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Rapid inactivation of cyclooxygenase activity after stimulation of intact platelets

(lipoxygenase/arachidonate/phospholipases/aggregation/self-regulation)

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ABSTRACT **Trypsin, thrombin, and ionophore A23187 activate phospholipid breakdown of platelets that have been labeled with [^{14}C]arachidonate, releasing their cyclooxygenase and lipoxygenase products. Intact platelets can also very effectively directly degrade low concentrations of exogenous, free [^{14}C]arachidonate. Pretreatment of platelets with trypsin, thrombin, or ionophore A23187 for a minimum time of 30 sec leads to complete inactivation of cyclooxygenase activity, as demonstrated by subsequent exposure to [^{14}C]arachidonate. Lipoxygenase activity is lost after 5 min. The thrombin-induced inactivation of cyclooxygenase and lipoxygenase is prevented by cyclic AMP (which inhibits the stimulated activity of phospholipase A_2), although cyclic AMP does not affect the degradation of exogenous [^{14}C]arachidonate. Exposure of platelets labeled with [^{14}C]arachidonate to unlabeled arachidonate under conditions that lead to use of the latter also results in a similarly rapid inhibition of cyclooxygenase activity, as determined by subsequent challenge with thrombin. Under these conditions lipoxygenase activity is much less markedly inactivated. The arachidonate-induced inhibition of cyclooxygenase activity is not prevented by cyclic AMP. Trypsin does not induce platelet aggregation, and platelets whose cyclooxygenase activity has been inactivated are intact insofar as they are still able to undergo aggregation. These studies demonstrate that operation in intact platelets of the cyclooxygenase pathway, through use of endogenous or exogenous substrate, leads to a very rapid, irreversible inactivation of this enzyme. The lipoxygenase pathway is also progressively impaired, but much less rapidly than the cyclooxygenase enzyme and much less markedly on use of exogenous compared to endogenous substrate. The possible consequences of these physiological processes of spontaneous inactivation are considered.**

Platelets contain virtually no free arachidonate (1). This fatty acid is bound chemically to phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol (2–6). Metabolism of arachidonate to prostaglandin and other oxygenated products is preceded by stimulation of specific phospholipases that cleave and release arachidonate from phospholipids. Indeed, thrombin, collagen, ionophore A23187, or trypsin causes a very rapid and marked activation of platelet phospholipase A_2 activity (2–13). The released arachidonate is then metabolized through the two known pathways which involve cyclooxygenase and lipoxygenase activities (2, 3, 11). Activation of phospholipase A_2 activity can be prevented by adenosine 3′:5′-cyclic monophosphate (cAMP) (4–6, 10–12).

Exposure of intact platelets to low concentrations of exogenous, free arachidonate leads to incorporation of this fatty acid into phospholipids or to metabolic oxygenation (2–6, 11, 13, 14). Higher concentrations produce lysis or aggregation of platelets along with oxidation of arachidonate (10, 11, 14–16).

Oxidation of arachidonate by *in vitro* preparations of cyclooxygenase from ram seminal vesicles leads spontaneously to an irreversible inactivation of the enzyme (17). This self-deactivation appears to result from a reductive breakdown of the 15-hydroperoxide group of the endoperoxide (PGG_2) and concomitant oxidation of the enzyme (18). It is not known whether a similar type of inactivation, leading to self-limiting activity, occurs when this enzyme pathway is operative under physiological conditions in intact platelets.

The present studies demonstrate that intact platelets can very effectively degrade both endogenous and exogenous nonesterified arachidonate, and that this is accompanied by a rapid inactivation of cyclooxygenase. Platelets that have undergone phospholipase activation and deactivation of cyclooxygenase are still capable of undergoing aggregation. If phospholipase activation and metabolism of arachidonate are causally related to the process of platelet aggregation, it is unlikely to be related in a critical way to the activity of the prostaglandin-producing cyclooxygenase system.

MATERIALS AND METHODS

Most methods have been essentially described elsewhere (4–6) or are described in the figure legends. Platelets were obtained from horse blood by centrifugation and were resuspended in Tris/saline/glucose/EDTA buffer (15 mM Tris·HCl, pH 7.4/134 mM NaCl/5 mM D-glucose/1 mM EDTA). Trypsin was obtained from Sigma. [1-^{14}C]Arachidonic acid (specific activity 56.2 mCi/mol) was obtained from Amersham/Searle.

RESULTS

Induction of Phospholipase Activation. When platelet-rich plasma is incubated with [^{14}C]arachidonate for 2 hr, there is effective labeling of phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol with virtually no detectable radioactivity in free arachidonate after the platelets are washed (refs. 4–6 and Fig. 1). As shown originally by Pickett *et al.* (8), trypsin can very effectively stimulate phospholipase A_2 activity (Fig. 1) in a manner very similar to that described for thrombin or ionophore A23187 (4–6). Free arachidonate was detectable shortly after stimulation, but virtually disappeared by 1 min, after which the radioactivity was detected as products of cyclooxygenase [12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) and thromboxane B_2 (TXB_2)] and lipoxygenase [12-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE)] activities; there was also increased labeling of phosphatidic acid. The time-courses for release and metabolism of arachidonate were very similar to those observed with thrombin and ionophore stimulation. Products of cyclooxygenase were produced maximally between 30 and 60 sec, and the lipoxygenase products after 3 min (refs. 5 and 6 and Fig. 1). A concentration of 1 μg of trypsin per ml

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Abbreviations: cAMP, adenosine 3′:5′-cyclic monophosphate; TX, thromboxane; HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid.

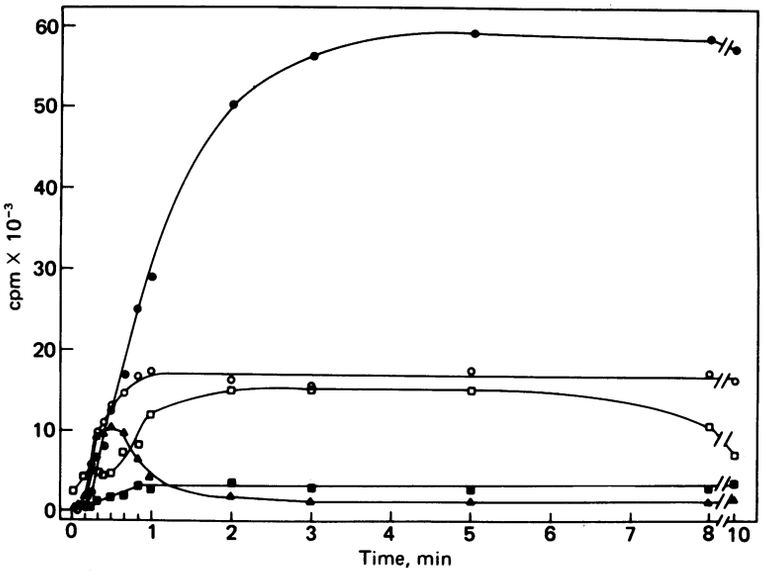

FIG. 1. Time-course of production of ^{14}C-labeled arachidonate products on stimulation of [^{14}C]arachidonate-labeled platelets by trypsin. All incubation mixtures had, in a final volume of 0.5 ml, 4.2 mg of protein and 5 μg of trypsin per ml. ●, HETE; ○, HHT; ▲, arachidonate; ■, TXB_2; □, phosphatidic acid.

was effective in stimulating phospholipase activity, and 2.5 μg per ml gave maximal production of cyclooxygenase products (data not shown).

Degradation of Exogenous [^{14}C]Arachidonate by Intact Platelets. Exposure of washed platelets to low concentrations (i.e., 1–20 μM) of [^{14}C]arachidonate resulted in its rapid degradation. HHT, TXB_2, and HETE were readily detected after 5 sec (Fig. 2), and there was virtually no further increase in HHT or TXB_2 after 60 sec. The continued production of HETE indicated that the substrate had not been exhausted. After 10 min of incubation, phospholipids had incorporated less than 3% of the total recovered radioactivity. At very early intervals the production of HHT was faster than that of HETE, whereas after 30 sec this ratio was reversed. The cessation of HHT and TXB_2 production after 1 min was not due to reduction of arachidonate to values below the K_m of the enzyme since the initial rates of production of products and the termination of activity were identical with 9 and 18 μM [^{14}C]arachidonate (not shown). The data suggest some self-limiting process in the use of exogenous substrate by the cyclooxygenase system.

The time-courses of arachidonate oxidation described here

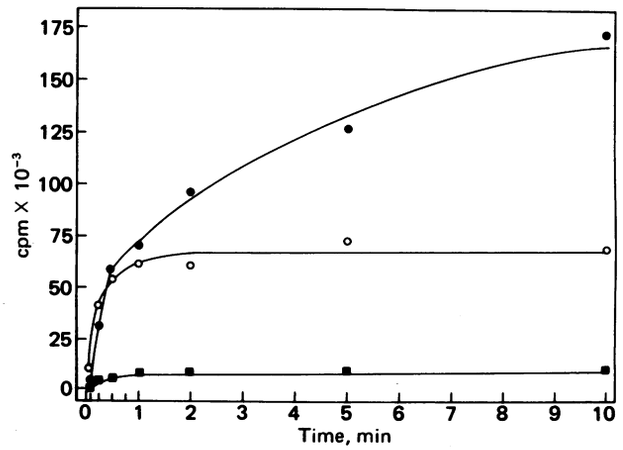

FIG. 2. Degradation of free, exogenous [^{14}C]arachidonate by unlabeled, intact platelets. Incubation mixtures of 0.5 ml had 1.4 mg of protein. [^{14}C]Arachidonate (17.6 μM) was added for different periods of time. Symbols, as in legend of Fig. 1.

were strikingly similar to those seen after stimulation of platelets labeled with [^{14}C]arachidonate and challenged with thrombin, ionophore A23187, or trypsin (refs. 5 and 6 and Fig. 1), although the latter also bring about enhanced labeling of phosphatidic acid, which is not seen with exogenous [^{14}C]arachidonate.

Pretreatment of Platelets with Trypsin, Thrombin, or Ionophore A23187 Inhibits Degradation of Exogenous [^{14}C]Arachidonate. The kinetics of the studies described above suggest that the ability of intact platelets to oxidize substrate may be lost rapidly as a consequence of processing of the substrate itself. In order to test this more directly under conditions of endogenous substrate utilization, we treated washed platelets for increasing times with trypsin, thrombin, and ionophore A23187 before examining their ability to metabolize exogenous [^{14}C]arachidonate added for 15 sec. In all cases there was nearly complete inhibition of cyclooxygenase activity after 30 sec of stimulation (Fig. 3). Lipoxygenase activity was also suppressed, but with a much slower time course; by 10 min this activity was inhibited nearly completely. Trypsin at 2.5 μg/ml was already sufficient for maximal inactivation of cyclooxygenase and lipoxygenase activities (Fig. 4).

The inactivation of lipoxygenase described in Fig. 3 occurs in an identical fashion if the washed platelets are first preincubated with 10 μM indomethacin to nearly complete inhibition of cyclooxygenase activity, indicating that the inactivation is probably independent of cyclooxygenase products.

cAMP Prevents Thrombin-Induced Inactivation of Cyclooxygenase. cAMP inhibits phospholipase activity induced by thrombin and ionophore A23187 in platelets (4–6, 10–12). The inactivation of cyclooxygenase produced by thrombin can be prevented by monobutyryl-cAMP plus aminophylline and methylisobutylxanthine (Fig. 5), suggesting that the inactivation occurs as a result of phospholipase activation and release of arachidonate. The slower rate of inactivation in this experiment (Fig. 5) reflects the proportionally slower rate of phospholipase activation obtained with this lower concentration (0.5 unit/ml) of thrombin (not shown).

Loss of Platelet Cyclooxygenase Activity on Exposure to Exogenous Arachidonate. Thrombin treatment of platelets prelabeled with [^{14}C]arachidonate and incubated for 10 min with exogenous, nonradioactive arachidonate (0.02 and 0.2 mM) demonstrated that 0.02 mM arachidonate produces a profound decrease of cyclooxygenase activity without affecting lipoxy-

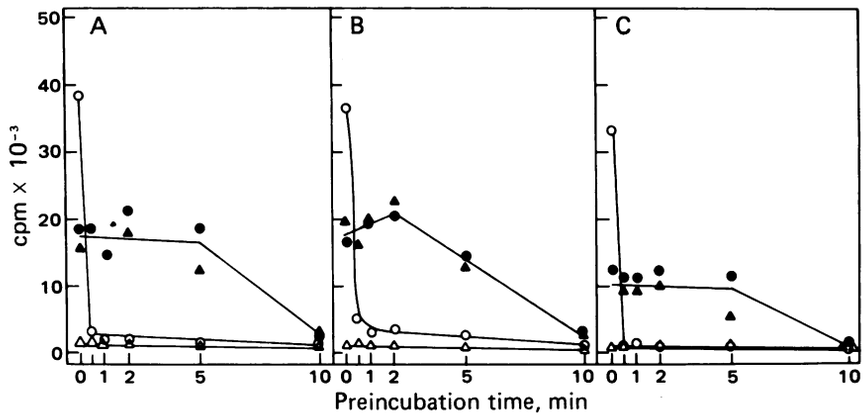

FIG. 3. Effect of pretreating platelets with (*A*) trypsin (5 μg/ml), (*B*) thrombin (1 unit/ml), or (*C*) ionophore A23187 (2 μM) on the metabolism of exogenous [^{14}C]arachidonate by unlabeled platelets. After the indicated time periods of treatment of platelets with trypsin, thrombin, or ionophore A23187, [^{14}C]arachidonate (8.8 μM) was added for 15 sec. ○, HHT; ●, HETE. The effect of incubating with indomethacin (10 μM) for 5 min before pretreatment with trypsin, thrombin, or ionophore A23187 is also shown: △, HHT; ▲, HETE. All assays (0.5 ml) contained 1.4 mg of protein.

genase activity (Fig. 6). This concentration of arachidonate does not interfere with the activation of phospholipase A_2 activity by thrombin. However, with higher concentrations of arachidonate (0.2 mM), thrombin is almost unable to exert its effect on phospholipid breakdown (with the exception of increase in phosphatidic acid labeling). Such high concentrations of arachidonate probably affect platelet integrity, which is necessary for observation of the activation of phospholipase A_2 by thrombin (unpublished observation). When the incubation time of platelets with exogenous, nonradioactive arachidonate (0.02 mM) is increased from 10 to 30 min, some inactivation of lipoxygenase (30%) is observed in parallel to loss of cyclooxygenase (data not shown).

cAMP Does Not Prevent Loss of Cyclooxygenase Activity by Exogenous Arachidonic Acid. Incubation of platelets with monobutyryl-cAMP plus cAMP phosphodiesterase inhibitors before addition of arachidonate (0.02 mM) does not prevent the loss of cyclooxygenase activity observed on incubation with thrombin (Fig. 7). The inhibitory effect of 0.02 mM arachidonate on cyclooxygenase varied from 50 to 80% in different experiments (Figs. 6 and 7). Higher arachidonate concentrations could not be used reliably because they probably affect platelet integrity. The inability of 0.02 mM arachidonate to completely inhibit cyclooxygenase may be due to difficulty of the exogenously added fatty acid in reaching all the cyclooxygenase molecules. In these experiments the platelets were thoroughly washed after incubation with nonradioactive arachidonate before stimulation with thrombin.

Inhibition of Cyclooxygenase Activity Does Not Prevent Platelet Aggregation. All the experiments described here have been done with washed platelets resuspended in EDTA buffer. All incubations have been carried out in a shaking-incubator bath in which tubes have been gently shaken. Under these conditions aggregation was not produced even when ionophore A23187 or thrombin was present. Washed platelets incubated with thrombin under conditions that inactivate cyclooxygenase activity can still be shown to be capable of aggregating many minutes later by simply adding a magnetic bar and stirring (Fig. 8). Thus, the platelets are still intact and capable of aggregating

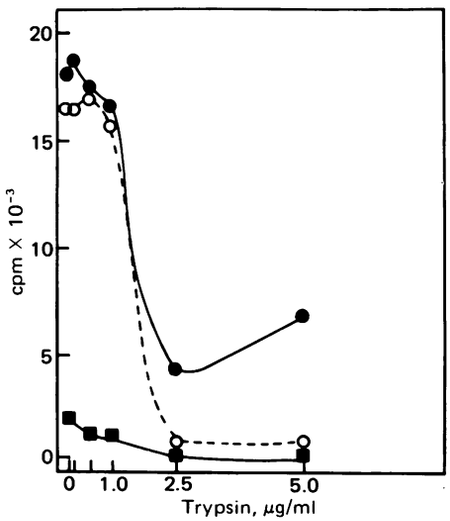

FIG. 4. Effect of different concentrations of trypsin on the degradation of exogenous [^{14}C]arachidonate by unlabeled platelets. Protein per 0.5-ml assay was 3.8 mg. [^{14}C]Arachidonate (3 μM) was added for 15 sec after the platelets were exposed for 5 min to trypsin. Symbols, as in legend of Fig. 1.

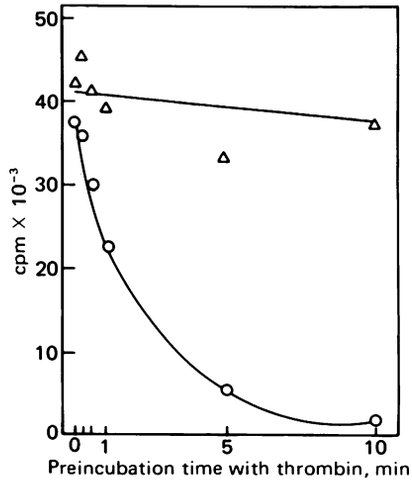

FIG. 5. Effect of cAMP on the thrombin-induced inactivation of cyclooxygenase, as measured by HHT production. Unlabeled platelets were incubated for 5 min with (△) or without (○) monobutyryl-cAMP (1 mM) plus aminophylline (0.50 mM) and methylbutylxanthine (44 μM) and then treated with thrombin (0.5 unit/ml) for different periods of time. After the indicated times, [^{14}C]arachidonate (8.8 μM) was added for 15 sec. All assays (0.5 ml) contained 2.0 mg of protein.

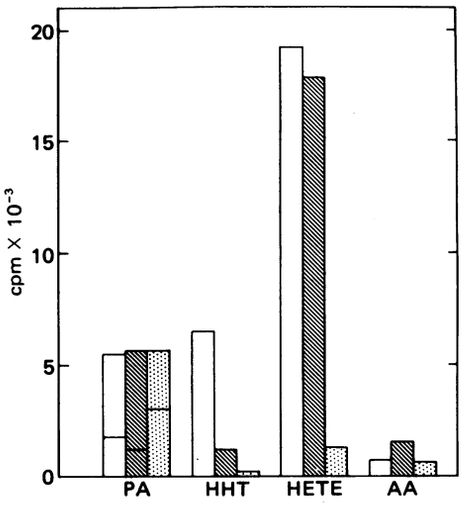

FIG. 6. Effect of pretreatment with unlabeled arachidonate on the stimulation by thrombin of [^{14}C]arachidonate-labeled platelets. [^{14}C]Arachidonate-labeled platelets were incubated with nonradioactive arachidonate (0.02 and 0.2 mM) for 10 min and then washed and resuspended with the buffer described in *Materials and Methods*. Control and arachidonate-treated platelets (0.5-ml assays) were then incubated with thrombin (1 unit/ml) for 2 min. Open bars, controls (1.9 mg of protein); slashed bars, 0.02 mM arachidonate (2.6 mg of protein); stippled bars, 0.2 mM arachidonate (2.1 mg of protein). PA, phosphatidic acid; AA, arachidonate. Lines dividing PA bars indicate unstimulated levels of PA.

in the absence of the continued formation of active cyclooxygenase products.

Trypsin at concentrations much higher (120 μg/ml) than those used in this paper does not produce aggregation, and it causes obvious platelet lysis (8). In addition, 2–10 μg of trypsin per ml also does not cause platelet aggregation, but rather can inhibit the aggregation induced by thrombin and ionophore A23187 (data not shown).

DISCUSSION

The involvement of phospholipases A_2 in prostaglandin biosynthesis by intact cells has been suggested for many years. Free fatty acid levels are very low in most tissues, and phospholipase activation is believed to be required for prostaglandin biosynthesis (1, 5, 16, 19–22). This activation can be brought about in different tissues through a variety of stimuli, such as bradykinin, angiotensin II, vasopressin, thrombin, collagen, serum, ionophore A23187, and trypsin (2–12, 19–22).

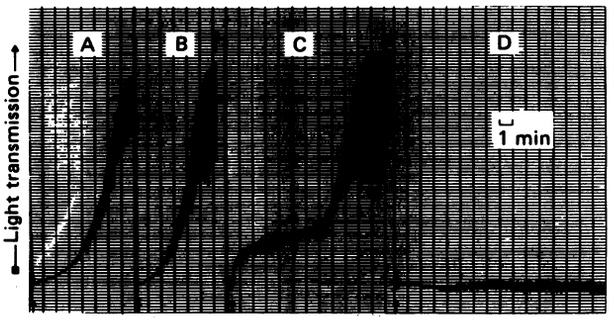

FIG. 8. Aggregation of washed platelets after cyclooxygenase activity is inactivated. Horse platelets from 50 ml of platelet-rich plasma were resuspended in 50 ml of EDTA buffer. Samples of 2.5 ml (5.1 mg of protein) were used for each assay. (*A*) Platelets were incubated at 37°C for 5 min in a shaking-incubator bath with thrombin (0.5 unit/ml); then a magnetic bar was added and aggregation was followed in the aggregometer. (*B*) As in *A*, but 1 unit of thrombin per ml was used. (*C*) Platelets were preincubated as in *A* but without thrombin; then a magnetic bar and thrombin (1 unit/ml) were added and the tube was placed in the aggregometer. (*D*) No aggregation of platelets stirred for 16 min without thrombin.

Although exogenous arachidonate can be incorporated into phospholipids and although this intermediate serves as a substrate precursor for prostaglandin synthesis, the present studies demonstrate that even very low concentrations of exogenous, nonesterified arachidonate can be used extremely rapidly and effectively by both the cyclooxygenase and lipoxygenase pathways. The qualitative, quantitative, and kinetic patterns of production of products are strikingly similar whether exogenous or endogenous substrate was used. In contrast to exogenous substrate, utilization of endogenous substrate requires stimulation of phospholipase activity and is inhibited by cAMP, indicating that the exogenous substrate can directly and effectively reach the metabolic pathways of the platelet. The possibility must be considered that, at least under certain circumstances, the physiological production of prostaglandins (or lipoxygenase products) may proceed from exogenously derived substrate and not be exclusively under the control of agents that

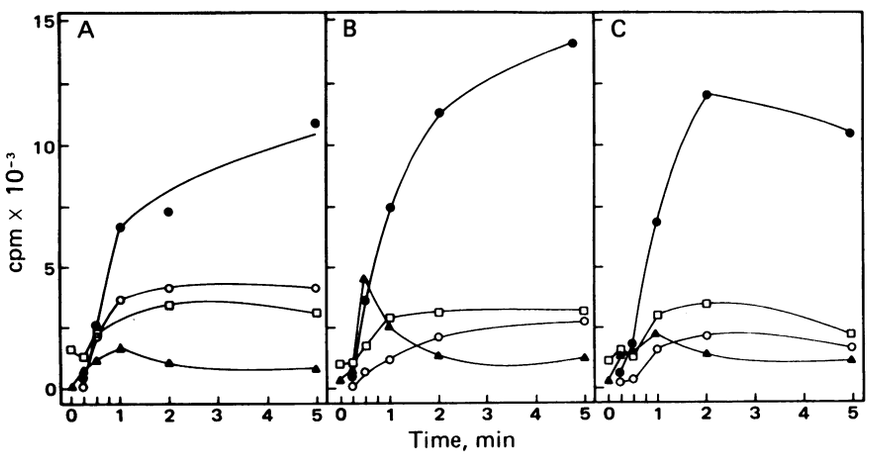

FIG. 7. Effect of cAMP on arachidonate-induced inactivation of cyclooxygenase in [^{14}C]arachidonate-labeled platelets. The labeled platelets from 1 unit of blood were divided into three samples and were incubated with 1 mM monobutyryl-cAMP plus 0.50 mM aminophylline and 0.044 mM methylbutylxanthine for 5 min and then with 0.02 mM arachidonate for 10 min (*C*), or only with 0.02 mM arachidonate for 10 min (*B*), or not pretreated (*A*). In all cases platelets were then washed and resuspended in the EDTA buffer. Samples (0.5 ml) were then incubated with thrombin (1 unit/ml) for different periods of time as indicated. Protein content per assay: (*A*) 3 mg, (*B*) 2.8 mg, (*C*) 2.6 mg. Symbols, as in legend of Fig. 1.

regulate the phospholipase activity of the same cell. Thus, it is possible that locally released arachidonate may serve as an effective, direct precursor for prostaglandin production by other cells.

The possible *in vivo*, physiological significance of the inactivation of the cyclooxygenase and lipoxygenase activities of intact platelets upon utilization of endogenous or exogenous substrate should be considered seriously on the basis of the studies presented here. It is possible that this may be a mechanism whereby platelets can respond to certain stimuli only once, but it is not known whether spontaneous recovery of the enzymatic activities would occur on more prolonged periods of incubation under physiological conditions. It will be important to determine whether similar "inactivations" occur in other tissues and whether the processes described here may be involved in mechanisms of self-regulation, tachyphylaxis, or desensitization. Enzymatic inactivation, dependent on stimulation of the same enzyme pathway as described here, could provide the basis for desensitization of prostaglandin (or other) biosynthesis. In tissues capable of protein synthesis, recovery could occur at least by *de novo* synthesis of new enzyme.

The enzymatic inactivations described here may be the corollary in intact cells (and possibly *in vivo*) of certain analogous properties observed with the isolated enzymes (17, 18). Cyclooxygenase isolated from sheep vesicular glands has been studied extensively. The oxygenation of polyunsaturated fatty acids for prostaglandin biosynthesis is an activatable, product-dependent, self-destructive enzymatic process. Once the enzyme begins to function, it produces a finite amount of hydroperoxide which serves as a precursor for prostaglandin biosynthesis (17). The mechanism of this irreversible self-deactivation seems to be based on the reductive breakdown of the endoperoxide PGG_2, which leads to the formation of the endoperoxide PGH_2, and oxidation of the enzyme (18). It is possible that similar processes occur in intact cells.

The fact that [^{14}C]arachidonate can be incorporated into platelet phospholipid and subsequently released and metabolized upon stimulation of phospholipase must be reconciled with the observations that exogenous arachidonate is metabolized directly with consequent inactivation of cyclooxygenase. Since the ^{14}C-labeling procedures use plasma, very low concentrations (0.7 μM) of [^{14}C]arachidonate, and relatively long (120 min) periods of incubation, it is possible that the plasma or actual substrate concentration may prevent or substantially decrease the rate of direct use of arachidonate or that under these conditions there is slow but significant recovery of the enzyme activity. Further studies are required to determine the kinetics of [^{14}C]arachidonate metabolism and enzyme inactivation under these conditions.

Pretreatment of platelets with thrombin produces degranulation (23). Platelets that have been induced to release 99% of the contents of their amine storage granules by exposure to 2 units of thrombin per ml are still able to undergo shape changes and aggregation when incubated with sodium arachidonate. Moreover, this aggregation is blocked by PGE_1, indomethacin, and acetylsalicylic acid (23). In light of the findings reported here, thrombin-degranulated platelets should also be devoid of cyclooxygenase activity. The action of arachidonate in the process of aggregation is therefore difficult to explain. If arachidonate metabolism is related in a causative manner to aggregation, it may occur through a pathway other than oxidation via cyclooxygenase. Oxidation through the lipoxygenase pathway must be considered seriously since this pathway is much more slowly and less readily inactivated. Alternatively, arachidonate could induce platelet aggregation by mechanisms independent of the presently known pathways for its metabolism.

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