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INHIBITION OF COLLAGEN-INDUCED PLATELET ACTIVATION BY ARACHIDONYL TRIFLUOROMETHYL KETONE

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Abstract—Collagen-induced platelet activation is associated with, and markedly potentiated by, the release of arachidonic acid and its subsequent conversion to thromboxane A_2. The precise mechanism of arachidonic acid release is unknown. An inhibitor of isolated cytosolic phospholipase A_2 ($cPLA_2$), arachidonyl trifluoromethyl ketone ($AACOCF_3$), was used to examine the role that $cPLA_2$ plays in this process. $AACOCF_3$ inhibited platelet aggregation in response to collagen and arachidonic acid but not to thrombin, calcium ionophore, phorbol ester, or a thromboxane mimetic. Thromboxane formation stimulated by thrombin or collagen was inhibited by $AACOCF_3$. However, $AACOCF_3$ did not inhibit collagen-induced [^{14}C]arachidonic acid release. These data are consistent with the inhibitory effects of $AACOCF_3$ on collagen-induced aggregation involving an action on the conversion of arachidonic acid to thromboxane.

Key words: platelets; collagen; arachidonic acid; cyclooxygenase; phospholipase A_2

Human platelets play a pivotal role in the haemostatic process, and abnormal platelet function is a major contributing factor to thrombotic disorders [1, 2]. Various agonists have been shown *in vitro* to initiate a multi-step platelet activation process that culminates in the formation of a platelet clump or aggregate [1, 3, 4]. Receptors for thrombin, TxA_2†, platelet-activating factor, and vasopressin have all been cloned [5–9]. Each of these receptors has seven transmembrane domains consistent with G-protein-linked receptors. Agonist interaction with these receptors induces phospholipase C-mediated phosphoinositide hydrolysis, second messenger production, and protein phosphorylation, which, in turn, mediates platelet activation [3, 10].

There are several functional and physical changes involved in the activation process, which, in a complementary manner, lead to the generation of a stable platelet aggregate [1, 11]. Shape change, pseudopod extension, and the expression of adhesive receptors on the platelet surface are all well documented. These effects are accompanied by the release of pro-aggregatory compounds (such as ADP) and adhesive proteins (including fibrinogen and fibronectin) from intracellular dense granules and alpha-granules, respectively.

In addition, TxA_2, which is synthesized in, and released from, activated platelets is an important pro-aggregatory and vasoconstricting mediator *in vivo* [1, 11, 12]. The pathway leading to the synthesis of TxA_2 involves the initial liberation of arachidonic acid from the *sn*-2 position of membrane phospholipids. Arachidonic acid is converted subsequently, by cyclooxygenase and thromboxane synthetase, to the active TxA_2 [13]. Cyclooxygenase is a widely studied enzyme that is inhibited by non-steroidal anti-inflammatory agents. In contrast, however, relatively little is known about the mechanism and regulation of arachidonic acid release, which is the rate-limiting step in the synthesis of TxA_2. Several pathways have been implicated in the generation of arachidonic acid. A minor component is liberated by the sequential actions of phospholipase C, to generate diacylglycerol, and diglyceride lipase [14, 15]. Most studies, however, indicate that the principal mechanism is the direct action of a PLA_2 on phospholipids [16–19]. Several forms of PLA_2 have been identified, although interest has centered on a recently cloned cytosolic form of PLA_2 ($cPLA_2$), which has a molecular weight of 70–90 kDa [20–22]. Platelets contain significant amounts of this calcium-dependent $cPLA_2$ [23, 24]. Furthermore, thrombin-induced platelet activation is associated with phosphorylation, and activation, of $cPLA_2$, and TxA_2 generation ([24]; McNicol A, unpublished results).

Street and colleagues [25] reported the synthesis of $AACOCF_3$, a potent and selective slow binding inhibitor of isolated $cPLA_2$. Subsequently, $AACOCF_3$ was shown to block arachidonic acid release from platelets stimulated with thrombin or A23187 [26, 27], two agonists known to act, at least in part, by the elevation of cytosolic free calcium levels.

The mechanism underlying collagen-induced platelet activation differs from that employed by other agonists. A tyrosine kinase-dependent phosphorylation of phospholipase Cγ-2 has been reported [28, 29]. Further, many of the biochemical effects stimulated by collagen (i.e. phosphatidic acid formation, calcium changes, protein kinase C activity) are aspirin sensitive and therefore likely to be TxA_2 dependent [30, 31]. This suggests that arachidonic acid release is a relatively early event in collagen-stimulated platelets [32].

In the present study, we have evaluated the effects of $AACOCF_3$ on collagen-induced platelet activation.

MATERIALS AND METHODS

Materials

$AACOCF_3$ was synthesized by the method of Street and colleagues [25], resuspended in DMSO, and stored

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† Abbreviations: TxA_2, thromboxane A_2; PLA_2, phospholipase A_2; $cPLA_2$, cytosolic PLA_2; $AACOCF_3$, arachidonyl trifluoromethyl ketone; and PMA, phorbol myristate acetate.

in a nitrogen environment. Collagen was obtained from Helena Laboratories (Beaumont, TX). [1-^{14}C]Arachidonic acid (59 mCi/mmol) and [^{32}P]orthophosphate were obtained from Amersham. Thrombin and PMA were from Sigma (St. Louis, MO), and U46619 and arachidonic acid from Cayman Chemicals (Ann Arbor, MI). All other chemicals were of the highest grade available.

Preparation of platelets

Blood was collected into acid citrate dextrose (3.8 mM citric acid, 7.5 mM trisodium citrate, 125 mM dextrose; 1.9 mL anticoagulant/8.1 mL blood) by venipuncture of healthy human volunteers who had not taken medication known to interfere with platelet function within the previous 2 weeks. Platelet-rich plasma was obtained by centrifugation at 800 *g* for 5 min [33].

Platelet aggregation

Plasma-free platelet suspensions were obtained by centrifugation of platelet-rich plasma at 800 *g* for 15 min, and the resultant pellet was resuspended in the plasma volume of HEPES-buffered Tyrodes (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM Na_2HPO_4, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM dextrose, 0.3% BSA; pH 7.4) [34]. Aliquots (0.4 mL), containing 1 mM $CaCl_2$, were dispensed into aggregometer cuvettes. Aggregation in response to agonists, in the presence or absence of inhibitor, was monitored photometrically in a Payton dual channel aggregometer at 37° with continuous stirring [33].

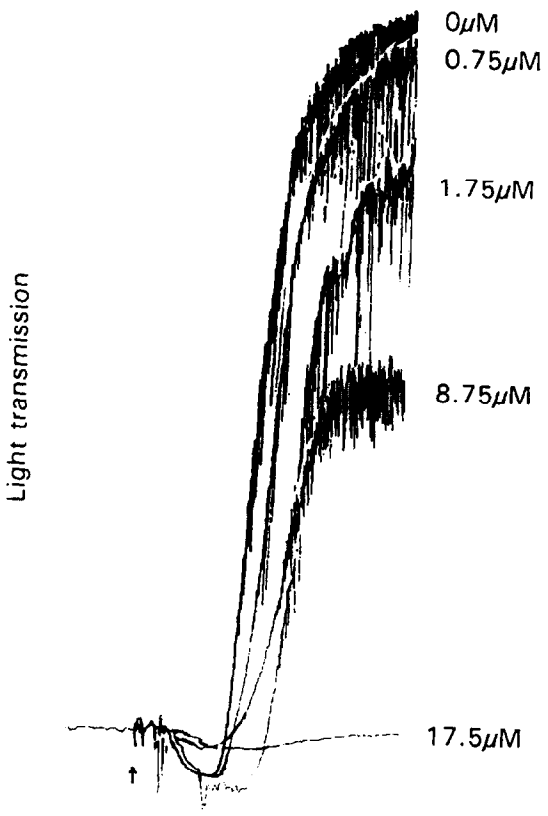

Fig. 1. Effects of $AACOCF_3$ on collagen-induced platelet aggregation. Washed human platelets were preincubated with 0.75 to 17.5 μM $AACOCF_3$, or 0.25% DMSO vehicle control, for 2 min prior to the addition of 1 μg/mL collagen (↑). Aggregation was monitored continuously as an increase in light transmission.

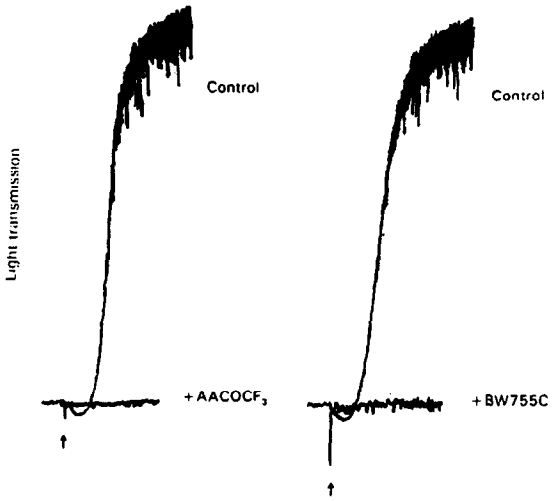

Fig. 2. Effects of $AACOCF_3$ and BW755C on arachidonic acid-induced platelet aggregation. Washed human platelets were preincubated with 17.5 μM $AACOCF_3$, 80 μM BW755C, or 0.25% DMSO vehicle control, for 2 min prior to the addition of 32 μM arachidonic acid (↑). Aggregation was monitored continuously as an increase in light transmission.

[^{14}C]Arachidonic acid release

Platelets were concentrated by centrifugation of platelet-rich plasma at 800 *g* for 15 min, and the resultant pellet was resuspended in 2 mL of the platelet-depleted plasma. The platelets were incubated with 1 μCi of [^{14}C]arachidonic acid for 1 h at 37°. Excess radiolabel was removed by the addition of 2 mL of the platelet-depleted plasma and 1 mL of acid citrate dextrose, and centrifugation at 800 *g* for 15 min. The resultant pellet was resuspended in the plasma volume of HEPES-buffered Tyrodes [34]. Aliquots (0.4 mL) containing 1 mM $CaCl_2$ were dispensed into aggregometer cuvettes and were stirred continuously in a Payton dual channel aggregometer at 37° [33]. Agonists were added in the presence or absence of inhibitor and, at the appropriate time, release was terminated by transferring the entire sample to 1.8 mL of ethyl acetate. The aqueous phase was extracted twice with ethyl acetate, acidified, and re-extracted with ethyl acetate, as previously described [33]. The combined organic phase was removed, evaporated under nitrogen, resuspended in 50 μL of ethyl acetate, applied to heat-activated silica gel 60 TLC plates, and separated by using a mobile phase of chloroform:methanol:acetic acid:water (90.0:8.0:1.0:0.8, by vol.). The plates were subjected to radiochromatographic scanning.

Thromboxane production

Platelet aggregation was carried out as outlined above. At the time indicated, release was terminated by the addition of 0.4 mL of ice-cold acid citrate dextrose. Platelets were removed by centrifugation, and the released material was diluted and analysed for TxB_2 by ELISA by the method of Docherty and colleagues [35, 36].

Phosphoinositide metabolism

Platelet-rich plasma was centrifuged at 800 *g* for 15 min as outlined above. The platelets were resuspended in

2 mL of phosphate-free, calcium-free HEPES buffer (150 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 10 mM dextrose, 0.3% BSA; pH 7.4) and incubated with 150 μCi [^{32}P]orthophosphate for 90 min at 37° [37]. Excess radiolabel was removed by the addition of 2 mL of phosphate-free, calcium-free HEPES buffer and 1 mL of acid citrate dextrose, and centrifugation at 800 *g* for 15 min. The resultant pellet was resuspended in the plasma volume of HEPES-buffered Tyrodes [34]. Aliquots (0.4 mL) containing 1 mM $CaCl_2$ were dispensed and incubated at 37° with agonists in the presence or absence of inhibitor. At the time indicated, the entire sample was transferred to 2 mL of chloroform: methanol:10 N HCl (25:50:4; by vol.). The phospholipids were extracted [37], separated [38], and subjected to radiochromatographic scanning [33]. Under the experimental conditions employed, [^{32}P]orthophosphate is preferentially incorporated into the phosphoinositides, and the formation of [^{32}P]phosphatidic acid reflects the action of phosphoinositide specific phospholipase C. [^{32}P]Phosphatidic acid production is, therefore, an indirect index of phosphoinositide specific phospholipase C activity [39].

RESULTS

The effect of $AACOCF_3$ preincubation was examined on agonist-induced platelet aggregation. Pretreatment of platelets for 2 min with 0.75 to 17.5 μM

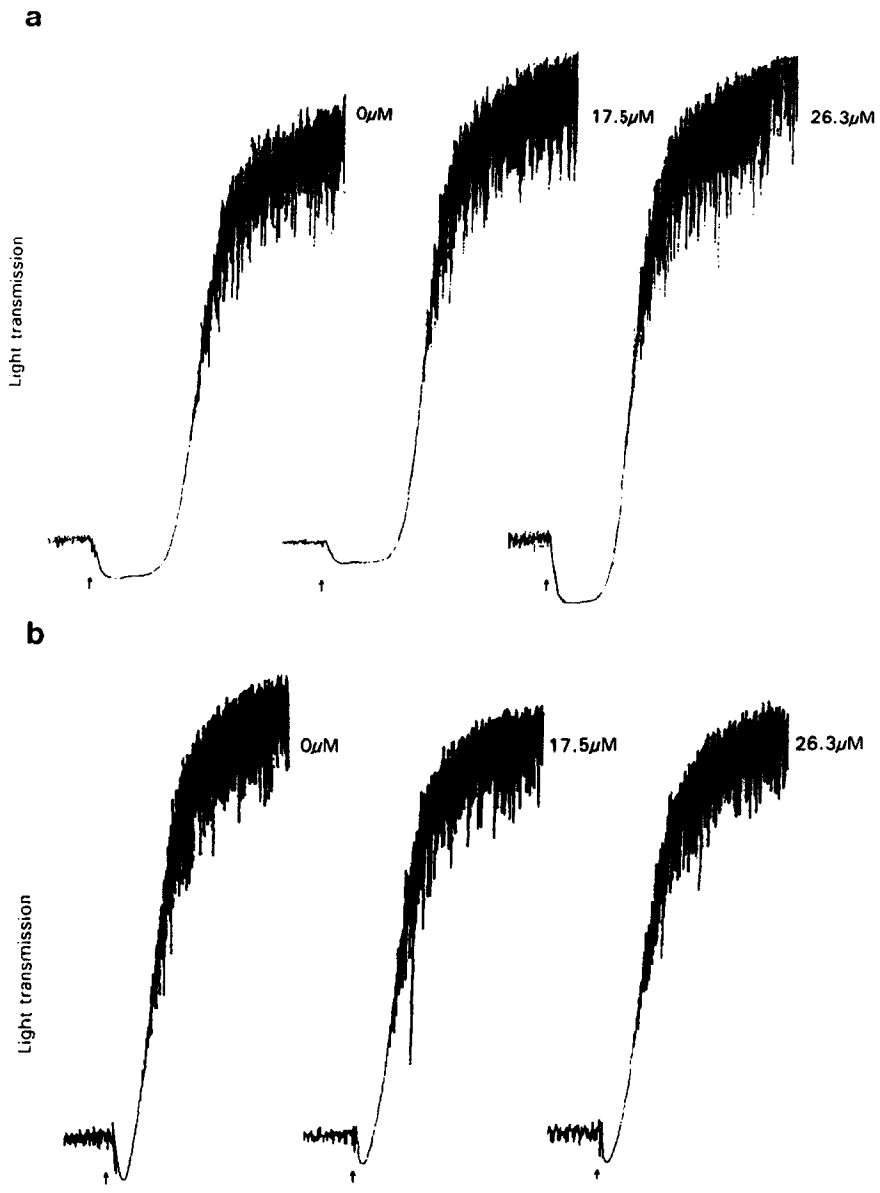

Fig. 3. Effects of $AACOCF_3$ on thrombin- and U46619-induced platelet aggregation. Washed human platelets were preincubated with 17.5 to 26.3 μM $AACOCF_3$, or 0.25% DMSO vehicle control, for 2 min prior to the addition (↑) of 0.1 U/mL thrombin (a) or 1 μM U46619 (b). Aggregation was monitored continuously as an increase in light transmission.

$AACOCF_3$ inhibited, in a concentration-dependent manner, aggregation in response to 1 μg/mL collagen (Fig. 1). $AACOCF_3$ (17.5 μM) also inhibited arachidonic acid-induced aggregation, similar to that observed with the dual cyclooxygenase/lipoxygenase inhibitor BW755C (80 μM) (Fig. 2).

In contrast, $AACOCF_3$ at concentrations up to 26.3 μM failed to inhibit aggregation induced by either thrombin (Fig. 3a) or the stable thromboxane analogue U46619 (Fig. 3b). Concentrations in excess of 26.3 μM $AACOCF_3$ caused platelet aggregation when added alone.

Platelet activation is associated with the stimulation of two synergistic biochemical pathways: an increase in cytosolic calcium and the activation of protein kinase C. The effects of $AACOCF_3$ on platelet aggregation stimulated by these respective mechanisms were examined. Aggregation in response to either the calcium ionophore A23187 (Fig. 4a) or to the protein kinase C activator PMA (Fig. 4b) was unaffected by pretreatment of the platelets with $AACOCF_3$ (17.5 to 26.3 μM).

In [^{14}C]arachidonic acid-prelabelled platelets, collagen caused the release of [^{14}C]arachidonic acid, which was not inhibited significantly by pretreatment of platelets with 8.75 to 26.3 μM $AACOCF_3$ (Fig. 5). In contrast, thrombin-induced [^{14}C]arachidonic acid release was inhibited to a small, but significant, extent by 26.3 μM $AACOCF_3$ (Fig. 5) consistent with previous observations [26]. Collagen and thrombin both caused substantial formation of TxB_2. Pretreatment of platelets with either $AACOCF_3$ or aspirin inhibited TxB_2 generation in response to either thrombin or collagen (Table 1).

The production of [^{32}P]phosphatidic acid in [^{32}P]orthophosphate-prelabelled platelets is an index of phospholipase C activation [39]. Collagen stimulated a small but significant increase in [^{32}P]phosphatidic acid levels. The effects of collagen were decreased by 8.75 μM $AACOCF_3$ and abolished by 26.3 μM $AACOCF_3$ (Fig. 6a). In contrast, the effects of thrombin, a more potent phospholipase C stimulant, on [^{32}P]phosphatidic acid production were affected minimally by pretreatment of the platelets with $AACOCF_3$ (Fig. 6b).

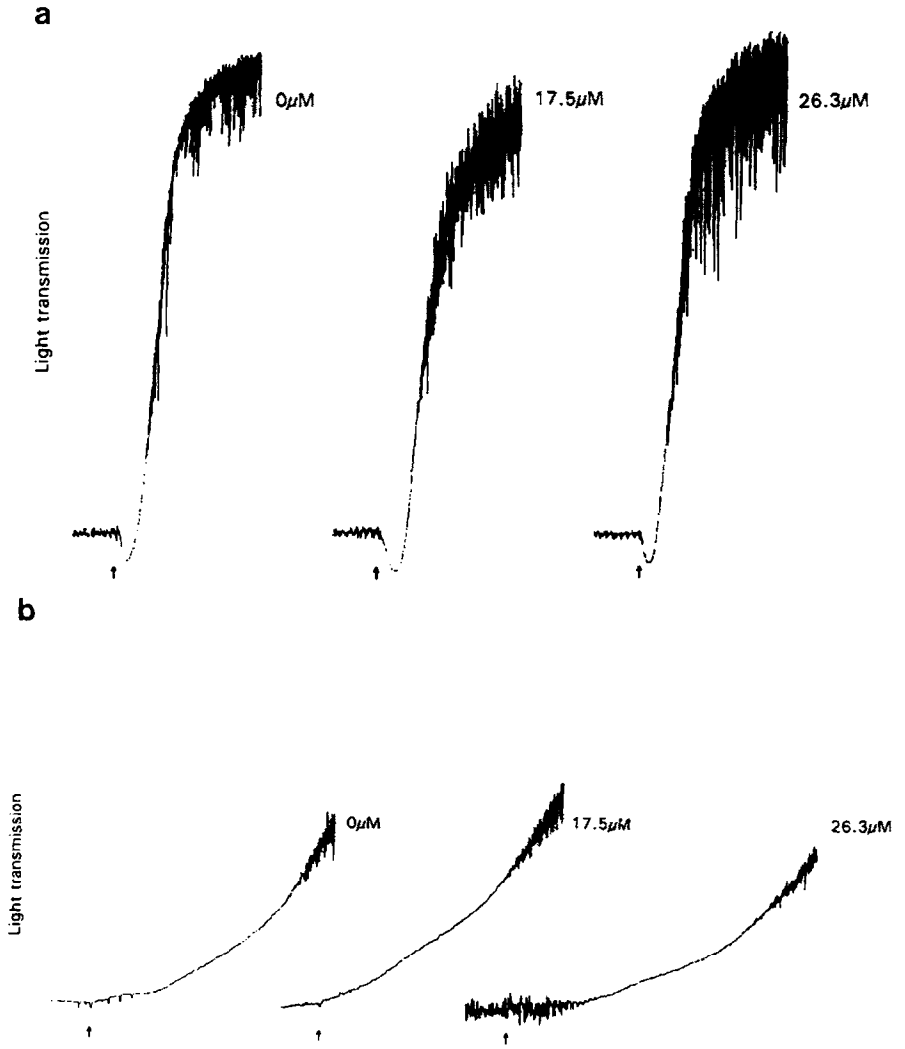

Fig. 4. Effects of $AACOCF_3$ on A23187- and PMA-induced platelet aggregation. Washed human platelets were preincubated with 17.5 to 26.3 μM $AACOCF_3$, or 0.25% DMSO vehicle control, for 2 min prior to the addition (↑) of 1 μM A23187 (a) or 30 nM PMA (b). Aggregation was monitored continuously as an increase in light transmission.

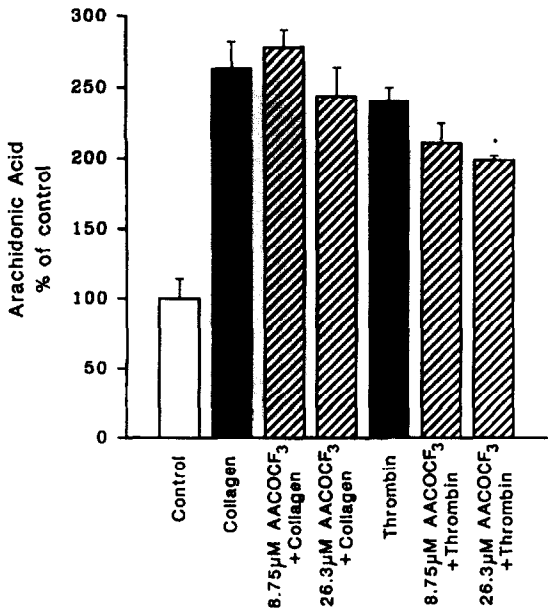

Fig. 5. Effects of $AACOCF_3$ on collagen- and thrombin-induced [^{14}C]arachidonic acid release. Human platelets were prelabelled with [^{14}C]arachidonic acid and subsequently washed in a physiological buffer. Aliquots were stirred with 0.25% DMSO, 8.7 μM $AACOCF_3$ or 26.3 μM $AACOCF_3$ for 2 min prior to the addition of saline, 10 μg/mL collagen or 0.1 U/mL thrombin for an additional 2 min. Release was terminated, and the [^{14}C]arachidonic acid was extracted, separated by TLC, and quantified by radiochromatographic scanning. Data (means ± SEM; N = 7 for collagen and N = 5 for thrombin) are given as a percentage of the DMSO/saline control. The mean control value was 3840 dpm. Key: (*) $P < 0.05$ vs thrombin alone.

DISCUSSION

Several agonists stimulate the events that constitute platelet activation. The biochemical mechanisms, from receptor occupancy to final response, associated with some of these agonists have been the subject of intense interest for several years [3, 10, 12, 40, 41]. Receptors, such as those for thromboxane A_2, thrombin, platelet-activating factor, and vasopressin, are linked via a G-protein to the activation of phosphoinositide specific phospholipase C. The resultant formation of second messengers mediates the platelet responses, such as shape change, granule release, eicosanoid production, and aggregation.

Collagen is arguably the most physiologically relevant platelet agonist, although the underlying mechanism remains to a large degree unclear. In common with other agonists, collagen causes phosphoinositide hydrolysis and calcium fluxes; however, this may be mediated by phospholipase C-γ2 rather than the phospholipase C-β associated with G-protein linked receptors [28]. Many of the biochemical effects, as well as platelet aggregation, in response to collagen are aspirin inhibitable and, therefore, TxA_2 dependent [30–32]. The release of arachidonic acid, and its subsequent conversion to TxA_2, are early and pivotal events in the platelet response to collagen. In platelets, this release generally is believed to occur via a calcium-mediated PLA_2 activity. Aspirin inhibits calcium changes in collagen-stimulated platelets [32], indicating that arachidonic acid release occurs independently of calcium changes, although this has been disputed [42]. Therefore, the precise mechanism of collagen-induced arachidonic acid release has not been identified.

In the present study, a novel potent inhibitor of cytosolic phospholipase A_2, $AACOCF_3$ [25], was used to examine the role that $cPLA_2$ plays in collagen-induced platelet activation. $AACOCF_3$ inhibited collagen-induced aggregation of washed human platelets. In contrast, $AACOCF_3$ had no effect on aggregation in response to thrombin, the thromboxane mimetic U46619, the calcium ionophore A23187, or the phorbol ester PMA. The data clearly support the specifically TxA_2-dependent nature of collagen-induced platelet aggregation in comparison with other agonists. $AACOCF_3$ inhibited collagen-induced thromboxane formation but not the release of arachidonic acid. Furthermore, aggregation in response to arachidonic acid, which requires conversion to TxA_2, was also inhibited by $AACOCF_3$. Taken together these data are consistent with a cyclooxygenase inhibitory effect of $AACOCF_3$.

$AACOCF_3$ inhibits arachidonic acid release in response to thrombin ([26]; Fig. 5) and A23187 [27] but not to collagen (Fig. 5). Several possibilities may account for this apparent anomaly. Under the conditions used, multiple pools of arachidonate-containing phospholipid may be labelled with [^{14}C]arachidonic acid. These pools may be non-uniformly labelled [43–45] in which case labelling studies do not reflect released arachidonic acid mass. Therefore, inhibitory effects of $AACOCF_3$ on collagen-induced arachidonic acid release may not be detected.

Furthermore, the multiple pools of phospholipid may be accessible to differential enzymatic hydrolysis stimulated by collagen and thrombin. Indeed, Takamura *et al.* [46], using mass analysis, proposed this possibility and suggested different mechanisms for phospholipid breakdown stimulated by the two agonists.

Thrombin elevates cytosolic calcium levels [3, 10, 41] and, therefore, presumably stimulates the calcium-dependent $cPLA_2$ [24]. This is believed to be the principal method of arachidonic acid liberation and would be sensitive to inhibition by $AACOCF_3$ [26]. Collagen releases arachidonic acid without elevating cytosolic calcium [32]. Therefore, the liberation of arachidonic acid by the $AACOCF_3$-inhibitable, calcium-mediated $cPLA_2$ would be less in collagen-stimulated, than in thrombin-stimulated, platelets. In this case, arachidonic acid liberating mechanisms insensitive to $AACOCF_3$ may be employed by collagen.

Table 1. Effects of $AACOCF_3$ on collagen- and thrombin-induced thromboxane production

Additions	Thromboxane*
DMSO + saline	440
DMSO + collagen	3,500
17.5 μM $AACOCF_3$ + collagen	520
Aspirin + collagen	340
DMSO + thrombin	65,000
17.5 μM $AACOCF_3$ + thrombin	10,000

Values are means of duplicate experiments.
* Data are expressed as pg/mL released thromboxane B_2.

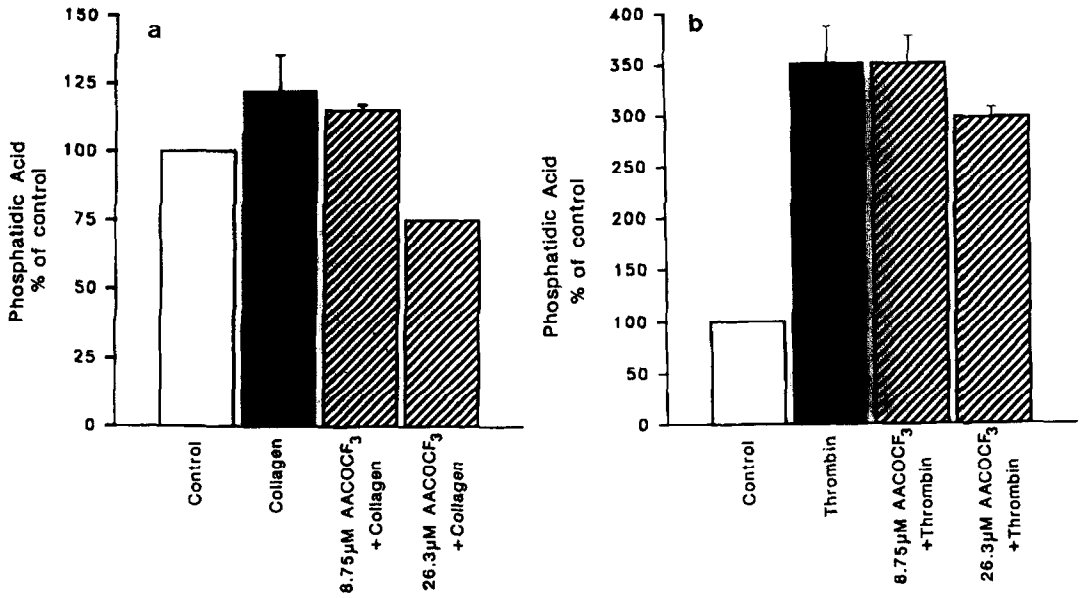

Fig. 6. Effects of $AACOCF_3$ on collagen- and thrombin-induced [^{32}P]phosphatidic acid formation. Human platelets were prelabelled with [^{32}P]orthophosphate and subsequently washed in a physiological buffer. Aliquots were incubated with 0.25% DMSO, 8.7 μM $AACOCF_3$ or 26.3 μM $AACOCF_3$ for 2 min prior to the addition of: (a) saline or 10 μg/mL collagen; or (b) saline or 0.1 U/mL thrombin, for an additional 2 min. The reaction was terminated, and the [^{32}P]phosphatidic acid extracted, separated by TLC, and quantified by radiochromatographic scanning. Data (means ± SEM; N = 3) are given as a percentage of the DMSO/saline control. The mean control value was 760 dpm. Statistical analysis: DMSO + saline vs DMSO + collagen, $P < 0.05$; DMSO + collagen vs 26.3 μM $AACOCF_3$ + collagen, $P < 0.05$; and DMSO + saline vs DMSO + thrombin, $0.05 > P > 0.01$.

Diacylglycerol lipase has been reported to play a small but significant role in collagen-induced arachidonic acid release [23, 47]. There is no evidence that diacylglycerol lipase is sensitive to $AACOCF_3$, and, therefore, arachidonic acid release via this route may not be inhibited. However, the source of diacylglycerol remains unclear. Collagen-induced diacylglycerol formation, presumably as a result of phospholipase C activity, is aspirin sensitive [30]. Similarly $AACOCF_3$ inhibited collagen- but not thrombin-induced phospholipase C activity. This suggests that the formation of diacylglycerol is a consequence, rather than a source, of TxA_2 in collagen-stimulated platelets.

A 29-kDa member of the 14-3-3 family of proteins has been identified in human platelets. This protein was reported to be a non-calcium-dependent PLA_2, associated with the high affinity thrombin receptor glycoprotein Ib/IX complex [48], although this observation has been challenged [49]. If confirmed, however, an analogous enzyme activated distal to the collagen receptor would account for both the calcium-independent nature of collagen-induced arachidonic acid release [32], and the ineffectiveness of the specific $cPLA_2$ inhibitor to influence collagen-induced arachidonic acid release.

The inhibitory effects of $AACOCF_3$ on collagen-induced thromboxane formation and aggregation could, therefore, only be explained by an action on the conversion of arachidonic acid to TxA_2 by cyclooxygenase. Consistent with such an effect on cyclooxygenase, $AACOCF_3$ inhibited arachidonic acid-induced platelet aggregation. Indeed, Riendeau and colleagues [27] reported some cyclooxygenase inhibitory actions of $AACOCF_3$.

In conclusion, the present study supports the concept that there is an early release of arachidonic acid in collagen-stimulated platelets. This arachidonic acid is converted to TxA_2, which is critical to platelet activation. The mechanism of collagen-induced arachidonic acid release differs from that employed by thrombin and, initially, probably does not involve the calcium-dependent $cPLA_2$ that is inhibitable by $AACOCF_3$.

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