High concentrations of exogenous arachidonate inhibit calcium mobilization in platelets by stimulation of adenylate cyclase

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1. Exposure of platelets to exogenous arachidonic acid results in aggregation and secretion, which are inhibited at high arachidonate concentrations. The mechanisms for this have not been elucidated fully. In our studies in platelet suspensions, peak aggregation and secretion occurred at 2–5 μM -sodium arachidonate, with complete inhibition around 25 μM . 2. In platelets loaded with quin2 or fura-2, the cytoplasmic Ca^{2+} concentration, $[\text{Ca}^{2+}]_i$, rose in the presence of 1 mM- CaCl_2 from 60–80 nM to 300–500 nM at 2–5 μM -arachidonate, followed by inhibition to basal values at 25–50 μM . Thromboxane production was not inhibited at 25 μM -arachidonate. Cyclic AMP increased in the presence of theophylline, from 3.5 pmol/ 10^8 platelets in unexposed platelets to 8 pmol/ 10^8 platelets at 50 μM -arachidonate; all platelet responses were inhibited with doubling of cyclic AMP contents. 3. The adenylate cyclase inhibitor 2',5'-dideoxyadenosine attenuated the inhibitory effect of arachidonate, suggesting that it is mediated by increased platelet cyclic AMP and that it is unlikely to be due to irreversible damage to platelets. 4. Aspirin or the combined lipoxygenase/cyclo-oxygenase inhibitor BW 755C did not prevent the inhibition by arachidonate of either $[\text{Ca}^{2+}]_i$ signals or aggregation induced by U46619. 5. Thus high arachidonate concentrations inhibit Ca^{2+} mobilization in platelets, and this is mediated by stimulation of adenylate cyclase. High arachidonate concentrations influence platelet responses by modulating intracellular concentrations of two key messenger molecules, cyclic AMP and Ca^{2+} .

INTRODUCTION

Exposure of platelets to exogenous arachidonic acid results in aggregation and secretion of the dense- and α -granule contents (Smith *et al.*, 1975; Linder *et al.*, 1979; Kaplan *et al.*, 1979). These responses are inhibited by cyclo-oxygenase inhibitors (Linder *et al.*, 1979; Kaplan *et al.*, 1979), indicating that they are mediated by the conversion of arachidonic acid into endoperoxides and thromboxane A_2 . Several studies have shown that the dose/response curves for arachidonate-induced platelet aggregation and secretion are biphasic, with inhibition at increasing concentrations of arachidonate (Linder *et al.*, 1979; Fratanoti & Poindexter, 1981; Aharony *et al.*, 1982; Linder & Goodman, 1982; Cattaneo *et al.*, 1982). The concentrations of arachidonic acid at which maximal aggregation and inhibition were observed vary depending on the albumin concentration in the suspending medium (Linder *et al.*, 1979). Several mechanisms have been considered to explain the inhibitory effects of high arachidonic acid concentrations, including that they: (a) stimulate production via the lipoxygenase pathway of 12-hydroperoxyeicosatetraenoic acid, which is reported to inhibit platelet responses (Hammarstrom & Falardeau, 1977; Siegel *et al.*, 1979; Aharony *et al.*, 1982); (b) induce a state of refractoriness in the enzymes responsible for synthesis of thromboxane A_2 and endoperoxide (Lapetina & Cuatrecasas, 1979); (c) increase platelet cyclic AMP (Linder & Goodman, 1982), which is known to inhibit platelet responses; (d) induce alterations in the platelet membranes, resulting in an inability of agonists to induce exposure of the fibrinogen receptors (MacIntyre

et al., 1981). Despite these studies, the mechanisms by which arachidonic acid inhibits platelet aggregation and secretion still remain to be fully clarified. For example, although Linder & Goodman (1982) noted increased cyclic AMP at high concentrations of arachidonic acid, other investigators (Cattaneo *et al.*, 1982; Rao *et al.*, 1981) have concluded that increased platelet cyclic AMP is unlikely to be responsible for the persistent inhibitory effect. Moreover, the effect of increasing arachidonic acid concentrations on other key platelet responses, such as intracellular Ca^{2+} mobilization, has not been carefully examined.

Cytoplasmic Ca^{2+} has been proposed as a key regulator of several cellular processes. An early response of platelets to stimulation is a rise in the cytoplasmic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), which has been considered to mediate several of the subsequent platelet responses such as aggregation and secretion (Rink & Hallam, 1984). The effect of the inhibitory concentrations of arachidonic acid on this critical platelet response has hitherto not been reported. In the present study we report that the rise in $[\text{Ca}^{2+}]_i$ noted at the stimulatory concentrations is inhibited at high arachidonate concentrations, and demonstrate that this is related to an increase in platelet cyclic AMP owing to stimulation of the adenylate cyclase.

MATERIALS AND METHODS

Materials

Arachidonic acid was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and was converted into its

Abbreviations used: DDA, 2',5'-dideoxyadenosine; $[\text{Ca}^{2+}]_i$, intracellular Ca^{2+} concentration.

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sodium salt by addition of 300 mM- Na_2CO_3 . It was stored at -70°C as multiple batches of 50 mM stock solution. Ester forms of the Ca^{2+} indicators quin2 and fura-2, and ionomycin, were obtained from Calbiochem (San Diego, CA, U.S.A.). BW 755C was obtained from Wellcome Research Laboratories (Beckenham, Kent, U.K.); the luciferin-luciferase reagent was purchased from DuPont Instrument Co. (Wilmington, DE, U.S.A.). All other reagents were from Sigma or Aldrich Chemicals (Milwaukee, WI, U.S.A.) and were of the highest grade available. 2',5'-Dideoxyadenosine (DDA) was obtained from P-L Biochemicals (Milwaukee, WI, U.S.A.). U46619 was obtained from Cayman Chemicals (Ann Arbor, MI, U.S.A.). The stable prostaglandin I_2 analogue ZK 36374 was kindly provided by Berlex Laboratories (Cedar Knolls, NJ, U.S.A.).

Platelet preparation

Human blood was obtained from healthy volunteers who gave informed consent and had not taken any drugs, especially aspirin, for at least 10 days. The blood was drawn in $\frac{1}{8}$ vol. of acid/citrate/dextrose (Aster & Jandl, 1964). Platelet-rich plasma was obtained by centrifugation at 150 g for 20 min at room temperature.

Studies with quin2 and fura-2

The platelets were loaded with quin2 by incubating platelet-rich plasma with 20 μM -quin2 acetoxymethyl ester at 37°C for 30 min as described previously (Rao & Kowalska, 1987). The platelets were resuspended in Hepes buffer, pH 7.4 (10 mM-Hepes, 145 mM- NaCl , 5 mM- KCl , 1 mM- MgSO_4 , 5 mM-glucose) to a concentration of 2×10^8 cells/ml, and apyrase was added to a final concentration of 10 $\mu\text{g}/\text{ml}$. Before each experiment the cells (2 ml portions) were equilibrated at 37°C for 3 min. The external Ca^{2+} concentration was adjusted to 1 mM- CaCl_2 followed by incubation for 2 min at 37°C . Small portions (5–10 μl) of agents were added, and fluorescence was recorded with time. During this time, the platelet suspensions were continuously stirred at 37°C . The fluorescence signal was recorded at 492 nm (excitation at 340 nm) with a Perkin-Elmer LS-5 spectrofluorimeter. The calibration of the fluorescence signal as a function of $[\text{Ca}^{2+}]$ was performed by the method of T sien *et al.* (1982) as described previously (Rao & Kowalska, 1987). For experiments using the second Ca^{2+} indicator fura-2, the platelet-rich plasma was incubated with fura-2 ester (3 μM) for 30 min at 37°C and platelets were resuspended in buffer as described above for quin2-loaded platelets. $[\text{Ca}^{2+}]_i$ was calculated as described by Pollock *et al.* (1986), by taking the effective dissociation constant (K_d) of fura-2 to be 224 nM (Gryniewicz *et al.*, 1985).

Treatment with inhibitors

For studies on the effect of aspirin, portions of platelet suspensions were preincubated for 15–20 min at room temperature with 200 μM -acetylsalicylic acid. For studies with the cyclo-oxygenase and lipoxygenase inhibitor BW 755C (Higgs *et al.*, 1979), platelets were incubated with the drug (100 μM) for 2 min before addition of agonist (Smith *et al.*, 1985). BW 755C and aspirin were dissolved in ethanol; the final concentration of ethanol in platelet suspensions did not exceed 0.1%. The adenylate cyclase antagonist DDA was dissolved in Hepes buffer (pH 7.4) to a concentration of 20 mM. Platelets were

incubated for 2 min with 200 μM -DDA before addition of agonists.

Aggregation and secretion

Platelet aggregation was monitored in the presence of CaCl_2 (1 mM) as a change in the light transmission, by using a Lumi-aggregometer (Chrono-Log, Havertown, PA, U.S.A.). Platelet dense-granule secretion was monitored simultaneously as described by Feinman *et al.* (1977). Portions (25 μl) of luciferin-luciferase reagent (31 mg/ml) were added to 500 μl portions of platelet suspensions. The amount of ATP released was quantified by using ATP standards and expressed as a percentage of the maximal secretion observed after stimulation with thrombin (0.5 unit/ml).

Measurement of thromboxane B_2 and cyclic AMP

Platelet suspensions were incubated with arachidonate for 3 min, centrifuged for 1 min at 1500 g, and thromboxane B_2 was measured in the supernatants by a radioimmunoassay (Lewy *et al.*, 1979). The antibody was kindly provided by Dr. J. Bryan Smith (Thrombosis Research Center, Temple University, Philadelphia, PA, U.S.A.). For measurement of cyclic AMP, samples (500 μl) of platelets suspended in Hepes buffer were incubated with arachidonate for 3 min at 37°C in the presence of theophylline (7 mM); the reaction was terminated by adding cold trichloroacetic acid (final concn. 10% w/v). Cyclic AMP was measured in the supernatants, after freeze-drying and resuspension in buffer, with a radioimmunoassay kit (DuPont, Wilmington, DE, U.S.A.) (Frandsen & Krishna, 1976).

All human investigations were performed after approval of the Institutional Human Research Review Committee.

RESULTS

Effect of arachidonate on aggregation, Ca^{2+} mobilization and thromboxane production

Platelet aggregation showed a dose-dependent biphasic response, with inhibition at higher arachidonate concentrations (Fig. 1). In most experiments, maximum aggregation was noted at 2–10 μM -arachidonate. At concentrations above 15 μM , aggregation was inhibited, with maximum inhibition at 25–50 μM -arachidonate. The dose/response curve for the rise in $[\text{Ca}^{2+}]_i$ was similar to that of aggregation. The basal $[\text{Ca}^{2+}]_i$ was 60–80 nM and rose to a peak value of about 300–500 nM at 2–10 μM -arachidonate, followed by a decline to basal values at higher concentrations (Fig. 1). These studies demonstrate that Ca^{2+} mobilization in platelets is also inhibited at the higher arachidonate concentrations. It was consistently noted that when the peak $[\text{Ca}^{2+}]_i$ was less than 200 nM no aggregation was present. In contrast, thromboxane synthesis continued to increase even when the aggregation and Ca^{2+} mobilization were inhibited (Fig. 1). The highest concentrations of thromboxane B_2 were noted at arachidonate concentrations at which aggregation and the Ca^{2+} responses were strikingly suppressed, indicating that the inhibition of the responses is unrelated to an effect on thromboxane synthesis. Pre-treatment of platelets with aspirin completely inhibited all responses, including Ca^{2+} mobilization, at both high and lower arachidonate concentrations (results not shown).

Fig. 1. Effect of increasing arachidonate concentration on peak $[\text{Ca}^{2+}]_i$, aggregation, and thromboxane B_2 synthesis in platelets with quin2

Portions (2 ml) of quin2-loaded platelet suspensions were equilibrated for 3 min at 37 °C, and then with CaCl_2 (1 mM) for a further 2 min. Fluorescence was recorded after addition of different concentrations of arachidonate, and the $[\text{Ca}^{2+}]_i$ (●) was calculated as described in the Materials and methods section. Aggregation (○) was recorded in the presence of 1 mM- CaCl_2 and 100 μg of fibrinogen/ml as change in optical density, and was expressed as percentage aggregation, taking light transmission through the buffer to be 100%. Thromboxane B_2 (Δ) was measured in the supernatants by a radioimmunoassay. The result shown is representative of at least five experiments.

Because of the evidence that quin2 may cause buffering of $[\text{Ca}^{2+}]_i$ and that cells need to be loaded with relatively high indicator concentrations to demonstrate Ca^{2+} fluxes (Grynkiewicz *et al.*, 1985; Rao *et al.*, 1985), we performed similar studies with platelets loaded with a second Ca^{2+} indicator, fura-2. The results of these studies on the effect of arachidonate on Ca^{2+} responses were similar to those in quin2-loaded platelets (results not shown).

Because of the methodology used for the measurement of $[\text{Ca}^{2+}]_i$, it was possible to assess the effect of arachidonate in inducing cell damage or lysis with the release of intracellular constituents. Agents such as prostaglandins I_2 and E_1 increase platelet cyclic AMP and completely inhibit the agonist-induced rise in $[\text{Ca}^{2+}]_i$ (Feinstein *et al.*, 1983). In our experiments, the stable prostacyclin analogue ZK 36374 (Schorr *et al.*, 1981) completely inhibited the rise in $[\text{Ca}^{2+}]_i$ induced by the stimulatory concentrations of arachidonate (Fig. 2). Further, lysis or membrane injury of quin2-loaded cells on addition of arachidonate would result in the release of the quin2 into the medium and lead to a fluorescence signal from the interaction of the indicator with the Ca^{2+} in the buffer. We therefore monitored the

Fig. 2. Effect of ZK 36374 on arachidonate-induced rise in $[\text{Ca}^{2+}]_i$

Quin2-loaded platelet suspensions were preincubated for 2 min with 20 nM-ZK 36374 (○) or buffer (●), equilibrated as described in the legend for Fig. 1, and $[\text{Ca}^{2+}]_i$ was measured after addition of arachidonate.

Fig. 3. Platelet responses and cyclic AMP contents at increasing arachidonate concentrations

Peak $[Ca^{2+}]_i$ (●), aggregation (○), dense-granule secretion (△) and cyclic AMP contents (▲) are shown. Secretion was measured with a lumi-aggregometer and expressed as percentage of maximal ATP secretion noted on addition of thrombin (0.5 unit/ml). For measurement of cyclic AMP, separate platelet suspensions (quin2-loaded) were incubated in the presence of theophylline (7 mM) with arachidonate for 3 min; the reaction was stopped with 10% trichloroacetic acid, and cyclic AMP was measured in the supernatants by a radioimmunoassay.

fluorescence after addition of increasing arachidonate concentrations to quin2-loaded platelets preincubated with ZK 36374. Under these conditions, any increase in fluorescence would indicate substantial membrane damage. At arachidonate concentrations up to 50 μ M, there was no increase in fluorescence in the presence of ZK 36374 (five experiments). At 100 μ M-arachidonate there was an abrupt increase in fluorescence from basal (Fig. 2), thereby providing evidence for substantial platelet injury or lysis at this concentration. To quantify the extent of membrane injury, as reflected by the extracellular appearance of quin2, and demonstrate an ability to detect even minimal lysis by the above method, we performed the following experiments. Addition of high concentrations (> 400 nM) of ionomycin to platelets results in equilibration of the Ca^{2+} indicator in the platelets with the Ca^{2+} in the ambient fluid and elicits maximum fluorescent signals. Fluorescence signals were measured after addition of ionomycin (400–800 nM) to platelet suspensions consisting of mixtures, in graded proportions, of quin2-loaded and non-loaded platelets from the same donor and that had been handled identically. There was a good linear correlation ($r=0.97$) between the fluorescence noted on addition of ionomycin and the percentage of quin2-loaded cells present in the

mixture (results not shown). In the presence of 1 mM external Ca^{2+} , it was possible to demonstrate release of quin2 from as little as 2.5% of cells. By this method, no evidence of such membrane damage was detected at arachidonate concentrations below 50 μ M. The extent of membrane injury or lysis at 50 μ M-arachidonate was 0% of the cells in four experiments and 12% in one experiment. In the latter case the inhibitory concentrations of arachidonate were much lower than in the other four experiments, indicating a shift in the dose/response curve to the left. At 100 μ M-arachidonate, the cell damage occurred in 9 and 55% of the cells in two experiments. These observations provide evidence that arachidonate concentrations which consistently inhibit platelet responses in our studies do not induce significant cell damage and liberation of cell contents.

Effect of arachidonate on platelet cyclic AMP and $[Ca^{2+}]_i$

Because elevated concentrations of cyclic AMP can inhibit platelet responses to stimulation with various agonists (Feinstein *et al.*, 1983), we measured cyclic AMP production in platelets exposed to increasing arachidonate concentrations. Fig. 3 shows the rise in $[Ca^{2+}]_i$, aggregation, dense-granule secretion and the changes in platelet cyclic AMP concentrations. Dense-

Fig. 4. Effect of DDA on the inhibitory effect of arachidonate

Shown are the responses to 15 μM (left panel)- and 25 μM (right panel)-arachidonate (AA) in the absence (a) and the presence (b) of DDA (200 μM , 2 min). The upper traces show aggregation responses, and lower traces reflect the changes in $[\text{Ca}^{2+}]_i$.

granule secretion also demonstrated a biphasic response, being essentially parallel to the aggregation response. The platelet cyclic AMP concentrations, measured in separate samples in the presence of theophylline, rose with increasing arachidonate concentration from 3.5 pmol/ 10^8 platelets in untreated platelets to 8 pmol/ 10^8 platelets at 50 μM -arachidonate (Fig. 3). Even at arachidonate concentrations that were not inhibitory to aggregation, some increase over baseline values was noted in cyclic AMP. At arachidonate concentrations inducing a 2-fold increase over basal concentrations of platelet cyclic AMP, Ca^{2+} mobilization, aggregation and secretion were abolished or markedly impaired. Because cyclic AMP concentrations, but not the other responses, were studied in the presence of theophylline, it is not possible to make direct correlations between the observed inhibition of the responses and specific absolute cyclic AMP concentrations.

Effect of adenylate cyclase inhibitor DDA

DDA is a potent inhibitor of adenylate cyclase (Haslam *et al.*, 1978). To address the relationship between the elevated cyclic AMP concentrations and the inhibition of platelet responses, we examined the effect of DDA on the responses to arachidonate. In control experiments, prior exposure of platelets to 200 μM -DDA for 2 min reversed the inhibitory effect of 0.5 μM -prostaglandin E_1 on platelet aggregation response to 15 μM -ADP, attesting to the efficacy of DDA. Moreover, by itself DDA did not alter $[\text{Ca}^{2+}]_i$ in quin2-loaded platelets. On exposure of platelets to 15 μM -arachidonate the aggregation response was better in the presence of 200 μM -DDA than in its absence (Fig. 4). The effect was more striking on the rise in $[\text{Ca}^{2+}]_i$; in the absence of DDA, the peak $[\text{Ca}^{2+}]_i$ at 15 μM -arachidonate was 205 nm, compared with 294 nm in its presence (Fig. 4). The improvement on the Ca^{2+} response by DDA suggests that even at 15 μM -arachidonate there is a minimal, but demonstrable, inhibitory effect related to increased cyclic AMP. At 25 μM -arachidonate, there was marked attenuation of the inhibitory effect by DDA. In the absence of DDA the aggregation response was absent, with a peak $[\text{Ca}^{2+}]_i$ of

only 126 nm. In the presence of DDA aggregation was restored, with a peak $[\text{Ca}^{2+}]_i$ of 205 nm. Thus inhibition of adenylate cyclase ameliorates the inhibitory effect of high [arachidonate] on both Ca^{2+} mobilization and aggregation, thereby providing the crucial link between the increased cyclic AMP and platelet inhibition by arachidonate.

Effect of aspirin and BW 755C on platelet inhibition by arachidonate

At the higher concentrations, arachidonate inhibits the responses to other agonists too (Linder & Goodman, 1982; Fratantoni & Poindexter, 1981). To determine if the inhibitory effect of arachidonate is related to production of inhibitory compounds via the cyclo-oxygenase pathway (such as prostaglandin D_2 , which can increase cyclic AMP), we studied the effect of aspirin (200 μM) on arachidonate inhibition of aggregation and Ca^{2+} mobilization induced by U46619, a thromboxane/endoperoxide analogue (Fig. 5). In control experiments, this concentration of aspirin completely abolished arachidonate-induced thromboxane B_2 production when measured by a radioimmunoassay. Pre-treatment of platelets with aspirin abolished the rise in $[\text{Ca}^{2+}]_i$ and aggregation induced by 5 μM - and 25 μM -arachidonate, but not the responses to 5 μM -U46619 alone (Fig. 5). In the absence of aspirin, exposure of platelets to 25 μM -arachidonate inhibited U46619-induced aggregation and the rise in $[\text{Ca}^{2+}]_i$. Pre-treatment of the platelets with aspirin did not prevent the inhibition by arachidonate of the responses to U46619. In other experiments, the cyclic AMP concentrations after exposure of platelets to 50 μM -arachidonate were similar in aspirin-treatment (12 pmol/ 10^8 platelets) and untreated (13 pmol/ 10^8 platelets) platelets. Together, these findings indicate that the inhibitory effect of arachidonate is not mediated by products of the cyclo-oxygenase pathway.

BW 755C is a combined cyclo-oxygenase and lip-oxygenase inhibitor (Higgs *et al.*, 1979). Exposure of platelets to BW 755C (100 μM) has been shown to block completely production of both thromboxane A_2 and 12-hydroxyicosatetraenoic acid (Smith *et al.*, 1985). In our

Fig. 5. Effect of aspirin on arachidonate-induced inhibition of platelet responses to U46619

Aggregation (upper traces) and changes in $[Ca^{2+}]_i$ (lower traces) are presented in untreated platelets (a) and aspirin ($200 \mu M$)-treated platelets (b). Shown are the responses to $5 \mu M$ - and $25 \mu M$ -arachidonate (AA) alone (left panel), to $5 \mu M$ -U46619 alone (middle panel) and to $25 \mu M$ -arachidonate followed at 30 s by $5 \mu M$ -U46619 (right panel).

Fig. 6. Effect of BW 755C on arachidonate-induced inhibition of platelet responses to U46619

Aggregation and $[Ca^{2+}]_i$ signals are presented in untreated platelets (a) and BW 755C ($200 \mu M$)-treated platelets (b). Shown are responses to $5 \mu M$ - and $25 \mu M$ -arachidonate (AA) alone (left panel), $5 \mu M$ -U46619 alone (middle panel), and $25 \mu M$ -arachidonate followed at 30 s by $5 \mu M$ -U46619 (right panel).

control studies, incubation of platelets with BW 755C ($100 \mu M$) for 2 min inhibited thromboxane B_2 production induced by $2.5 \mu M$ - and $20 \mu M$ -arachidonate by over 93% and 87% respectively. BW 755C ($200 \mu M$; 2 min) inhibited the rise in $[Ca^{2+}]_i$ and aggregation caused by arachidonate, but not that by U46619 alone (Fig. 6). Pre-treatment of platelets with BW 755C did not prevent the inhibition by $25 \mu M$ -arachidonate of U46619-induced responses (Fig. 6).

DISCUSSION

An important mechanism by which agonists and antagonists can induce changes in platelet function is by altering the intracellular concentrations of one or more of the key messenger molecules. These second messengers include Ca^{2+} ions, cyclic nucleotides (cyclic AMP), diacylglycerol and inositol trisphosphate (Rink & Hallam, 1984); they interact with specific intracellular

targets and initiate or alter cellular mechanisms. For example, elevated $[\text{Ca}^{2+}]_i$ can activate phospholipase A_2 (Jesse & Franson, 1979), thus mobilizing free arachidonic acid and leading to formation of thromboxane A_2 . Another target for Ca^{2+} ions is the myosin light-chain kinase, which phosphorylates myosin light chain, a process linked to several platelet responses (Daniel, 1985). As an intracellular messenger, cyclic AMP has an inhibitory effect on platelet responses, including the rise in $[\text{Ca}^{2+}]_i$ during activation (Feinstein *et al.*, 1983). To understand better the mechanisms by which higher concentrations of exogenous arachidonate, a compound widely used in studies on platelets, inhibit platelet function, we studied its effect on changes in intracellular Ca^{2+} and cyclic AMP. We demonstrate that low concentrations of arachidonate (1–5 μM) induce a rise in $[\text{Ca}^{2+}]_i$ from basal to peak values of about 300–500 nM, but at higher concentrations this rise is inhibited (Figs. 1 and 3). The dose/response curves for this effect were parallel to those noted for aggregation and secretion. Thus a major mechanism by which high concentrations of arachidonate impair platelet responses may be through its effect in regulating intracellular Ca^{2+} .

Increased platelet cyclic AMP inhibits the rise in $[\text{Ca}^{2+}]_i$ during activation and the subsequent platelet responses (Feinstein *et al.*, 1983). In our studies, platelet cyclic AMP was found to increase with increasing arachidonate concentrations (Fig. 3), and there was inhibition of cytoplasmic Ca^{2+} mobilization, aggregation and secretion, with a doubling of the resting cyclic AMP concentrations. These findings are similar to those by Linder & Goodman (1982), who also noted a 2-fold increase in platelet cyclic AMP at the inhibitory arachidonate concentrations. However, those authors did not examine Ca^{2+} mobilization or directly demonstrate the relationship of the elevated cyclic AMP to the platelet inhibition. Our experiments with the adenylate cyclase inhibitor DDA provided the crucial, hitherto undemonstrated, evidence for the link between the arachidonate-induced increase in cyclic AMP and the inhibition of the Ca^{2+} signals and aggregation. Pre-incubation of platelets with the adenylate cyclase inhibitor enhanced the Ca^{2+} responses and aggregation elicited by 15 μM -arachidonate, and strikingly ameliorated the marked inhibition noted with 25 μM -arachidonate (Fig. 4). In contrast with the conclusions reached by Cattaneo *et al.* (1982), that the increased cyclic AMP is not likely to be responsible for the inhibitory effect, our observations establish that the elevated concentrations of cyclic AMP are in fact causally related to the inhibitory effects on platelet responses.

Cyclic AMP concentrations may be elevated either by stimulation of the adenylate cyclase or by inhibition of the phosphodiesterases responsible for its degradation. The experiments with DDA suggest that the mechanism of increased cyclic AMP involves stimulation of adenylate cyclase. Although attention has been focused previously on increased production of prostaglandin D_2 to explain this, it is unlikely, because the inhibitory effects of arachidonate are not prevented by prior treatment of platelets with cyclo-oxygenase inhibitors (Figs. 5 and 6), and since cyclic AMP concentrations were similar in aspirin-treated and untreated platelets exposed to arachidonate. The results of the experiments with BW 755C make it unlikely that production of the lipoxygenase product, 12-hydroperoxyeicosatetraenoic

acid, is the major mechanism for platelet inhibition at high arachidonate concentrations, as previously suggested (Aharony *et al.*, 1982).

Several groups (Aharony *et al.*, 1982; Cattaneo *et al.*, 1982; Fratanoti & Poindexter, 1981) have concluded that the inhibitory effects of arachidonate occur at concentrations below those inducing substantial cell lysis. This is supported by our ability to suppress completely the fluorescence in quin2-loaded platelets with ZK 36374, even at 50 μM -arachidonate (Fig. 2), and by our studies quantifying the extent of cell injury associated with release of cellular constituents. Strong evidence for this is provided also by the ability of DDA to maintain platelet responsiveness at even high arachidonate concentrations (Fig. 4); thus irreversible membrane damage is not the major mechanism, as previously considered (Cattaneo *et al.*, 1982; Lapetina & Cuatrecasas, 1979).

In summary, our studies demonstrate that high arachidonate concentrations inhibit not only platelet aggregation and secretion, but also the key regulatory process of intracellular Ca^{2+} mobilization. We provide evidence that inhibition of this process is related to elevated platelet concentrations of cyclic AMP, owing to stimulation of adenylate cyclase. We propose that high arachidonate concentrations influence platelet responses of aggregation and secretion by modulating the cytoplasmic concentrations of two important second messengers in platelets, Ca^{2+} and cyclic AMP.

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