text{L}$  were removed at various times for the assay of enzyme activities. Line A, incubation mixture alone; line B, with 0.2 mg/mL membranes; line C, with 1 mM free  $\text{Ca}^{2+}$ ; line D, with 1 mM free  $\text{Ca}^{2+}$  and 0.2 mg/mL membranes.

Using a fixed amount of membrane fraction (0.2 mg of protein) and increasing cytosolic protein (0.5–4 mg), we found a linear increase in the amount of membrane-associated enzyme. Binding saturated at 15 mg of cytosolic protein/mg of membrane protein.

**Inactivation of 5-Lipoxygenase by Preincubating with  $\text{Ca}^{2+}$ .** We examined the effects of  $\text{Ca}^{2+}$  and membrane fraction on the stability of 5-lipoxygenase. When the cytosolic fraction was incubated at room temperature for 2 min prior to being assayed for enzyme activity, a slight decrease in activity was observed (Figure 5, curve A). If the membrane fraction was included in the incubation mixture, 5-lipoxygenase activity was increased by approximately 50–70%, which is similar to the results obtained in Figure 4. The stimulatory effect of the membrane fraction was maintained throughout the period of preincubation (curve B). On the other hand, preincubation of the enzyme with  $\text{Ca}^{2+}$  resulted in a rapid inactivation of the enzyme. A decrease of 90% was observed within 1 min (curve C). However, preincubation of the enzyme with  $\text{Ca}^{2+}$  and membranes for 1 min resulted in only a 40% decrease in activity (curve D). These data suggest that (1)  $\text{Ca}^{2+}$  alone may enhance inactivation of 5-lipoxygenase and that (2) membrane fraction stimulates enzyme activity and reduces the inactivation of the enzyme by  $\text{Ca}^{2+}$ .

**Binding of Cytosolic 5-Lipoxygenase with the Mitochondrial and Microsomal Membrane-Enriched Fractions.** The RBL-1 cell mitochondrial and microsomal membrane-enriched fractions did not exhibit detectable 5-lipoxygenase by immunoblot. After incubation with the cytosolic fraction in the presence of  $\text{Ca}^{2+}$ , some mitochondrial and microsomal membrane-associated enzyme was detected. Similar to the observation with membrane fraction, the binding of enzyme to these fractions was  $\text{Ca}^{2+}$  dependent. No enzyme was detected in these fractions if  $\text{Ca}^{2+}$  was eliminated.

**Binding of Purified 5-Lipoxygenase to the Membrane Fraction.** Highly purified 5-lipoxygenase bound to the membrane fraction even in the absence of  $\text{Ca}^{2+}$ . The purified enzyme was also found to adhere to a SpheroGel TSK column and glass tubes. Addition of  $\text{Ca}^{2+}$  increased the binding 4–5-fold. Whether the binding to membranes in the presence and absence of  $\text{Ca}^{2+}$  is mediated by the same mechanism remains to be studied.

**Binding of 5-Lipoxygenase to the Trypsin and Phospholipase  $\text{A}_2$  Modified Membranes.** Membrane fraction was treated with trypsin or phospholipase  $\text{A}_2$ , and the  $\text{Ca}^{2+}$ -induced binding of 5-lipoxygenase to the treated membranes was determined. Trypsin treatment of the membrane fraction did

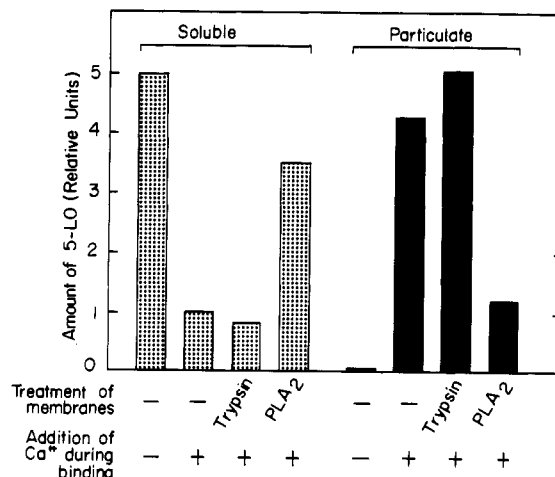


FIGURE 6: Binding of 5-lipoxygenase to trypsin and phospholipase  $\text{A}_2$  modified membranes. A 1-mL aliquot of isolated membranes (0.4 mg/mL) was incubated with trypsin (50  $\mu\text{g}/\text{mL}$ ) or phospholipase  $\text{A}_2$  (10  $\mu\text{g}/\text{mL}$ ) for 1 h at 25  $^{\circ}\text{C}$ .  $\text{Ca}^{2+}$  (2 mM) was included in the phospholipase  $\text{A}_2$  treatment. After extensive washing of the membranes, the  $\text{Ca}^{2+}$ -induced membrane binding of lipoxygenase was determined.

not affect binding with 5-lipoxygenase. This was demonstrated in Figure 6 that the same amount of enzyme was bound to control membrane fraction and to trypsin-treated membrane fraction, and also a similar percentage decrease in the soluble enzyme in both incubations (Figure 6). In contrast, phospholipase  $\text{A}_2$  treatment resulted in an approximately 70% reduction in the amount of membrane-associated 5-lipoxygenase.

**Reversibility of the Membrane-Associated 5-Lipoxygenase.** The membrane-associated enzyme was stable to chelator (10 mM EDTA) and high salt (2 M NaCl) but could be readily dissociated by extracting with detergents (0.5% SDS, 10 mM Chaps, 0.5% digitonin, 0.5% Triton X-100, or 1% Brij 35). These studies suggest a tight binding of the 5-lipoxygenase to the membrane fraction.

## DISCUSSION

During the course of our studies, other investigators demonstrated that 5-lipoxygenase in the presence of  $\text{Ca}^{2+}$  is membrane-associated (Rouzer & Samuelsson, 1987). In the present study, the interactions between 5-lipoxygenase and the membrane fraction were examined in the same incubation mixture that was used for enzyme assay. Results show that in the presence of  $\text{Ca}^{2+}$  the enzyme became membrane-associated and the enzyme activity of the incubation mixture containing both the cytosolic and membrane fractions was higher than that for the cytosolic fraction alone. These *in vitro* studies suggest the possibility that in intact cells, the interactions between cytosolic 5-lipoxygenase and membranes are involved in the regulation of enzyme activity.

When the RBL-1 cells were stimulated with the calcium ionophore A23187, which permeabilized cell membranes to  $\text{Ca}^{2+}$ , there was a decrease in the cytosolic 5-lipoxygenase, and the enzyme became associated with the particulate fraction. Coupled with this was an increase in  $\text{LTC}_4$  release. The stimulating effects of A23187 suggest that  $\text{Ca}^{2+}$ -sensitive component(s) is(are) involved in the synthesis of  $\text{LTC}_4$ . It has been demonstrated that in both RBL-1 cells (Jakschik et al., 1977, 1978) and mouse peritoneal macrophages (Tripp et al., 1985), the  $\text{LTC}_4$  production is stimulated by an increase in intracellular  $\text{Ca}^{2+}$  concentration, which is mediated by an activation of 5-lipoxygenase. Our results indicate that in intact cells, an increase in the intracellular concentration of  $\text{Ca}^{2+}$



induces the association of 5-lipoxygenase to the membranes, and a shift of the enzyme from the soluble to the particulate form is accompanied by the production of LTC<sub>4</sub>.

It is perplexing that no particulate-associated enzyme activity could be obtained. There are four possible explanations: (1) Although Ca<sup>2+</sup> is required to induce the membrane association of 5-lipoxygenase, it also inactivates the enzyme, as demonstrated by the results in Figure 5. Since inactivation enzyme binds to the membranes to the same extent as the active enzyme, it is possible that the 5-lipoxygenase is inactivated by Ca<sup>2+</sup> before it binds to the membranes. (2) Another possibility is that in the presence of Ca<sup>2+</sup>, the enzyme binds to the membranes, presumably to membrane phospholipids. It may utilize the phospholipid-associated arachidonic acid as substrate and undergo self-inactivation during conversion of arachidonic acid to LTA<sub>4</sub>. (3) 5-Lipoxygenase activity may be masked in the membranes by interfering components, similar to the decrease in activity of the translocated protein kinase C that has been reported (Gopalakrishna et al., 1986). To examine this possibility, further experiments should be performed to recover the enzyme activity by solubilizing the particulate-associated enzyme. (4) Rouzer and Samuelsson (1987) have shown that the membrane-associated enzyme required a cytosolic factor(s) for its activity. They recovered moderate particulate-associated enzyme activities by the addition of a 60–90% saturated ammonium sulfate cytosolic fraction. However, the 60–90% saturated ammonium sulfate fraction we obtained from the RBL-1 cytosolic fraction was contaminated with a significant amount of 5-lipoxygenase (demonstrated by immunoblot). Therefore, it could not be used in the add-back experiment to stimulate the particulate-associated enzyme.

Under the optimal conditions described above, Ca<sup>2+</sup>-induced binding of 5-lipoxygenase saturated at 10–15 mg of cytosolic protein/mg of membrane protein. On the basis of the amount of purified enzyme obtained from the cytosolic fraction (0.14 µg of enzyme/mg of cytosolic protein) (Hogaboam et al., 1986), we calculate that approximately 2 µg of purified 5-lipoxygenase was associated with 1 mg of membrane protein. As a moderate amount of enzyme is able to bind to the membrane fraction, these results suggest that the membrane association may not take place at highly specific receptor site(s) in the membrane fraction. Moreover, our studies demonstrate that there is little change in Ca<sup>2+</sup>-induced binding of 5-lipoxygenase with the trypsin-treated membranes, indicating that the trypsin-sensitive membrane proteins may not be involved. The decrease noted in membrane binding after treatment with phospholipase A<sub>2</sub> suggests that membrane phospholipids are involved. The observations that 5-lipoxygenase may bind to the membrane fraction as well as to mitochondrial and microsomal membrane-enriched fractions, and Ca<sup>2+</sup> may also induce binding of the enzyme to the TSK-5PW column (Wong and Hwang, unpublished observations), suggest that the Ca<sup>2+</sup>-induced binding of the enzyme to subcellular fractions may not be specific.

In our laboratory, we have recently cloned and sequenced the cDNA clone encoding rat 5-lipoxygenase (Balcerek et al., 1988). It exhibits over 95% sequence homology with the cloned human 5-lipoxygenase (Mitumoto et al., 1988; Dixon et al., 1988). The enzyme possesses 2 copies of a 17 amino acid consensus sequence which represents a new type of Ca<sup>2+</sup> binding site or a lipid binding domain (Geisow et al., 1986). This feature is common to a family of Ca<sup>2+</sup>-dependent phospholipid binding proteins, including calelectrin from the electric organ of *Torpedo marmorata* (Sudhof et al., 1985),

endonexin (Geisow et al., 1984), protein II (Gerke & Weber, 1985), p36 from pig mesenteric lymph nodes (Hexham et al., 1986), and p68 from B-lymphoblastoid cells (Crompton et al., 1988). Functionally, 5-lipoxygenase appears to share a common property with the above proteins: in the presence of Ca<sup>2+</sup>, each of the protein becomes membrane-associated or binds to liposomes and hydrophobic affinity matrices. Further study is under way in our laboratory to examine the interactions of 5-lipoxygenase with liposomes, the specificities toward various phospholipids, and the position of the Ca<sup>2+</sup>-phospholipid binding domain.

In summary, both in intact cells and in *in vitro* incubations, a shift of 5-lipoxygenase from the soluble to the particulate form is accompanied by an activation of enzyme. However, the finding that the membrane-associated enzyme exhibits no activity raises an unsolved paradox. Our studies also show that Ca<sup>2+</sup> induced the association of enzyme to both membranes and mitochondrial and microsomal membrane-enriched fractions and also to other hydrophobic matrices, suggesting that the binding may not be specific. It is possible that in the cells, this binding may not occur solely in association with the activation of 5-lipoxygenase but may also serve as a sequestering mechanism to remove the enzyme from the cytosol to attenuate its activity. Although further studies are required to obtain conclusive evidence, the possibility that the membranes and microsomes may play an important role as a mechanism regulating activity of cytosolic 5-lipoxygenase is proposed.

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**Registry No.** Ca, 7440-70-2; 5-lipoxygenase, 80619-02-9.

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## Anion Binding to Neutral and Positively Charged Lipid Membranes<sup>†</sup>

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**ABSTRACT:** Aqueous anion binding to bilayer membranes consisting of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was investigated by using deuterium and phosphorus-31 nuclear magnetic resonance (NMR) spectroscopy. Only those anions that exhibit chaotropic properties showed significant binding to POPC membranes. A detailed investigation of thiocyanate binding to neutral POPC and to positively charged mixed POPC/dihexadecyldimethylammonium bromide (DHDMA) (8:2 mol/mol) membranes revealed changes in the <sup>2</sup>H NMR quadrupole splittings from POPC specifically deuterated at either the  $\alpha$ -segment or the  $\beta$ -segment of the choline head group which were consistent with a progressive accumulation of excess negative charge at the membrane surface with increasing SCN<sup>-</sup> concentration. Both the <sup>2</sup>H and <sup>31</sup>P NMR spectra indicated the presence of fluid lipids in a bilayer configuration up to at least 1.0 M NaSCN with no indication of any phase separation of lipid domains. Calibration of the relationship between the change in the <sup>2</sup>H NMR quadrupole splitting and the amount of SCN<sup>-</sup> binding provided thiocyanate binding isotherms. At a given SCN<sup>-</sup> concentration the positively charged membranes bound levels of SCN<sup>-</sup> 3 times that of the neutral membranes. The binding isotherms were analyzed by considering both the electrostatic and the chemical equilibrium contributions to SCN<sup>-</sup> binding. Electrostatic considerations were accounted for by using the Gouy-Chapman theory. For 100% POPC membranes as well as for mixed POPC/DHDMA (8:2 mol/mol) membranes the thiocyanate binding up to concentrations of 100 mM was characterized by a partition equilibrium with an association constant of  $K \approx 1.4 \pm 0.3 \text{ M}^{-1}$ . Hence, the greater levels of thiocyanate binding occurring in the presence of positively charged membranes were primarily the result of electrostatic effects. The <sup>2</sup>H NMR results further indicated that the anions were binding in the plane of the POPC choline head group quaternary nitrogen. The factor critical to the ability of an aqueous anion to bind to lipid membrane surfaces appeared to be the ease with which its hydration shell water molecules could be removed.

**L**ipid bilayers bind a variety of aqueous cations [cf. McLaughlin (1977)], anions (Tatullian, 1983), hydrophobic ions [cf. Honig et al. (1986)], polyamines (Chung et al.,

1985b), and proteins (Deveaux & Seigneuret, 1985). Most of these studies have been performed by measuring the electrophoretic mobility of phospholipid vesicles from which it was possible to calculate the so-called  $\zeta$  potential, i.e., the average electrostatic potential at the plane of shear. The  $\zeta$  potential could then be related to the electric surface charge density, in terms of the Gouy-Chapman theory, and, in turn, also to

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