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SENSITIVITY OF IMMUNOAFFINITY-PURIFIED PORCINE 5-LIPOXYGENASE TO INHIBITORS AND ACTIVATING LIPID HYDROPEROXIDES

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Abstract—The requirement for hydroperoxide activation and the effect of inhibitors from different structural classes on 5-lipoxygenase activity were determined on the immunoaffinity-purified enzyme from porcine leukocytes. The 5-lipoxygenase activity was measured using a continuous spectrophotometric assay monitoring the increase in conjugated diene formation (A_{235}) upon incubation of the enzyme with arachidonic acid. Under standard assay conditions, the reaction progress curves showed little or no lag phase, with a rapid first-order decay in enzyme activity ($T_{1/2}$ = 0.7 to 1.1 min). Both the initial rate of the reaction and total product formation were stimulated by the addition of ATP, Ca^{2+} and phosphatidylcholine (PC). PC (24 μg/ml) was also found to increase the recovery of radiolabeled arachidonic acid from the assay mixture and thus part of the stimulation may be due to an increase in substrate availability and reduction of surface adsorption effects. The requirement of hydroperoxides for the initiation of the reaction was shown by the induction of 0.1 to 1-min lag phases using $NaBH_4$ or glutathione peroxidase and by the reduction in lag times by 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and 13-hydroperoxyoctadecadienoic acid (13-HPOD). The following compounds were evaluated as inhibitors of the 5-lipoxygenase reaction and caused a 50% decrease in product accumulation (IC_{50}) at the indicated concentrations: quercetin, L-651,896, L-656,224, MTPPH and L-651,392 (0.3–0.5 μM); diphenyldisulfide (2–5 μM); phenidone (5–10 μM); AA861 (4–10 μM) and BW 755C (4–15 μM). In addition, the presence of inhibitors extended the initial lag phase of the reaction and increased the dependence of the initiation of the reaction on exogenous lipid hydroperoxides. The inhibition by phenidone was accompanied by a 2-fold increase in the rate of enzyme inactivation, whereas other compounds such as AA861 and L-656,224 did not show this effect. The results indicate that the presence of inhibitors can modify the kinetics of 5-lipoxygenase at the levels of the initiation of the reaction and the rate of enzyme inactivation, with variations depending on the structural class of the inhibitor and the concentration of lipid hydroperoxides.

Leukotrienes constitute a group of arachidonic acid derivatives with potent biological activity as mediators of hypersensitivity and inflammation reactions [1]. The initial step in their biosynthesis involves the selective dioxygenation of the *cis,cis*-1,4-pentadiene unit at the 5-position to form 5(*S*)-hydroperoxyeicosatetraenoic acid (5-(*S*)-HPETE). The reaction is catalyzed by a 5-lipoxygenase which also exhibits LTA_4 synthase activity responsible for the further conversion of 5-HPETE to the unstable (5*S*)- *trans*- 5,6- oxido- 7,9- *trans*- 11,14- *cis*- eicosatetraenoic acid (LTA_4) [2–6]. LTA_4 then serves as a precursor for the neutrophil chemotactic factor (5*S*,12*R*)- 5,12 dihydroxy- 6,14-*cis*- 8,10-*trans*-eicosatetraenoic acid (LTB_4) and a series of peptido-leukotrienes [1].

The identification of specific and potent 5-lipoxygenase inhibitors is of importance for defining the role of leukotrienes in normal and pathological situations. A number of substrate analogs, antioxidants and iron chelators have been developed as enzyme inhibitors using LTB_4 release by intact cells or 5-lipoxygenase accumulation products in cell lysates to evaluate their potencies [7–15]. Unfortunately, very limited information is available on the effects of these inhibitors on the reaction catalyzed by purified 5-lipoxygenases since the purification of this highly unstable enzyme with a good recovery of activity has been achieved only recently [5, 6]. Furthermore, a spectrophotometric assay for the continuous measurement of product accumulation has been described, using ammonium sulfate precipitates from neutrophil cytosols to investigate the mechanism of the reaction [16], but has not been applied to the study of purified 5-lipoxygenases. In the present paper, we describe the effects of inhibitors from different structural classes on the activity, the rate of self-catalyzed inactivation and the hydroperoxide requirement of the reaction catalyzed by immunoaffinity-purified 5-lipoxygenase from porcine leukocytes.

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MATERIALS AND METHODS

Materials. Arachidonic acid, ATP (crystalline, 99–

100%), quercetin, and L-α-phosphatidylcholine (Type III-E, from egg yolk) were obtained from the Sigma Chemical Co. (St Louis, MO). Diphenyldisulfide and phenidone (1-phenyl-3-pyrazolidinone) were purchased from the Aldrich Chemical Co. (Milwaukee, WI). 5(*S*,*R*)-HPETE was obtained from Biomol (Philadelphia, PA). The 5-lipoxygenase inhibitor 2,3-dihydro-6-[3-(2-hydroxymethyl)-phenyl-2-propenyl]-5-benzofuranol (L-651,896) [8] was obtained from Merck, Sharp & Dohme Research Laboratories (Rahway, NJ). The other lipoxygenase inhibitors were synthesized at the Department of Medicinal Chemistry, Merck-Frosst Canada Inc.: 3-amino-1-[3-(trifluoromethyl)phenyl]-2-pyrazoline (BW 755C) [9]; 2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone (AA861) [10]; *N*-methyl-2-[4-(2,4,6-trimethylphenyl)phenyl]-propenehydroxamic acid (MTPPH, Compound No. 51 in Ref. 11); 7-chloro-2-[(4-methoxyphenyl)-methyl]-3-methyl-5-propyl-4-benzofuranol (L-656,224) [12]; and 4-bromo-2,7-dimethoxy-3*H*-phenothiazin-3-one (L-651,392) [15].

Phosphatidylcholine (PC) solutions were prepared by evaporating the hexane stock solution under N_2 and redissolving in absolute ethanol at a concentration of 6 mg/ml. Aliquots from the ethanol solution were then diluted 250-fold in the reaction mixture (no sonication). 13-Hydroperoxyoctadecadienoic acid (13-HPOD) was prepared by reaction of linoleic acid with soybean lipoxygenase (Sigma) as previously described [3] with the omission of the borohydride reduction step. The 13-HPOD (in hexane/diethyl ether, 90:10) was purified by chromatography on a Bio-Sil A column (100–200 mesh, Bio-Rad) and eluted with hexane/diethyl ether (70:30). The concentrations of 13-HPOD and other hydroperoxides containing conjugated dienes were determined using an ε of 23,000 $M^{-1}\,cm^{-1}$ at 235 nm [17].

Purification of 5-lipoxygenase. 5-Lipoxygenase was purified from porcine leukocytes by immunoaffinity chromatography using the monoclonal anti-5-lipoxygenase antibody 5 Lox-6 as previously described [5]. The enzyme was eluted from the immunoaffinity column with 50 mM sodium carbonate, pH 10, containing 0.2% sodium deoxycholate, 0.5 mM dithiothreitol and 1 mM EDTA. The pH of the eluting fractions was lowered by the addition of one-sixth volume of 0.5 M Tris–HCl, pH 7.4. Active fractions were pooled and stored frozen at −70° [5].

Assay of 5-lipoxygenase activity. 5-Lipoxygenase activity was measured from the increase in A_{235} following incubation of the enzyme with arachidonic acid, ATP and calcium. The standard reaction mixture contained 0.05 M Tris–HCl, pH 7.4, 0.2 mM ATP, 0.4 mM $CaCl_2$, 20 or 25 μM arachidonic acid (5 μl of a 100-fold concentrated solution in ethanol), 24 μg/ml PC, and an aliquot of the enzyme preparation (5–75 μl) in a final volume of 0.5 ml. The volume of enzyme was completed to 100 μl using the chromatography elution buffer containing Tris as described above to ensure identical conditions for all assays. The buffer solution containing $CaCl_2$ and PC was filtered through 0.2 μm Nalgene filters. The reactions were performed in semi-micro cuvettes (1.4 ml capacity, 100 mm path length and 4 mm internal width) and initiated by the addition of the enzyme to the assay mixture. The reaction mixture was gently mixed with a Pasteur pipette (15 sec) before recording the variation in A_{235} as a function of time at room temperature using a Perkin–Elmer Lambda 5 spectrophotometer.

Measurement of 5-lipoxygenase inactivation. Under conditions of fully activated enzyme where no initial lag phases were observed, the progress curves were analyzed as first-order changes in instantaneous velocities with time according to the equation:

$$V = V_0\, e^{-k_{obs}t}$$

where V = velocity at a given time t, V_0 = velocity at $t = 0$ and k_{obs} = first-order rate constant for enzyme inactivation under the conditions used. Integrating this equation for $V = dA/dt$ gives the equation:

$$A = \frac{V_0\,(1 - e^{-k_{obs}t})}{k_{obs}} + A_0$$

where A and A_0 correspond to absorbance at 235 nm at time t and zero respectively. The total amount of product formed during the reaction (plateau level) is equal to V_0/k_{obs} and the k_{obs} related to the half-time of inactivation ($T_{1/2}$) by $k_{obs} = (0.693/T_{1/2})$. Similar kinetic models have been used previously to characterize the reactions catalyzed by soybean lipoxygenase [18] and partially purified 5-lipoxygenase from human leukocytes [19]. The inactivation constants were estimated from data points collected at 10-sec intervals and fit to the integrated equation given above by non-linear regression analysis (Asystant, Macmillan Software Co.). In cases where initial lag phases were observed, k_{obs} were determined using the portion of the curve after the lag phase where no systematic deviation in the residuals of A_{235} was observed as a function of time.

Measurement of 5-lipoxygenase activity. 5-Lipoxygenase activity was calculated from the optimal rate of the conjugated diene formation obtained after the initial lag phase [20]. In the absence of lag phase, the optimal velocity corresponded to the velocity at $t = 0$ as determined by curve fitting. Total product formation was determined directly from the maximal absorbance change (plateau level) or calculated from the parameters obtained by curve fitting (V_0/k_{obs}).

RESULTS

Kinetics of the 5-lipoxygenase reaction. The oxidation of arachidonic acid catalyzed by 5-lipoxygenase was monitored using the spectrophotometric assay to define the characteristics of the reaction under a variety of experimental conditions. A series of progress curves was initially generated at different enzyme concentrations under optimal assay conditions in the presence of ATP, Ca^{2+}, PC and hydroperoxide. A few examples of these curves are given in Fig. 1 to illustrate that product formation was non-linear with time ($T_{1/2}$ = 0.9 min) and rapidly reached a maximum value of A_{235} that depended on the enzyme concentration.

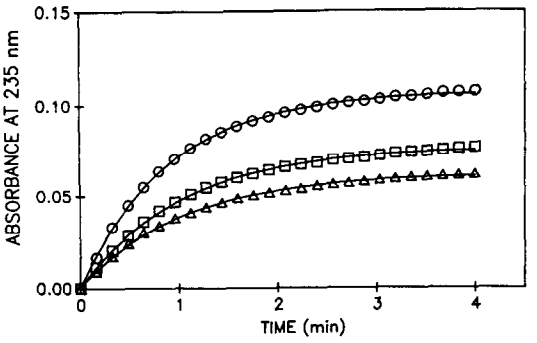

Fig. 1. Progress curves of the 5-lipoxygenase reaction at different enzyme concentrations. Immunoaffinity purified 5-lipoxygenase was assayed for activity under optimal assay conditions in the presence of 1 μM 13-HPOD (20 μM arachidonic acid). Data points from the spectrophotometric recordings were collected at 10-sec intervals and fitted into the integrated equation for absorbance changes vs time as described under Materials and Methods. Assays were performed using enzyme aliquots of 10 μl ($\triangle$), 15 μl ($\square$) and 20 μl ($\bigcirc$) with the continuous line showing the fitted curve obtained by non-linear regression analysis of the data.

For each curve, the data points from spectrophotometric measurements were collected every 10 sec and fitted by non-linear regression analysis into the integrated equation for A_{235} vs t given in the preceding section. Figure 1 shows the excellent correspondence between the fitted curves (continuous lines) and the experimental measurements at all enzyme concentrations examined, suggesting that the equation provides an adequate description of the variation in enzyme activity during the course of the reaction. The apparent first-order rate constant for enzyme inactivation ranged from 0.7 to 1.1 min^{-1} (twenty different enzyme preparations). The rapid cessation of the reaction was due to irreversible enzyme inactivation rather than substrate depletion since less than 20% of the arachidonic acid was oxidized at low enzyme concentrations and more product could be formed by adding fresh enzyme after completion of a first reaction (data not shown).

Figure 2 shows the variations in optimal velocity and total product formation as a function of enzyme concentration. A linear relationship was obtained for both parameters, with specific activities for different enzyme preparations ranging from 0.9 to 2.0 μmol/min/mg protein and 1.1 to 2.1 μmol/mg protein, respectively, under the conditions used.

Stimulation by ATP, calcium and phosphatidylcholine. The effect of phosphatidylcholine on the reaction was investigated since this phospholipid had been shown previously to stimulate rat 5-lipoxygenase activity [2] and to stabilize the enzyme during purification [2, 6]. Figure 3 shows that PC stimulated both the initial rate of the reaction and total product formation (1.6- to 2.2-fold) without significant effect on the half-time of inactivation. Maximal stimulation was obtained at a PC concentration of 24 μg/ml, and no activity was detected in control experiments with

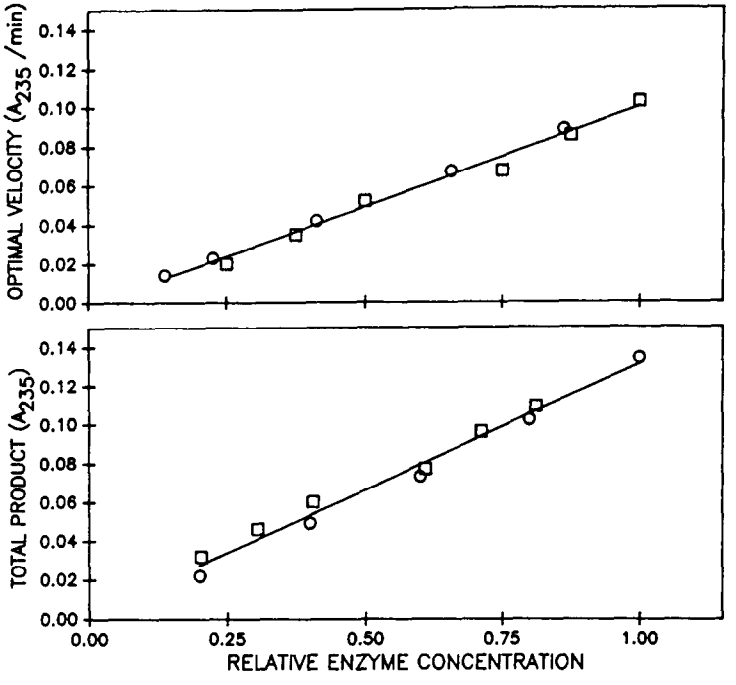

Fig. 2. Variation in optimal velocity and product accumulation as a function of enzyme concentration. 5-Lipoxygenase activity was measured in the presence of 1 μM 13-HPOD and 20 μM arachidonic acid using the spectrophotometric assay described under Materials and Methods. Optimal velocities and maximal absorbance changes were determined at various enzyme dilutions for two different enzyme preparations ($\bigcirc$, $\square$).

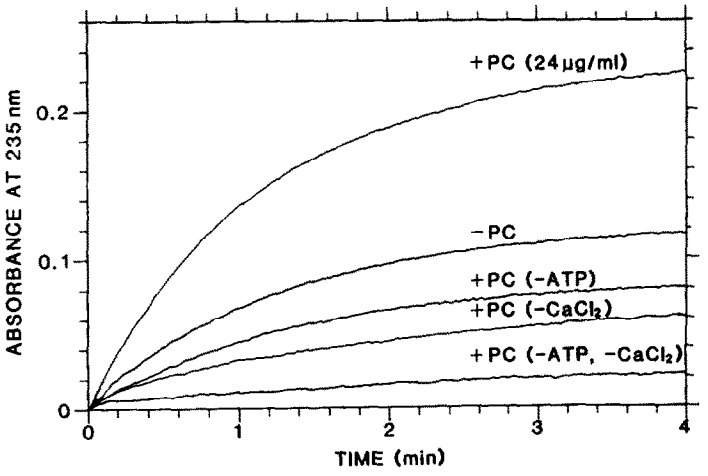

Fig. 3. Stimulation of the 5-lipoxygenase reaction by ATP, calcium and PC. Progress curves of the 5-lipoxygenase reaction were determined using the spectrophotometric assay under optimal conditions or in the absence of the indicated components.

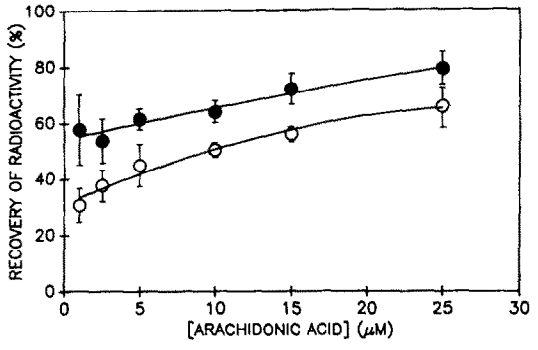

Fig. 4. Effect of PC on the recovery of arachidonic acid from reaction mixtures. Five microliters of [^{14}C]arachidonic acid (5×10^4 dpm) in ethanol was added to the reaction mixture containing ATP, Ca^{2+} and elution buffer as described for the spectrophotometric assay except that arachidonic acid concentrations were varied as indicated in the absence (○) or in the presence of 24 μg/ml PC (●). The amount of radioactivity present after mixing of the samples was determined by liquid scintillation counting, using 100-μl aliquots, and is reported as a percentage of the added radioactivity.

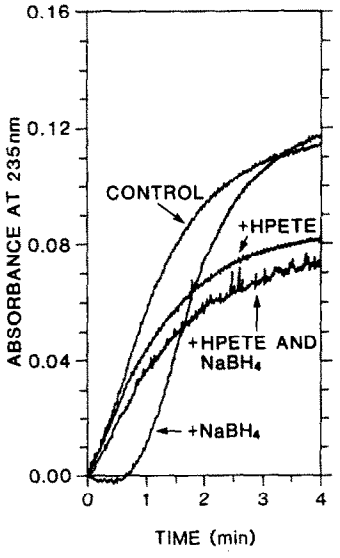

Fig. 5. Effect of hydroperoxide removal on the kinetics of the 5-lipoxygenase reaction. Enzyme activity was measured using 25 μM arachidonic acid as described for the spectrophotometric assay in Materials and Methods. $NaBH_4$ was added from a freshly prepared 10% solution (4°) to obtain a final concentration of 0.1%. The borohydride was included in the assay mixture immediately before the addition of substrate and initiation of the reaction with enzyme (± 5-HPETE). 5(*S*,*R*)-HPETE was added as a 500-fold concentration solution in ethanol to obtain a final concentration of 1 μM.

PC in the absence of arachidonic acid or with PC and substrate in the absence of enzyme (data not shown). PC did not substitute for the ATP and calcium requirements of the enzyme reaction, as shown by much lower levels of activity when either $CaCl_2$ or ATP was omitted from the incubation mixture (Fig. 3). Direct confirmation for the stimulation of product formation by PC was obtained using the radioactivity assay (1.6-fold), which also indicated that 5-HPETE was the major reduction product in the absence or in the presence of PC (data not shown).

During the comparison of the spectrophotometry and radioactivity assays, we observed that the stimulation by PC was dependent on the volume of the reaction mixture, suggesting that surface effects influence the activity. This aspect was investigated further by determining the effect of PC on the recovery of radiolabeled arachidonic acid from assay mixtures containing ATP and Ca^{2+} in the absence of enzyme (Fig. 4). The recovery of radioactivity in the absence of PC was dependent on arachidonic acid concentration, ranging from 31 to 65% for concentrations of 1 to 25 μM. The addition of PC significantly improved the recovery of radioactivity (54–

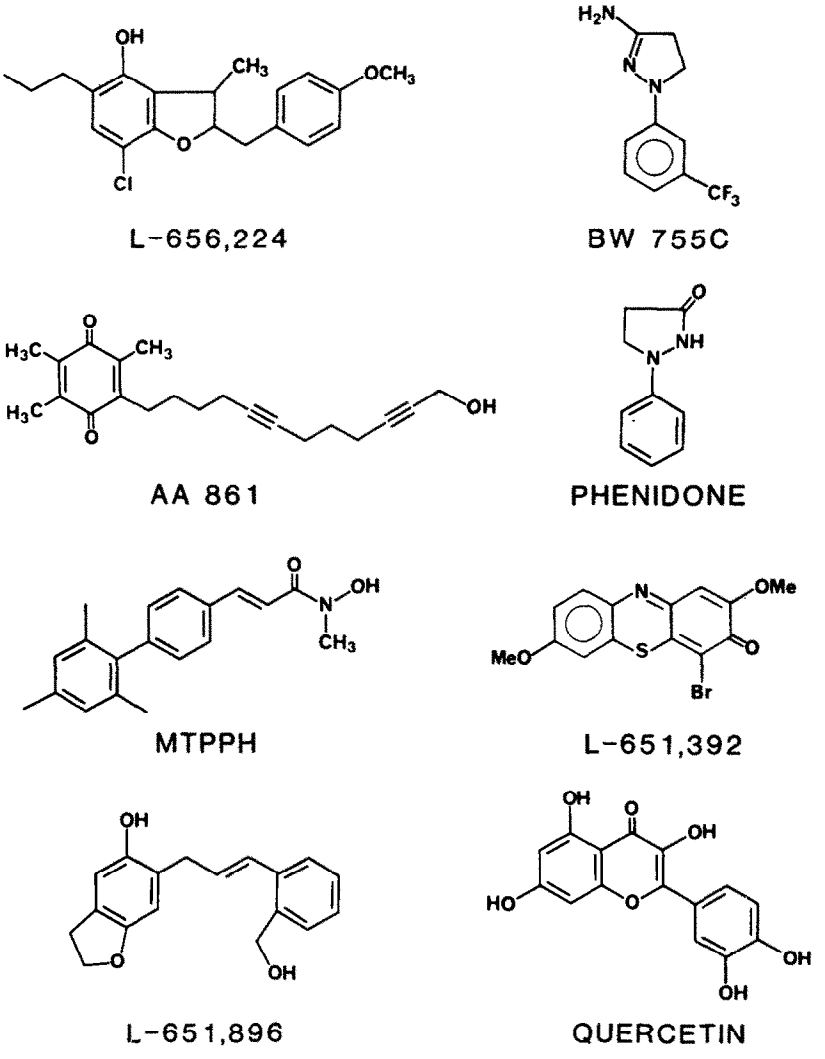

Fig. 6. Structures of the lipoxygenase inhibitors.

79% over the same range of arachidonic acid concentrations). This result suggests that a fraction of the added arachidonic acid may be adsorbed on the cuvette wall or unevenly distributed at the air/water interface and that the presence of PC increased the concentration of arachidonic acid in the bulk phase.

Enzyme activation by hydroperoxides. The progress curves of the 5-lipoxygenase reaction using arachidonic acid as substrate show little or no kinetic lag phase under the standard assay conditions. Although some lag phase can be seen by using arachidonic acid solutions freshly treated with $NaBH_4$ to remove hydroperoxides [21], more pronounced effects were observed when $NaBH_4$ was added to the assay mixture (0.1% final concentration) prior to enzyme addition (Fig. 5). The onset of the reaction was delayed by about 40 sec in this case, but the amount of product formed by the enzyme reached the same level as that obtained in the absence of borohydride after 3 min. The lag phase induced by $NaBH_4$ was eliminated by the addition of 1 μM 5-HPETE, which also caused a decrease in product formation comparable to that observed in the absence of borohydride (40 and 30% inhibition at 4 min respectively) (Fig. 5). Initial lag phases could also be introduced by the enzymatic reduction of hydroperoxides with glutathione peroxidase (0.01 units/ml, 1 mM glutathione), although the effects were less pronounced than those seen with $NaBH_4$ (data not shown). These results indicate that the 5-lipoxygenase reaction can be promoted by 5-HPETE and suggest that endogenous hydroperoxides and/or the 5-HPETE produced by the reaction contribute to the activation of the enzyme under standard assay conditions.

Effects of inhibitors on enzyme activation and optimal velocity of the reaction. The effects of inhibitors on the time course of the 5-lipoxygenase reaction were studied for a variety of compounds from different structural classes (Fig. 6). The spectrophotometric data for L-656,224 and AA861 are shown in Fig. 7 to illustrate the changes in the kinetics of product formation introduced by several inhibitors. Increasing amounts of these compounds

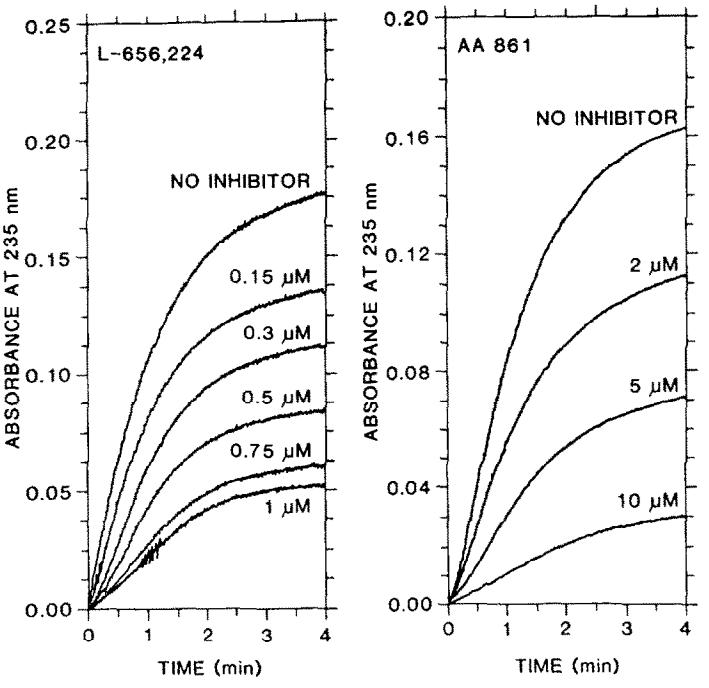

Fig. 7. Inhibition of the 5-lipoxygenase reaction by L-656,224 and AA861. Product formation was followed with time using the conditions of the spectrophotometric assay (no 13-HPOD) in the presence of the indicated concentration of inhibitor.

reduced both the optimal rate of the reaction and the total amount of product released, with L-656,224 being about 10-fold more potent than AA861. The most striking observation was that the inhibition was accompanied by an increase in the initial lag phase of the reaction, indicating that these compounds interfered with the activation process of the enzyme. The presence of a lag phase was not due to a direct effect of the drugs on hydroperoxides at the concentrations used since a stable A_{235} baseline was observed when the inhibitor was incubated with 13-HPOD and enzyme in the absence of substrate.

The effects of varying the concentration of L-656,224 on the percentage of inhibition of the optimal velocity was determined for different enzyme preparations and compared to that obtained using the total product formation as an index of enzyme activity (Fig. 8). The inhibition profiles were similar using either parameter, with IC_{50} values of 0.23 ± 0.05 μM and 0.34 ± 0.07 μM for rate and plateau measurements (mean ± SD, N = 4) respectively. A parallel decrease in rate and product accumulation could be obtained upon inhibition by L-656,224 because the inhibitor had a negligible effect on the decay of enzyme activity during the reaction. This was also the case for AA861 (Fig. 7).

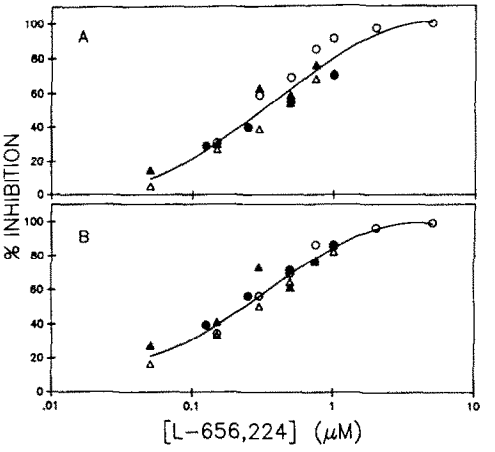

Fig. 8. Inhibitory effects of L-656,224 on optimal velocity and product accumulation of the 5-lipoxygenase reaction. The percentage of inhibition of the optimal velocity (A) and total product formation (B) was determined at various inhibitor concentrations for four different enzyme preparations as indicated by the different symbols.

Stimulation of the rate of enzyme inactivation by certain inhibitors. The most noticeable changes on enzyme stability during the course of the reactions were obtained with BW 775C and phenidone (Fig. 9). The effect of phenidone on the rate of enzyme inactivation can be readily observed at low concentrations (e.g. 2.5 μM) where a 40% decrease in product accumulation was detected without a significant effect on the initial rate of the reaction. Thus, under these conditions, the inhibition of product formation was due to a more rapid enzyme inactivation, with a doubling in the k_{obs} (± SD from the fit to the integrated equation) from 0.79 ± 0.01 min^{-1} to 1.58 ± 0.03 min^{-1} for the experiment shown in Fig. 9. At higher phenidone concentrations, the rate of the reaction was also inhibited (IC_{50} = 10 μM),

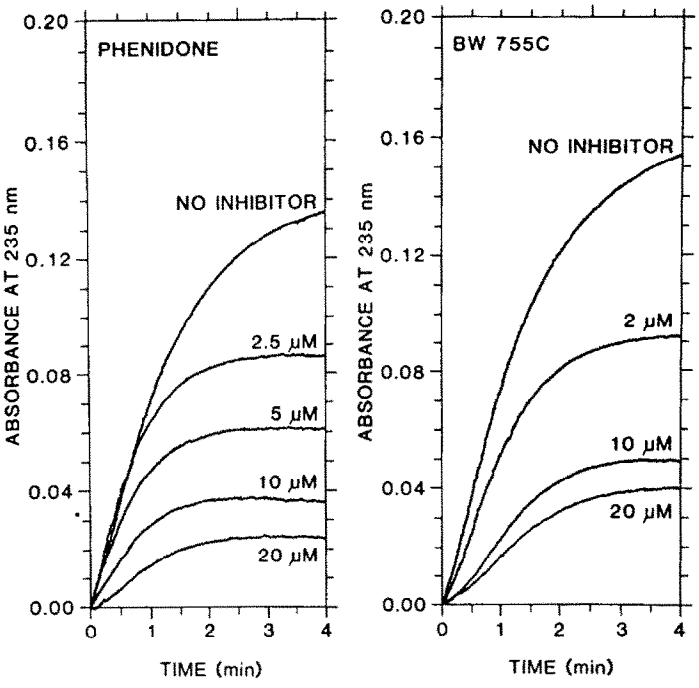

Fig. 9. Effects of phenidone and BW 755C on the 5-lipoxygenase reaction. 5-Lipoxygenase activity was measured using the spectrophotometric assay (no 13-HPOD) in the presence of indicated amounts of the inhibitor.

Table 1. Effects of inhibitors on the 5-lipoxygenase reaction

Inhibitor	IC_{50} (μM)
L-656,224	0.3–0.4
L-651,896	0.3–0.4
Quercetin	0.3–0.5
MTPPH	0.5
L-651,392 (+1 mM NADH)	0.3–0.5
Diphenyldisulfide	2–5
Phenidone	5–10
AA861	4–10
BW 755C	4–15

The range of inhibitor concentrations required to cause a 50% decrease in product accumulation (IC_{50}) was determined for a minimum of two different enzyme preparations. Product accumulation was measured from the maximal increase in A_{235} except in the case of L-651,392 and diphenyldisulfide from which oxidation of [^{14}C]arachidonic acid was used. The inhibition by L-651,392 was dependent on the presence of NADH, as previously observed in crude preparations [15].

together with the increase in enzyme inactivation. Inhibition of 5-lipoxygenase activity by BW 755C was also accompanied by an increase in the rate of enzyme inactivation (Fig. 9), although the variations were less pronounced than for phenidone ($k_{obs} \pm$ SD increased from 0.73 ± 0.01 to 1.09 ± 0.02 min^{-1} by the addition of 2 μM BW 755C).

Effects of lipid hydroperoxides on the duration of initial lag phases. The data of Figs 7 and 9 indicated that the presence of inhibitors delayed the 5-lipoxygenase reaction. The duration of initial lag phases was found to be strongly dependent on the concentration of lipid hydroperoxides. As shown in Fig. 10, the initial lag phase introduced by the presence of a 0.5 μM concentration of the iron chelator MTPPH (~ 25 sec) was almost completely eliminated by the addition of 1 μM 13-HPOD. The presence of low amounts of this hydroperoxide caused a slight decrease in product formation in the absence of inhibitor (Fig. 10). The reduction in lag phase by 13-HPOD was also observed for the enzyme reaction inhibited by L-656,224, quercetin, L-651,896 and BW 755C (data not shown).

Sensitivity of the 5-lipoxygenase to inhibitors. The relative potencies of various lipoxygenase inhibitors to decrease product formation by 50% were investigated at a saturating concentration of arachidonic acid (20 μM) (Table 1). Quercetin and the recently developed biarylhydroxamate, phenothiazinone and benzofuranols [8, 11, 12, 15] were the most effective inhibitors, about 10-fold more potent than other standard inhibitors such as diphenyldisulfide, phenidone, AA861 and BW 755C.

DISCUSSION

The reaction of immunoaffinity-purified 5-lipoxygenase is characterized by a rapid self-catalyzed inactivation and hydroperoxide activation, two of the typical features of arachidonic acid oxidizing enzymes [7]. The half-time of inactivation (0.7 to 1.1 min) is similar to that reported for various 5-lipoxygenases in crude preparations [16, 22]. The

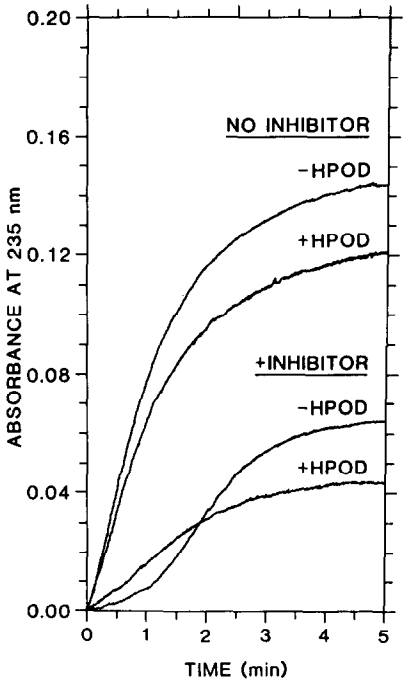

Fig. 10. Modification of the time course of the inhibited 5-lipoxygenase reaction by lipid hydroperoxides. The effect of 1 μM 13-HPOD on the 5-lipoxygenase reaction was determined in the absence of inhibitor or in the presence of 0.5 μM MTPPH (see Fig. 6).

dependence on hydroperoxide, however, is considerably lower than that observed in several other systems [23, 24], a result which can probably be explained by a more effective activation of the purified enzyme by the 5-HPETE reaction product after the removal of peroxidase activity and other contaminating proteins.

Previous studies on 5-lipoxygenase from rat basophilic leukemia cells have shown that PC stabilized the enzyme during purification [2, 6] but was not required for enzyme activity [6]. Our results with the purified enzyme show that the addition of PC stimulated the activity by about 2-fold without any significant effect on the rate of enzyme inactivation during the short time of the assay. The presence of PC was also found to improve the recovery of [^{14}C]arachidonic acid from the bulk phase of the assay mixture and part of the stimulation may thus be due to an increase in substrate availability for the reaction. This effect was not specific for 5-lipoxygenase since PC also stimulated the porcine 12-lipoxygenase (unpublished data) and the 15-lipoxygenase from human eosinophils [25]. With regard to assay conditions, it should be noted that ATP and calcium concentrations were adjusted to 0.2 and 0.4 mM, respectively, for the spectrophotometric assay since a time-dependent increase in A_{235} was observed (no enzyme) when the concentration of either $CaCl_2$ (0.4 mM) or arachidonic acid (25 μM) was raised in the incubation mixture (data not shown). This is presumably due to the formation of calcium–arachidonate complexes in agreement with previous studies demonstrating the slow binding of calcium to palmitic acid and a decrease in exchangeable calcium at these concentrations [26]. These observations suggest that changes in free concentrations of arachidonic acid and calcium may also occur during incubation under typical assay conditions used to measure 5-lipoxygenase with radiolabeled substrate.

There has been considerable interest in identifying potent inhibitors of 5-lipoxygenase in order to investigate their roles as therapeutic agents for the treatment of inflammatory diseases. Most of the inhibitor characteristics have been determined in cell lysates using product accumulation to measure the amount of active enzyme [8–15]. In this study, the maximal amount of product (A_{235}) released by the purified enzyme varied linearly with the enzyme concentration and was inhibited to the same extent as the optimal velocity by both L-656,224 and AA861, indicating that either parameter provides an adequate measurement of the relative amount of active enzyme present under these conditions. Other inhibitors that increased the rate of enzyme inactivation (e.g. phenidone) were more effective in reducing product accumulation than in inhibiting the initial rate of the reaction.

The results of the present study show that the presence of inhibitors extended the initial lag phase of the reaction and increased the dependence of the initiation of the reaction on exogenous lipid peroxides. These effects were detected with all inhibitors tested in the spectrophotometric assay (see Table 1), although the duration of the lag phases (up to 45 sec) was variable from one set of experiments to another. Such variability has also been observed with soybean lipoxygenase and was ascribed to differences in the levels of contaminating peroxides and/or content of ferric enzyme in the reaction mixtures [27]. Experiments with soybean lipoxygenase have also provided evidence that the increase in lag phase caused by *N*-alkylhydroxylamines [27] and nordihydroguaiaretic acid [28] was due to the reduction of the catalytically active ferric form of the enzyme to the inactive ferrous form. A similar mechanism involving the reduction of 5-lipoxygenase or an interference in the generation of the oxidized form of the enzyme may also explain the effects of inhibitors, although the iron content of 5-lipoxygenase remains to be determined.

Fatty acid hydroperoxides are believed to play a role in the activation of prostaglandins and leukotriene biosynthesis and have been implicated in the pathophysiology of various diseases [29]. The present results suggest that variation in lipid hydroperoxide levels may also modify the kinetics of 5-lipoxygenase inhibition and that inhibitors may be more effective in delaying the production of leukotrienes at limiting concentrations of hydroperoxide. The ability of certain inhibitors to stimulate the rate of enzyme inactivation may provide an additional mechanism to reduce 5-lipoxygenase activity under conditions where the enzyme is highly activated.

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