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On the nature of the 5-lipoxygenase reaction in human leukocytes: Characterization of a membrane-associated stimulatory factor

(5-hydroperoxyicosatetraenoic acid/5-hydroxyicosatetraenoic acid/leukotriene/lipoxin/inflammation)

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ABSTRACT **When 10,000 × *g* supernatants of human leukocyte homogenates were subjected to centrifugation at 100,000 × *g* for 75 min, the activity of 5-lipoxygenase decreased by 30–60%, even though no enzyme was detectable in the resuspended 100,000 × *g* pellet. Recombination of the 100,000 × *g* supernatant and pellet resulted in a restoration of the lost enzymatic activity, indicating the presence of a 5-lipoxygenase stimulatory factor in the microsomal membrane preparation. Dialysis of human leukocyte supernatants resulted in an apparent decrease in 5-lipoxygenase activity, but only in samples that contained the membrane-associated stimulatory factor, suggesting that the factor required a small molecular weight component for optimal function. The 5-lipoxygenase stimulatory activity was highly unstable to washing of the 100,000 × *g* pellet or to incubation (16–20 hr) at 4°C. In contrast, the activity was remarkably stable to heat (100°C for 40 min). The responses of the 12- and 15-lipoxygenases in human leukocyte homogenates to the membrane-associated factor and to dialysis were notably different from that of the 5-lipoxygenase. These results demonstrate, therefore, that the 5-lipoxygenase is unique among the human lipoxygenases, not only in its requirement for Ca^{2+} and ATP but also in its regulation by a membrane-associated stimulatory factor. The mechanism of action of this regulatory factor is of obvious interest for the understanding of the control of leukotriene and lipoxin biosynthesis.**

The enzyme 5-lipoxygenase has become the subject of increasing interest in recent years, after its role in the synthesis of leukotrienes and lipoxins was defined (1, 2). Because these potent arachidonic acid (20:4) metabolites most likely are involved in the regulation of inflammation and immunity as well as other physiological and pathophysiological processes, a detailed understanding of their biosynthetic pathways should provide useful information for a variety of disciplines in biology and medicine.

A number of lipoxygenases from various sources have been purified and studied. These include enzymes from soybeans and potatoes, which produce predominantly 15-hydroperoxyicosatetraenoic acid (15-HPETE) and 5-HPETE from 20:4, respectively. A lipoxygenase with specificity for the C-12 and C-15 positions of 20:4 has been isolated from rabbit reticulocytes, and the characterization of a 20:4 15-lipoxygenase from rabbit leukocytes has also been reported (3–6). In each of these cases, the isolated enzyme was found to be a cytosolic protein that was not dependent upon other proteins or membrane components for activity.

In the past, similar efforts to isolate and characterize the mammalian 20:4 5-lipoxygenase have met with limited success due to a high degree of instability of the protein. Recently, however, the purification of the 5-lipoxygenase from human leukocytes was reported (7). These studies showed that maximal activity of the human leukocyte enzyme requires not only Ca^{2+} and ATP but also the presence of at least two high molecular weight cytosolic factors and one membrane-bound component. These findings suggest that the human leukocyte 5-lipoxygenase possesses a unique and complex regulatory system that may play a key role in the control of leukotriene and lipoxin formation. In this report we describe in detail the results of investigations of the membrane-associated 5-lipoxygenase stimulatory factor of human leukocytes.

MATERIALS AND METHODS

The preparation of human leukocyte homogenates has been described (7, 8).

The 5-, 12-, and 15-lipoxygenase activities were assayed at 37°C and pH 7.5 for an incubation period of 10 min with 100 nmol of [^{14}C]20:4 (1.5 mCi/mmol; 1 Ci = 37 GBq) in a total volume of 1 ml. Assay mixtures routinely contained 2 mM ATP, 4 mM $CaCl_2$, and 20 μM mixed, nonenzymatically generated HPETEs as stimulatory factors (7). Details of the assay conditions and the analysis of samples by extraction, silicic acid column chromatography, and high-pressure liquid chromatography have been published (7). One unit of enzyme activity is defined as the quantity that produces 1 nmol of HPETE under standard assay conditions. The data shown represent the results of multiple experiments in which single samples were assayed.

Preparation of Microsomal Membranes. After centrifugation of the leukocyte homogenate at 10,000 × *g* for 15 min, the resulting supernatant was subjected to further centrifugation at 100,000 × *g* for 75 min. The 100,000 × *g* supernatant was removed, and the pellet was resuspended to 1/10 the original volume in homogenization buffer (50 mM potassium phosphate containing 0.1 M NaCl, 2 mM EDTA, and 1 mM dithiothreitol, pH 7.1) by using a hand-driven glass homogenizer with a tightly fitting Teflon pestle. In some experiments, the centrifugation and resuspension procedure were repeated to produce a washed microsomal membrane preparation.

Preparation of Concentrated Ultrafiltrate. A 10,000 × *g* supernatant of leukocyte homogenate was concentrated at 4°C on an ultrafiltration membrane having a molecular weight cutoff of 10,000 (PM10, Amicon). The filtrate was collected and concentrated 10-fold under reduced pressure with warming in a 50°C water bath for ≈15 min.

Protein Assay. Protein concentrations were estimated by the method of Bradford with bovine serum albumin as a standard (9). The protein concentrations of 10,000 × *g* supernatants,

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Abbreviations: HPETE, hydroperoxyicosatetraenoic acid; 20:4, arachidonic acid.

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100,000 × *g* supernatants, and 100,000 × *g* pellets were 2.0–3.5 mg/ml, 1.5–2.0 mg/ml, and 1.5–3.0 mg/ml, respectively.

RESULTS

Effects of Microsomal Membrane on Leukocyte Lipoxygenase Activities. The 10,000 × *g* supernatants of leukocyte homogenates possessed 5-, 12-, and 15-lipoxygenase activities in varying proportions. As shown in Fig. 1, centrifugation of these preparations at 100,000 × *g* for 1 hr produced a decrease in the activity of all three enzymes in the resulting supernatant, although only 12-lipoxygenase activity could be detected in the resuspended 100,000 × *g* pellet. When the 100,000 × *g* supernatants and pellets were recombined before assay, a striking difference was noted in the behavior of the three enzymes. The activity of the 5-lipoxygenase was stimulated by the addition of the pellet fraction in a clearly dose-dependent manner. As little as 50 μl of the membrane preparation completely restored the enzyme activity to the level of the original 10,000 × *g* supernatant, and increasing quantities of membrane caused even greater stimulation. The 15-lipoxygenase was also stimulated by the 100,000 × *g* pellet fraction, but only at the lower concentrations tested. As the quantity of added membrane was increased from 100 to 200 μl, the stimulatory effect on the 15-lipoxygenase was lost, and an enzyme inhibition was observed. Although the activity of the 12-lipoxygenase appeared to increase upon addition of the pellet to the supernatant, the actual measured activity in the combined samples was never as high as the sum of the activities of the individual fractions. Thus, in the case of the 12-lipoxygenase, combination of the 100,000 × *g* supernatants and pellets effected an enzyme inhibition.

Effect of Dialysis on Leukocyte Lipoxygenase. Fig. 2 demonstrates the effect of dialysis on 5-, 12-, and 15-lipoxygenase activities. The 12- and 15-lipoxygenases were stimulated as a result of dialysis, regardless of whether a 10,000 × *g* or a 100,000 × *g* supernatant was studied. In contrast, dialysis always resulted in an inhibition of 5-lipoxygenase activity in 10,000 × *g* supernatants, whereas the enzyme in 100,000 × *g* supernatants was not affected by this procedure.

The fact that dialysis only inhibited the 5-lipoxygenase in 10,000 × *g* supernatants suggested that this effect may be dependent on the presence of microsomal membranes in the sample. This hypothesis was supported in recombination experiments in which microsomal membranes were added to dialyzed and untreated 100,000 × *g* supernatants. As shown in Fig. 2, the stimulatory activity of the membrane fraction was not as great when the 100,000 × *g* supernatant had been dialyzed previously.

To determine whether the 5-lipoxygenase stimulatory activity of the microsomal membrane was dependent on a small molecular weight factor that was removed by dialysis, the effects of a concentrated ultrafiltrate of the 10,000 × *g* supernatant were investigated. As shown in Fig. 2, addition of such a preparation to samples containing dialyzed 100,000 × *g* supernatants and 100,000 × *g* pellets resulted in small increases in detectable enzymatic activity. However, a much more striking effect was observed upon increasing the quantity of ultrafiltrate from 50 to 100 μl per assay sample, which resulted in a marked enzyme inhibition.

Effect of Ca^{2+} and ATP on 5-Lipoxygenase Activity. The requirement of the mammalian 5-lipoxygenase for Ca^{2+} and ATP is well documented and has been confirmed for the human enzyme (7, 10, 11). However, the precise role of these cofactors in the lipoxygenase reaction is poorly understood. To determine whether Ca^{2+} or ATP could be involved in the interaction between the 5-lipoxygenase and the membrane-associated stimulatory factor, the effect of omission of each of the cofactors was examined in the presence and absence of 100,000 × *g* pellet. As shown in Fig. 3, both Ca^{2+} and ATP effected an enzyme stimulation in the absence or presence of microsomal membranes. ATP was never stimulatory unless Ca^{2+} was also included in the reaction mixture. In contrast, 5-lipoxygenase activity was partially activated by Ca^{2+} in the

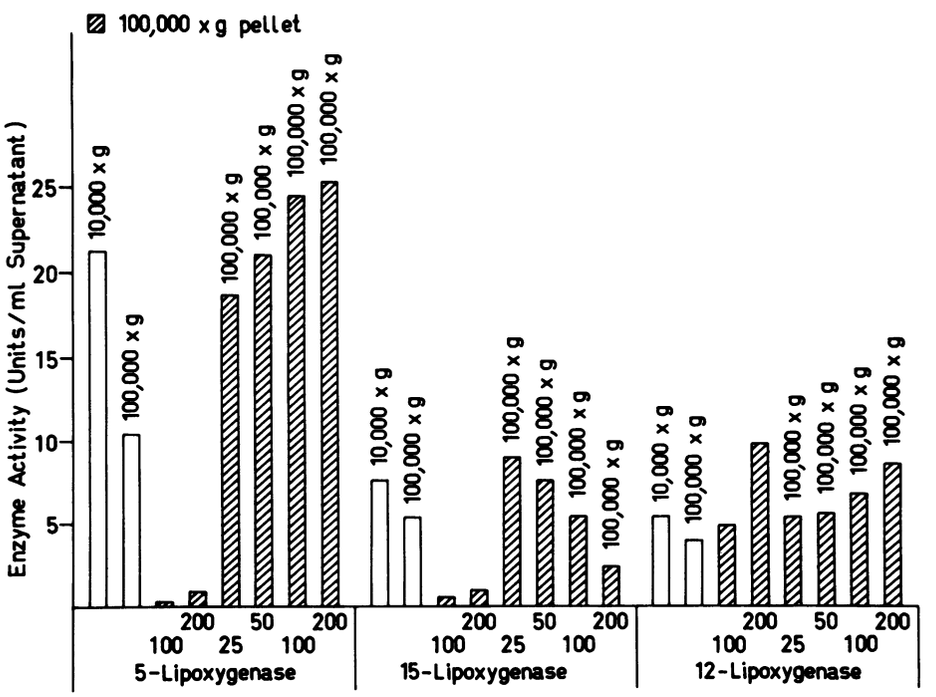

FIG. 1. Effect of high-speed centrifugation on leukocyte lipoxygenase activities. Leukocyte homogenates were subjected to centrifugation at 10,000 × *g* for 15 min followed by centrifugation at 100,000 × *g* for 75 min. The 100,000 × *g* pellets were resuspended in homogenization buffer. The 5-, 12-, and 15-lipoxygenase activities were assayed by using 500-μl aliquots of the 10,000 × *g* and 100,000 × *g* supernatants. The numbers below the bars indicate the quantities (μl) of 100,000 × *g* pellet that were included in the assay samples. The notations above the bars indicate the supernatants that were assayed.

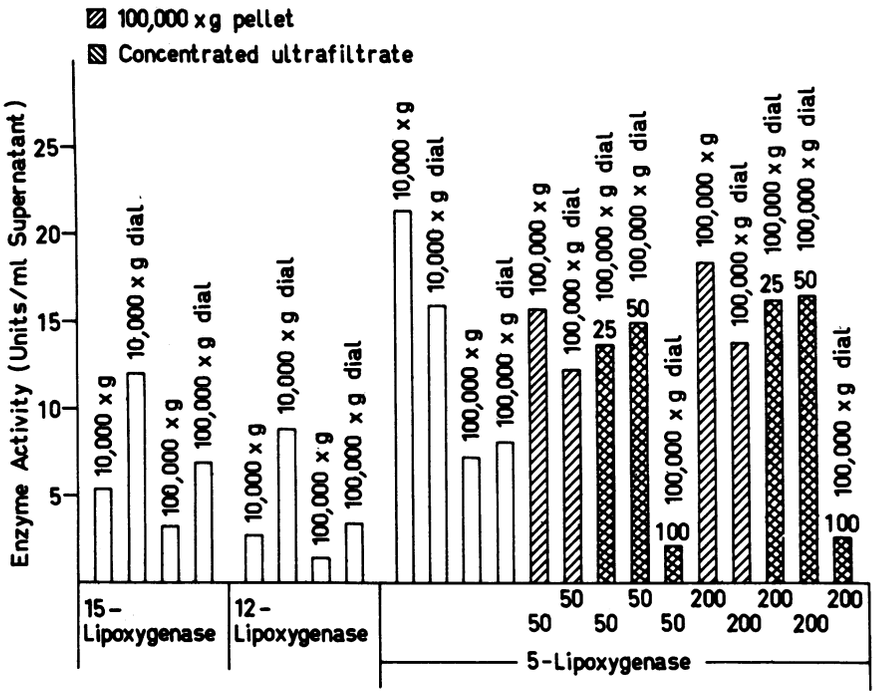

FIG. 2. Effect of dialysis on leukocyte lipoxygenase activities. The 10,000 × *g* and 100,000 × *g* supernatants of leukocyte homogenates were subjected to dialysis against 3 × 100 volumes of homogenization buffer for a total of 3 hr. Five hundred microliters of the dialyzed and undialyzed supernatants was assayed for 5-, 12-, and 15-lipoxygenase activities. The numbers below the bars indicate the quantities (μl) of 100,000 × *g* pellet that were added to the samples. The numbers above the bars are the quantities (μl) of a concentrated ultrafiltrate of the 10,000 × *g* supernatant that were included. The notations 10,000 × *g*, 100,000 × *g*, 10,000 × *g* dial, and 100,000 × *g* dial indicate the corresponding undialyzed and dialyzed supernatants.

absence of ATP. The latter finding was consistently observed in samples containing microsomal membranes but was variable for samples containing 100,000 × *g* supernatant alone.

Stability of Membrane-Associated 5-Lipoxygenase Stimulatory Factor: Effect of Pellet Washing. Fig. 4 shows the results obtained when a resuspended 100,000 × *g* pellet was subjected to a second centrifugation at 100,000 × *g* for 75 min and then reconstituted to its original volume. This procedure resulted in a total loss of the stimulatory activity of the membrane preparation, and the activity was not restored when the supernatant from the wash step was added back to the assay mixture (Fig. 4). It could be concluded, therefore, that the loss of activity resulting from the washing procedure was not due to removal of a loosely associated active component from the membrane. Fig. 4 also indicates that washing the membrane preparation did not alter its 12-lipoxygenase activity. Thus, the presence of this enzyme in the 100,000 × *g* pellet could be dissociated from the 5-lipoxygenase stimulatory activity.

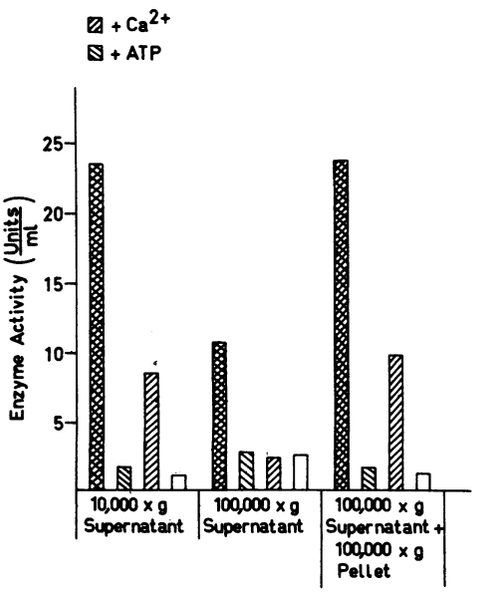

FIG. 3. Ca^{2+} and ATP dependence of leukocyte 5-lipoxygenase. Five hundred microliters of 10,000 × *g* and 100,000 × *g* supernatants of leukocyte homogenates was assayed for 5-lipoxygenase activity under standard assay conditions (4 mM Ca^{2+}, 2 mM ATP) and in the absence of Ca^{2+} or ATP, or both. Where indicated, 200 μl of 100,000 × *g* pellet was added to the 100,000 × *g* supernatant.

Stability of Membrane-Associated 5-Lipoxygenase Stimulatory Factor: Effect of Time. When 100,000 × *g* pellets were incubated at 4°C for a period of up to 20 hr, a marked decrease in their capacity to stimulate 5-lipoxygenase activity in 100,000 × *g* supernatants occurred. As shown in Fig. 5, the 5-lipoxygenase activity in the 100,000 × *g* supernatant was relatively stable, decreasing from 11.5 units/ml to 7.28 units/ml during the 20-hr incubation. Initially, addition of the 100,000 × *g* pellet to the supernatant immediately prior to assay resulted in a stimulation of 5-lipoxygenase activity to 21.9 units/ml of supernatant. In contrast, after 20 hr at 4°C, the membrane preparation had no effect on, or was slightly inhibitory to, the enzyme. This rapid decline in the activity of the 100,000 × *g* pellet could be prevented by mixing it with the 100,000 × *g* supernatant at the beginning of the 20-hr incubation. Samples treated in this way maintained activities approximately equal to those of the 10,000 × *g* supernatant and 2-fold greater than the 100,000 × *g* supernatant alone.

Stability of Membrane-Associated 5-Lipoxygenase Stimulatory Factor: Effect of Heat. Despite the apparently high degree of instability of the membrane-associated stimulatory factor with respect to washing and time, the activity of 100,000 × *g* pellets was remarkably stable during incubation at 100°C (Fig. 6). Even after 40 min of exposure to these conditions, microsomal membrane preparations retained significant,

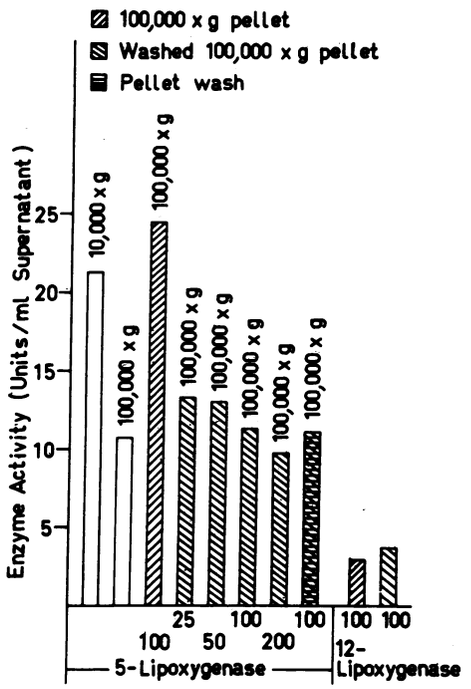

FIG. 4. Stability of membrane-associated 5-lipoxygenase stimulatory activity to pellet washing. A microsomal membrane preparation (100,000 × *g* pellet) in homogenization buffer was subjected to a second centrifugation (100,000 × *g*, 75 min) and resuspended again to its original volume. Five hundred microliters of 10,000 × *g* and 100,000 × *g* supernatants (indicated by notations above the bars) was assayed for 5-lipoxygenase activity. The numbers below the bars indicate the quantities (μl) of washed or unwashed 100,000 × *g* pellet included in the samples. Where designated, 200 μl of the supernatant from the pellet wash step was added. The washed and unwashed 100,000 × *g* pellets were also assayed for 12-lipoxygenase activity.

though somewhat reduced, 5-lipoxygenase stimulatory activity. This is in marked contrast to the 5-lipoxygenase enzyme itself, which is completely inactivated by 4 min of heating at 100°C.

DISCUSSION

These studies show that the 5-lipoxygenase from human leukocytes is dependent upon a membrane-associated stimulatory factor for maximal activity, despite the fact that the enzyme itself is cytosolic. Thus, 100,000 × *g* supernatants of leukocyte homogenates always contained 30–60% lower 5-lipoxygenase activity than their corresponding 10,000 × *g* supernatants, and this activity could be restored by combination with the resuspended 100,000 × *g* pellet. When assayed alone, the 100,000 × *g* pellet contained insignificant quantities of 5-lipoxygenase, although 12-lipoxygenase activity could be detected in these preparations. There was, however, no correlation between the membrane-associated 12-lipoxygenase and the 5-lipoxygenase stimulatory activities.

Maximal stimulation of 5-lipoxygenase by the membrane-associated factor appears to require a small molecular weight component, as indicated by the fact that dialysis resulted in decreased enzymatic activity, but only in samples that contained the microsomal membrane. However, attempts to overcome the effect of dialysis by the addition of the concentrated ultrafiltrate of a 10,000 × *g* supernatant were limited by the inhibitory nature of such preparations at higher concentrations. The potential role for a small molecular weight component thus requires further definition.

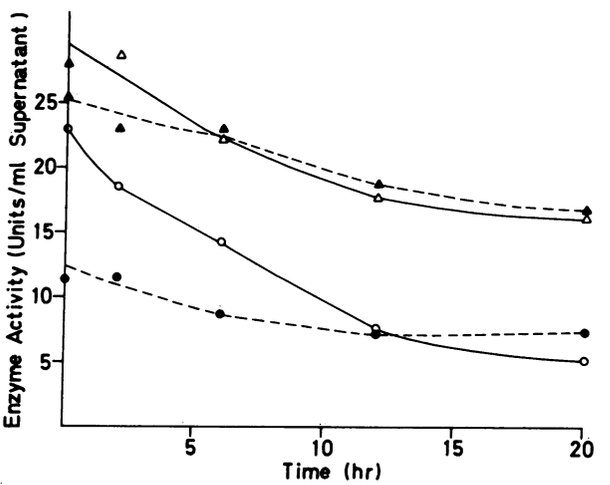

FIG. 5. Stability of membrane-associated 5-lipoxygenase stimulatory activity with respect to time. The 10,000 × *g* and 100,000 × *g* supernatants and 100,000 × *g* pellets were prepared from leukocyte homogenates. Five milliliters of 100,000 × *g* supernatant was recombined with 2 ml of 100,000 × *g* pellet at time = 0 hr, and the individual and recombined fractions were incubated at 4°C for the indicated times. 5-Lipoxygenase activity was then determined on samples containing 500 μl of the 10,000 × *g* supernatant (▲), 500 μl of the 100,000 × *g* supernatant (●), 500 μl of the 100,000 × *g* supernatant combined immediately before assay with 200 μl of the 100,000 × *g* pellet (○), and 700 μl of the 100,000 × *g* supernatant and pellet combined at time = 0 hr (△).

The membrane-associated 5-lipoxygenase stimulatory factor was highly unstable under some conditions. Attempts to wash the microsomal membranes by repeated centrifugation and resuspension consistently resulted in loss of the stimulatory activity, and its restoration could not be achieved by recombination with the wash supernatant. The 100,000 × *g* pellet preparations also rapidly lost their activity when incubated alone at 4°C. Stability was improved, however, if the microsomal membranes were combined with 100,000 × *g* supernatants at the beginning of the incubation period. These results suggest that the stability of the membrane-associated factor is dependent upon some component in the supernatant.

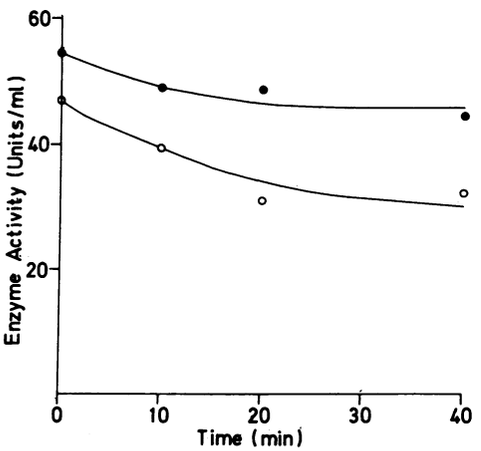

FIG. 6. Stability of membrane-associated 5-lipoxygenase stimulatory activity with respect to heat. Fifty-microliter or 100-μl aliquots of a microsomal membrane preparation (100,000 × *g* pellet) were incubated for the indicated times in a boiling water bath. The samples were cooled on ice, and 500 μl of 100,000 × *g* supernatant was added. 5-Lipoxygenase activity was then measured under standard assay conditions in the 100,000 × *g* supernatant alone (activity = 23.1 nmol/ml) and in samples containing the 100,000 × *g* supernatant with 50 μl (○) or 100 μl (●) of 100,000 × *g* pellet.

Washing the pellets could remove the small remaining quantities of this component, resulting in irreversible damage to the 5-lipoxygenase stimulatory activity. The instability of the membrane-associated factor with respect to time and pellet washing implies that the 5-lipoxygenase stimulation cannot simply be explained by a nonspecific interaction between the enzyme itself and the membrane lipid. However, the failure to destroy the stimulatory activity with incubation at 100°C is uncharacteristic of a membrane-bound enzyme or receptor.

The homogenates utilized for these investigations contained three lipoxygenase activities that are known to reside in different proteins. The 5- and 15-lipoxygenases, of predominantly granulocyte origin, have been separated by ion-exchange chromatography (7, 12). The human 12-lipoxygenase is distinguished by the fact that it is mainly a platelet-derived enzyme and, in the present studies, by its partial association with microsomal membranes. It is interesting to note that the 5-lipoxygenase differs from the other two enzymes in terms of its response to the membrane-associated stimulatory factor and dialysis. These findings, in addition to those reported previously (7), suggest that the 5-lipoxygenase reaction in human leukocytes is uniquely dependent upon a complex, multicomponent, regulatory system. In view of the central role of the 5-lipoxygenase in the synthesis of leukotrienes and lipoxins, the existence of such a regulatory system is highly significant, and the elucidation of its mechanism of action is an important future concern.

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