We have previously reported that arachidonic acid induced a biphasic pattern of platelet aggregation and the release of both dense and α-granule components. Low levels of arachidonate (0.025–0.1 mM) specifically induced aggregation and release, while high concentrations (0.15–0.35 mM) caused a progressive inhibition of these platelet responses in human gel-filtered platelets (GFP). We now report studies of the mechanism(s) responsible for this arachidonate-induced turn-off of platelet function. Electron micrographic studies demonstrated that there was no gross damage to the platelets during the turn-off. Active synthesis of malondialdehyde and thromboxane A₂ was seen at the high arachidonate levels, despite the inhibition of aggregation. Furthermore, GFP inhibited by 0.25 mM arachidonate were capable of undergoing aggregation and serotonin release in response to other stimuli, such as collagen or thrombin. Thus, GFP appeared to be metabolically intact and functional during the inhibition by high arachidonate levels. Thin-layer chromatographic studies revealed that prostaglandin metabolism was not changed at the high arachidonate levels. In addition, indomethacin (20 μM) did not abolish the arachidonate-induced inhibition of platelet function. Therefore, the inhibitory effect of high arachidonate did not depend on its conversion to other prostaglandin products. Platelet cyclic AMP levels increased twofold at the high arachidonate concentrations (1.3 ± 0.3 pmole/10⁶ platelets at peak aggregation, compared with 2.9 ± 0.4 pmole/10⁶ platelets at inhibition by 0.25 mM arachidonate, p < 0.001). Prostaglandin-D₂, a platelet inhibitor known to increase cyclic AMP, generated a similar rise (to 2.4 ± 0.2 pmole/10⁶ platelets). Thus, the magnitude of the arachidonate-induced increase in platelet cyclic AMP levels can account for the inhibition of aggregation and release.

Platelets contain several different populations of granules. The dense granules are known to contain serotonin, calcium, and a storage pool of adenosine triphosphate (ATP) and diphosphate (ADP). In contrast, the more numerous α-granules have been shown to contain platelet factor 4, β-thromboglobulin, the platelet-derived growth factor, fibrinogen, and thrombin-sensitive protein. It is well established that platelet aggregation and dense granule release are mediated, in part, by the metabolism of arachidonic acid to the prostaglandin endoperoxides and thromboxanes. Recent studies have demonstrated that prostaglandin-related pathways are also involved in the regulation of the release of α-granule constituents from platelets. Thus, previous studies from our laboratory showed that the platelet-derived growth factor and other α-granule constituents could be released from platelets specifically by arachidonate via an indomethacin-sensitive pathway, most probably involving the platelet cyclooxygenase and conversion of arachidonic to prostaglandin metabolites.

During the course of these latter studies, we observed that arachidonate induced a biphasic pattern of platelet-derived growth factor release from human gel-filtered platelets. Low levels of arachidonate (0.025–0.1 mM) specifically induced platelet-derived growth factor release, while high arachidonate levels (0.15–0.35 mM) caused a progressive inhibition of release. Similar concentration-dependent biphasic patterns of response were also observed for platelet aggregation and for the release of serotonin, platelet factor 4, and β-thromboglobulin.

We now report studies aimed at exploring the mechanism of the inhibition of platelet function by high levels of arachidonate.

**MATERIALS AND METHODS**

**Platelet Preparation**

Platelet-rich plasma was prepared, as described by Holmsen et al., from venous blood drawn from normal healthy donors who had not taken any medication within the preceding 2 wk. The platelet dense granule pool was labeled with [³⁵S]-labeled serotonin, and labeled gel-filtered platelets (GFP) were then prepared, as described previously. The procedure yielded 30–40 ml of labeled GFP containing 1–3 × 10⁹ platelets/ml.

**Platelet Aggregation and Serotonin Release**

Platelet aggregation was monitored photometrically at 37°C using a Payton dual channel aggregation module with a Riken-Denshi vertical two-channel recorder. In general, incubations were carried out with 0.9 ml of GFP and 0.1 ml of the aggregating agent preparation. Unless otherwise indicated, aggregation was allowed to proceed for 3 min following the addition of the aggregating agent and was terminated by immersing the samples in an ice-water bath.

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In inhibition studies, GFP were preincubated with the inhibitor (or the buffer in which the inhibitor was dissolved) for 1 min prior to the addition of the aggregating agent. Inhibitors were generally added in a volume of no greater than 20 µl.

At the end of each experiment, the samples were centrifuged at 10,000 rpm for 30 min at 4°C to pellet the platelets and the supernatant fluids were decanted and retained for assay of released products. The release of serotonin was determined by measuring 14C in platelet supernatants, as previously described.1

**Platelet-Aggregating Agents**

An aqueous solution of the potassium salt of arachidonic acid was prepared as described.10 Portions of the potassium arachidonate solution were sealed under nitrogen and stored at –20°C. On the day of an experiment, the solution to be used was thawed and the necessary dilutions were prepared in phosphate-buffered saline (PBS) just prior to addition to GFP. The potassium salt of 1-14C-arachidonic acid (56.5 mCi/m mole, New England Nuclear, Boston, Mass.) was prepared in a similar manner.

Prostaglandin-G2 (PGG2) was kindly supplied by Drs. Bengt Samuelsson and Sven Hammarström (Karolinska Institutet, Stockholm, Sweden). The endoperoxide, dissolved in dry acetone, was stored in a dessicator at –70°C. For addition to GFP, the acetone was evaporated with nitrogen, the PGG2 redissolved in 0.1 ml of PBS and added immediately to the GFP. This procedure was necessary because acetone was found to cause marked platelet inhibition.

Collagen (Collagenreagent Horm, Hormon-Chemic Munchen, Munich, West Germany) was obtained as a 1 mg/ml suspension of equine tendon collagen (pH 2.7) and was stored as such at –5°C. Desired dilutions of the collagen were made immediately prior to use in the buffer supplied with the collagen.

Purified human thrombin was provided by Dr. D. Aronson (Division of Biologics, Bethesda, Md.) via Dr. Karen Kaplan (Department of Medicine, Columbia, N.Y.). A stock solution of 5 U/ml in 95% ethanol was prepared in a similar manner.

Purified arachidonic acid (Upjohn Co., Kalamazoo, Mich.) was prepared in a similar manner.

Indomethacin (Sigma, St. Louis, Mo.) was prepared as a solution in 70% (v/v) ethanol. In all experiments, the final concentration of indomethacin was 20 µM and that of ethanol was no greater than 0.15%. A concentration of 20 µM indomethacin completely inhibited platelet cytoxygenase activity, as determined by assay for malondialdehyde synthesis (see below).

PGD2 was the generous gift of Dr. John Pike (Upjohn Co., Kalamazoo, Mich.). A stock solution of 1 mg/ml in 95% ethanol was stored at –20°C. Further dilutions were made in PBS immediately prior to use.

**Electron Microscopy**

Electron microscopy of platelets incubated with or without arachidonate was kindly carried out by Dr. Tuan Duc Pham (Department of Medicine, Columbia, N.Y.). Incubated GFP were centrifuged for 30 min at 3000 rpm. The supernatant was carefully removed and 10 ml of 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) was gently added to the platelet pellet. After 12–16 hr at 4°C, the pellets were rinsed 3 times with 0.1 M phosphate buffer (pH 7.4), divided into 1 x 1 mm pieces, and further fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 hr. After 2 rinses in phosphate buffer, the platelet samples were dehydrated in graded acetone and then embedded in Durcupan, which was polymerized at 60°C for several days. Thin sections of about 60 nm were cut using an LKB ultratome III with a diamond knife. The sections were mounted on formvar-coated 100-mesh grids and stained with 2% uranyl acetate in 30% ethanol for 30 min. The grids were washed with distilled water and stained with Reynold’s lead citrate for 10 min. Microscopy was carried out with a Philips EM 300 microscope at 80 kV.

**Assay for Malondialdehyde (MDA)**

Platelet MDA production was measured colorimetrically by its reaction with thiobarbiturate, using a modification of the method of Macfarlane et al.11 Two-milliliter aliquots of GFP were incubated with varying doses of arachidonate for 5 min. Samples were prepared for MDA assay by addition of 0.75 ml of 40% trichloroacetic acid in 1 N HCl to each sample. After centrifugation (12,000 rpm for 10 min at 4°C) to sediment protein, the supernatants were added to 0.4 ml of 0.1 M 2-thiobarbituric acid in 0.2 M Tris-HCl buffer, pH 7.0. The samples were incubated at 70°C for 30 min and absorbance measured at 532 nm. A linear standard curve (0.05–5.0 µM MDA) was generated for each assay by adding known, measured concentrations of MDA to 2-m1 aliquots of the buffer used in gel filtering the platelets. To prepare the standards, a 1-mM MDA solution was made by hydrolyzing 110 mg of malonaldehyde triethyl acetel (Eastem Kodak Co., Rochester, N.Y.) in 500 ml water at room temperature for 1 hr. All experimental samples fell within the range of values covered by the standard curve.

**Prostaglandin and Thromboxane Metabolites of 14C-Arachidonate**

Thin-layer chromatography was used to separate and measure the various prostaglandin-related products formed from labeled arachidonic acid. Two milliliters of GFP were incubated with varying concentrations of 1-14C-arachidonate for 3 min, and then acidified to pH 3.0 with 0.2 ml of 10% formic acid. Samples were extracted 3 times with 2 volumes of ethyl acetate. The extracts were evaporated to dryness in a rotary evaporator under reduced pressure and redissolved in chloroform-methanol (93:7, v/v). Ten micrograms of each prostaglandin standard (kindly provided by Dr. John Pike, Upjohn Co.) were added to the extracts, which were then applied to silica-gel-impregnated chromatographic paper (SG81, Whatman, Inc., Clifton, N.J.). The chromatogram was run in 1% glacial acetic acid in ethyl acetate; this solvent system effectively separated PGE2, PGE3, PGD2, and thromboxane B2 (TXB2). Following visualization of these standards by exposure to iodine vapor, the localization of the radioactivity on the chromatogram was determined by scanning with a Vanguard model 930 scanner. In order to quantitate the amount of each product formed from 14C-arachidonate, the visualized spots (corresponding to each standard compound) were cut out and counted in a Packard Model 3003 liquid scintillation counter.

**Measurement of Platelet Cyclic AMP**

Platelet intracellular cyclic adenosine 3',5'-monophosphate (AMP) was measured using a radiolimmunoassay kit from New England Nuclear. Samples were prepared for assay by centrifuging the experimental platelet samples at 10,000 rpm for 30 min at 4°C to pellet the platelets. The supernatants were decanted and the platelet pellets washed 3 times with ice-cold PBS. This procedure was carried out because preliminary experiments indicated that the presence of the buffer used in gel filtering the platelets interfered with the immunoassay. After washing, the platelet pellets were lysed with 0.5 ml of distilled water. The samples were then boiled for 15 min to inactivate phosphodiesterase and centrifuged at 3000 rpm for 10 min at 4°C to sediment protein. The supernatants were stored at –70°C until assayed. The assay was carried out as outlined in the instruction manual accompanying the kit, using the acetylation option to increase the sensitivity of the assay. Antibody-bound 125I-cyclic-
AMP was determined in a Packard model 5266 autogamma scintillation spectrometer. Verification of the assay was made by demonstrating destruction of the immunoreactive cyclic AMP after enzymatic hydrolysis by cyclic nucleotide phosphodiesterase (Sigma).

RESULTS

Biphasic Response of Platelets to Arachidonate

We previously reported that platelet responses (aggregation and the release of both dense granule and α-granule constituents) exhibited concentration-dependent biphasic (on-off) patterns in the presence of increasing concentrations of arachidonate. A similar biphasic pattern of platelet aggregation and release was observed with platelet-rich plasma at higher arachidonate concentrations but over a similar range of arachidonate to albumin mole ratios.

A total of 20 arachidonate dose-response studies with GFP were conducted in the course of this work (including the several experiments reported previously). A similar pattern of biphasic platelet responses was observed in every one of these experiments, although there was some variation from experiment to experiment in the precise arachidonate concentrations at which particular events occurred. Analysis of these 20 experiments confirmed the previous definitions of the two arachidonate concentration ranges that defined the biphasic dose-response pattern. These two ranges were: (1) low (0.03–0.1 mM) and (2) high (0.15–0.35 mM). In the low arachidonate concentration range, specific arachidonate-mediated platelet responses were observed. Release and aggregation declined and were abolished in the high arachidonate concentration range (see Fig. 1 for an example).

As previously reported, no leakage of lactate dehydrogenase (LDH) occurred during the turn-off of platelet aggregation and release induced by high levels (0.15–0.35 mM) of arachidonate. Thus, in 5 dose-response studies on the effects of arachidonate on GFP, LDH was not detectable in any of the supernatants of GFP exposed to arachidonate in the concentration range 0.01–0.35 mM. In contrast, supernatants from GFP samples that had been disrupted by repeated freeze-thawing or by addition of extremely high levels of fatty acid (1 mM) contained 200–300 U/ml of LDH activity, reflecting platelet membrane lysis and leakage of cytoplasmic components during these manipulations.

Malondialdehyde Synthesis

Although the lack of LDH leakage in the arachidonate concentration range associated with the turn-off argued against substantial platelet membrane damage, the possibility remained that the turn-off of platelet responses resulted from a functional injury to the platelet. MDA synthesis was, therefore, evaluated in three arachidonate dose-response studies to determine whether the enzymatic pathway required for the initial metabolism of arachidonate was intact in the presence of high levels of arachidonate.

The results of one of these experiments are shown in Fig. 1: similar results were obtained in the other two experiments. As indicated in Fig. 1, the synthesis of MDA continued to rise as the arachidonate level was increased, despite the fall-off in platelet aggregation. Preincubating the platelets with indomethacin (20 μM) before adding arachidonate (at any level, 0.05–0.25 mM) completely abolished both aggregation and MDA synthesis.

Responses to Collagen and Thrombin

Studies were conducted to determine whether platelets that were inhibited by high levels of arachidonate would be capable of responding to a different aggregating agent. Collagen and thrombin were selected for study, since they are known to induce platelet responses by both cyclooxygenase-dependent and by cyclooxygenase-independent pathways. In these experiments, a dose-response study was first performed with arachidonate to (1) establish that the GFP preparation used could be stimulated by low levels of arachidonate, and (2) determine the arachidonate concentration(s) that would inhibit platelet aggregation and the release of both dense granule and α-granule constituents.

Fig. 1. The effects of arachidonate on malondialdehyde synthesis. The data show the concentration-dependent effects of arachidonate on platelet aggregation and on the synthesis of MDA.
responses. In all cases, 0.25 mM arachidonate was found to completely abolish platelet aggregation and release. GFP were, therefore, first incubated with 0.25 mM arachidonate and were then exposed to increasing concentrations of collagen (in five experiments) or of thrombin (in one experiment). Aggregation and serotonin release were measured in all experiments.

The serotonin release in one of the experiments with collagen is shown in Fig. 2; similar results were obtained with platelet aggregation and in the other four experiments with collagen. At low levels of collagen (≤2.5 μg/ml), 0.25 mM arachidonate completely inhibited platelet aggregation and serotonin release. However, as the concentration of collagen was increased, these platelet responses were again observed. Similar results were obtained with thrombin. Thus, at low thrombin levels (≤0.05 U/ml), 0.25 mM arachidonate inhibited aggregation and release; however, higher thrombin levels (>0.05 U/ml) induced a concentration-dependent stimulation of both aggregation and release. No LDH leakage was associated with the collagen- or thrombin-induced stimulation of these platelets.

Fig. 2. The effects of a high concentration of arachidonate (20:4) on collagen-induced platelet release of serotonin. GFP were preincubated with either 0.25 mM arachidonate in PBS or with PBS alone (control).

Fig. 3. Electron micrographs of gel-filtered platelets. The left-hand panel shows platelets that were incubated with PBS alone (control); the right-hand panel shows platelets that were incubated with 0.25 mM arachidonate for 3 min (see Materials and Methods). No aggregation occurred in either sample. Magnification: × 7000.

CONTROL

0.25 mM 20:4
**Electron Microscopy of Arachidonate-Inhibited GFP**

GFP were incubated with an inhibitory concentration of arachidonate (0.25 mM) (dissolved in PBS) or with PBS alone. No aggregation occurred in either GFP-incubated preparation. Incubation of a separate portion of the same platelet sample with 0.1 mM arachidonate confirmed that the platelets actively responded to lower levels of arachidonate. The incubated platelets were then prepared for electron microscopy as described in Materials and Methods.

Figure 3 shows sections of representative micrographs of the control (i.e., PBS-incubated) and arachidonate-inhibited platelets. As seen in Fig. 3, right hand panel, the membranes of the arachidonate-inhibited platelets were intact; in addition, there did not appear to be any obvious changes in the granules or in any other cytoplasmic constituents. These micrographs thus reinforced the data, presented above, which indicated that no platelet damage occurred at the high levels of arachidonate that induced the inhibition of platelet function.

**Platelet Metabolism of 14C-Arachidonate**

Chromatographic separation and assay of various prostaglandin-related products was performed in order to assess the metabolism of arachidonate during the turn-off of platelet function by high levels of the fatty acid. In three experiments, aggregation was measured and correlated with the production of 14C-labeled TXB2 and of the stable prostaglandins (PGD2, PGE2, and PGF2α) after incubation of GFP with 14C-labeled arachidonate (0.01–0.25 mM).

![Figure 4](image-url)

*Fig. 4.* The effects of arachidonate concentration on platelet production of labeled prostaglandins and thromboxane A2 from 14C-labeled arachidonate. The top radioscanner tracing shows the product formation at 0.1 mM arachidonate (20:4), a concentration that induced maximal platelet aggregation. The middle scan shows product formation at 0.25 mM arachidonate, a concentration that caused complete inhibition of platelet aggregation and release. The bottom scan shows product formation with 0.1 mM arachidonate added after preincubation of the GFP with 20 µM indomethacin. The location of the products separated (PGF2α, PGE2, TXB2, PGD2, and unchanged 20:4) shown at the bottom of the scans was determined by visualization of the cochromatographed standards with iodine vapor.
As in all previous experiments, vigorous aggregation occurred upon addition of 0.1 mM arachidonate to GFP; this aggregation was abolished by preincubation with indomethacin (20 μM). No aggregation occurred on addition of very low levels of arachidonate (0.01 mM) or of an inhibitory concentration (0.25 mM) of arachidonate. As shown by the radioscan tracings in Fig. 4 (top two panels), active TXB₂ formation occurred at both 0.1 and at 0.25 mM arachidonate. No distinct formation of PGD₂, PGE₂, or PGF₂α was detected at either of these two arachidonate concentrations.

The proportional amount of 14C-TXB₂ formation from 14C-arachidonate was similar at both 0.1 and 0.25 mM arachidonate levels. Thus, TXB₂ formation at 0.1 mM arachidonate was 2.6% ± 0.5% (mean ± SD) of total radioactivity recovered from the chromatogram; TXB₂ formation at 0.25 mM arachidonate was 2.3% ± 0.4%.

**Inhibition by Arachidonate Plus Indomethacin**

The chromatographic results suggested that there was no significant difference in the pattern of arachidonate metabolism (by the cyclooxygenase and subsequent pathways) between those concentrations of arachidonate that stimulated and those that inhibited platelet responses. Experiments were therefore conducted to determine whether the inhibition of aggregation and release by arachidonate depended on its conversion by cyclooxygenase to other products. The results of three such experiments are summarized in Table 1.

In these experiments, GFP were preincubated with 0.25 mM arachidonate, or with indomethacin (20 μM) plus 0.25 mM arachidonate, and the GFP were then exposed to PGG₂ (1 μg/ml). In separate experiments it was determined that this level of PGG₂ produced maximal platelet aggregation and release. Preincubation of the platelets with 0.25 mM arachidonate completely inhibited aggregation and serotonin release in response to PGG₂. Preincubation of the platelets with indomethacin before addition of 0.25 mM arachidonate did not affect the arachidonate-induced inhibition of platelet responses to PGG₂. Thus, the arachidonate-mediated inhibition of platelet aggregation and release did not appear to depend on its conversion via cyclooxygenase to other prostaglandin products.

**Effect of Arachidonate on Platelet Cyclic AMP**

Four complete dose–response studies were conducted to examine the effects of arachidonate (0.01–0.25 mM) on platelet cyclic AMP levels. The production of cyclic AMP was measured and correlated with platelet aggregation; the results of two of these studies are shown in Fig. 5. In all four experiments, a rise in cyclic AMP levels was observed at the concentration of arachidonate that induced the turn-off of platelet aggregation.

In three additional experiments, the cyclic AMP level generated at 0.1 mM arachidonate, a level that induced maximal platelet aggregation, was compared with that at 0.2 mM arachidonate, a level that caused complete platelet inhibition. The observed platelet cyclic AMP levels at 0.2 mM arachidonate were 2.9 ± 0.4 pmole/10⁸ platelets (mean ± SD; range 2.3–3.4). At 0.1 mM arachidonate, the observed platelet cyclic AMP levels were 1.3 ± 0.3 pmole/10⁸ platelets (mean ± SD; range 0.9–1.7). Thus, at the inhibitory arachidonate concentration, platelet cyclic AMP levels were more than twice those found in the presence of 0.1 mM arachidonate (p < 0.001). Cyclic AMP levels in control platelets incubated without arachidonate were 1.7 ± 0.5 pmole/10⁸ platelets, which was not significantly different from the levels observed at 0.1 mM arachidonate.

**Studies with PGD₂**

To assess whether the twofold rise in cyclic AMP induced by high levels of arachidonate could account for the inhibition of platelet function, cyclic AMP levels were measured after incubating GFP with various concentrations of PGD₂. PGD₂ is known to inhibit platelet aggregation and release by stimulating platelet cyclic AMP production. In three experiments, GFP were preincubated with PGD₂ (or its vehicle, ethanol, as a control), and then exposed to either 0.075 mM arachidonate or 0.2 mM arachidonate. Cyclic AMP levels were measured and correlated with platelet aggregation.
let aggregation. The results of these studies are summarized in Table 2.

Incubation of GFP with a very low concentration of PGD₂ (0.1 ng/ml) did not affect aggregation in response to 0.075 mM arachidonate. Under these conditions, the platelet cyclic AMP levels (mean 1.2 pmoles/10⁸ platelets) were comparable to those observed in platelets treated with 0.075 mM arachidonate alone (mean 1.3 pmoles/10⁸ platelets). Incubation of GFP with 10 ng/ml PGD₂ completely abolished platelet aggregation in response to 0.075 mM arachidonate. No aggregation occurred in response to 0.2 mM arachidonate. In both these cases, cyclic AMP levels were found to be increased by 2–2.5-fold (p < 0.01) when compared with the levels found in the aggregating platelets.

Because it appeared that both PDG₂ and high levels of arachidonate (0.15–0.35 mM) could inhibit platelet function by causing an elevation in platelet cyclic AMP levels, studies were conducted to determine whether platelets that were inhibited by PGD₂ would be capable of responding to collagen. At low levels of collagen (<2.5 µg/ml), preincubating GFP with 10 ng/ml PGD₂ inhibited the response to collagen. However, as the concentration of collagen was increased above 2.5 µg/ml, platelet aggregation again occurred.

<table>
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<th>Table 2. Comparison of the Effects of Arachidonate (20:4) and PGD₂ on Platelet Cyclic AMP*</th>
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<td>Platelet Treatment</td>
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<td>0.075 mM 20:4 + 0.1 ng/ml PGD₂</td>
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<td>0.075 mM 20:4 + 10 ng/ml PGD₂</td>
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*Platelets were preincubated with PGD₂ (in ethanol) or with an identical volume of ethanol alone for 1 min prior to the addition of arachidonate.
†Data are expressed as mean ± standard deviation.
‡Platelets were preincubated with PGD₂ (in ethanol) or with an identical volume of ethanol alone for 1 min prior to the addition of arachidonate.
These results were comparable to those obtained in studies of collagen with arachidonate-inhibited platelets (see Fig. 2).

In addition, experiments were carried out to explore the combined effects of arachidonate and PGD₂ on platelet function. Three experiments were conducted in which GFP were preincubated with varying concentrations of PGD₂ (1–10 ng/ml) and then exposed to arachidonate (0.01–0.25 mM). The group of dose–response curves generated in one of these experiments is shown in Fig. 6. PGD₂ at 10 ng/ml completely abolished aggregation in response to low doses of arachidonate. Lower PGD₂ concentrations (1 or 5 ng/ml) allowed aggregation to occur, but delayed the onset and extent of stimulation. Furthermore, at these lower doses of PGD₂, the turn-off platelet function was observed at progressively lower arachidonate concentrations. Thus, arachidonate and PGD₂ appeared to interact synergistically in determining the state of reactivity of GFP.

DISCUSSION

The studies reported here were conducted to explore the mechanism(s) responsible for the inhibition of platelet aggregation and release by high (0.15–0.35 mM) concentrations of arachidonate. Although these studies were carried out with human gel-filtered platelets, we have previously shown that the arachidonate-induced turn-off of platelet function also occurs in platelet-rich plasma.¹⁰ These previous studies demonstrated that the turn-off is dependent on the relative concentrations of arachidonate and albumin, with inhibition occurring when the arachidonate to albumin mole ratio was high (>5:1). Recently, Fratantoni and Poindexter have extended these findings to a washed platelet system.¹⁸

A crucial question raised by the observation of the turn-off phenomenon concerned the physiologic status of the inhibited platelets. Thus, we previously asked whether the platelets are physiologically intact during this apparent inhibition, or whether the turn-off reflects platelet damage by high concentrations of arachidonate not bound to albumin. Although the lack of LDH leakage during the turn-off argued¹⁰ against severe platelet membrane damage at the high arachidonate levels, the possibility remained that the progressive decline in response represented an increasing functional injury to the platelet.

The experiments reported here rule out this possibility. First of all, electron micrographic studies confirmed that there was no gross damage to the platelets during the turn-off. Secondly, arachidonate-inhibited platelets were found to actively synthesize both malondialdehyde and thromboxane A₂, demonstrating that the platelets were metabolically intact. Third, the arachidonate-inhibited platelets were found capable of undergoing aggregation and release in response to other agonists, specifically collagen and thrombin. Although high levels of arachidonate completely inhibited aggregation and release at low levels of thrombin and collagen, higher concentrations of these agents, which are able to stimulate platelets by cyclooxygenase-independent mechanisms,¹⁵,¹⁶ were able to stimulate platelet responses. Taken together, these several lines of evidence indicate that the platelets were metabolically intact and functional during the inhibition by high arachidonate concentrations.

We previously considered two classes of possible mechanisms by which arachidonate could affect the turn-off of platelet function.¹⁰ These were: (1) inhibition of critical enzymes and (2) formation of inhibitory products. The possibility of inhibition of key enzymes was suggested by the work of several investigators,¹⁹,²¹ who have reported that the cyclooxygenase can be inactivated during the course of arachidonate metabolism. Furthermore, as suggested by the work of Hammarström and Falardeau,²² platelet thromboxane synthetase may be similarly sensitive to inactivation during the course of metabolism of arachidonate to other products.

The studies reported here rule out this mechanism as the explanation for the observed turn-off of platelet function by high arachidonate levels. Thus, vigorous synthesis of both MDA and TXA₂ occurred during the arachidonate-induced platelet inhibition, demonstrating continuing activity of both the cyclooxygenase and thromboxane synthetase under these conditions. Hence, the high levels of arachidonate were not inhibiting platelet responses by preventing the generation of those compounds (particularly TXA₂) that are critical to the platelet response.

The other possible mechanism of arachidonate-induced platelet inhibition that we considered previously involved the formation of inhibitory compounds, particularly of PGD₂. It seemed conceivable that, at the high arachidonate levels, increased substrate availability might lead to the formation of significant, and inhibitory, quantities of PGD₂. The studies reported here, however, indicate that PGD₂ production was not the mechanism responsible for the turn-off phenomenon. First of all, under the conditions of our experiments, no distinct peak of PGD₂ production from [¹⁴C]-labeled arachidonate was detected by radiochromatographic assay of the prostaglandin products formed after exposure of GFP to any concentration (0.01–0.25 mM) of [¹⁴C]-labeled arachidonate (Fig. 4). This finding did not, however, conclusively rule out the possible involvement of PGD₂ in the
turn-off phenomenon, since the chromatographic assay used might have been unable to detect small but inhibitory amounts (e.g., 5–10 ng/ml) of PGD₂ if such small amounts had been formed from the labeled arachidonate. Even more definitive evidence was obtained from the study with indomethacin and arachidonate (Table 1). Thus, preincubating the platelets with indomethacin at a level that completely inhibited the cyclooxygenase pathway, did not affect the subsequently observed arachidonate-induced inhibition of platelet responses to PGG₂. Hence, the inhibitory action of arachidonate did not appear to depend on its conversion by the cyclooxygenase to other products.

Another potential inhibitory compound that might be formed by the platelet is cyclic AMP. It has been well established that agents that elevate platelet cyclic AMP levels, including PGD₂, inhibit platelet aggregation and dense granule release. In the studies reported here, platelet cyclic AMP levels were measured by radioimmunoassay and were found to be increased approximately twofold at the arachidonate concentrations (0.2–0.25 mM) that induced the turn-off of aggregation. The cyclic AMP levels at these high arachidonate concentrations were significantly different (p < 0.001) from those observed at the arachidonate concentrations (0.075–0.1 mM) that caused maximal platelet stimulation. Furthermore, the studies reported here demonstrate that the magnitude of the rise in cyclic AMP levels is adequate to explain the inhibition of platelet function. Thus, in numerous experiments, both 0.2 mM arachidonate and 10 ng/ml PGD₂ were found to inhibit platelet aggregation and to stimulate quantitatively similar rises in platelet cyclic AMP levels.

These studies indicate that the high levels of arachidonate inhibit platelet aggregation and release by effecting a rise in cyclic AMP levels. The precise mechanism by which arachidonate induces this rise is not known. Future studies are needed to determine whether the rise in cyclic AMP levels reflects an effect on adenylate cyclase or on phosphodiesterase. It also remains to be determined whether arachidonate acts directly. The data presented here suggest that the inhibitory effect of arachidonate is independent of cyclooxygenase activity. In addition, the work of Frantoni and Poindexter suggests that inhibition of the lipoxigenase pathway of arachidonate metabolism also does not abolish the arachidonate-induced turn-off of platelet function. Finally, the specificity of arachidonate, as compared to other fatty acids, in effecting the turn-off phenomenon needs to be explored.

The physiologic significance of the arachidonate-induced turn-off of platelet function remains unknown. We have previously pointed out the possibility that the local concentrations of endogenously released arachidonic acid achieved during platelet stimulation might be within the concentration range for the turn-off. This possibility requires further investigation.

The role of cyclic AMP in platelet function is of great interest. Several investigators have suggested that cyclic AMP and TXA₂ may interact in determining platelet reactivity by affecting platelet Ca²⁺ influx. However, the precise role of Ca²⁺ in platelet physiology remains undefined, and the interaction of prostaglandins (including their precursor, arachidonic acid) and cyclic nucleotides in regulating platelet function is currently an area of active investigation in a number of laboratories.

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REFERENCES


Studies on the mechanism of the inhibition of platelet aggregation and release induced by high levels of arachidonate

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