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In-vitro Platelet Responses to Arachidonic Acid in the Rat

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Abstract

Using both the turbidimetric and the conductive methods to study aggregation of platelets, we found that arachidonic acid stimulated rat washed platelets in a dose-dependent manner (40 μM–0·5 mM).

Although a high concentration of arachidonic acid (0·5 mM) produced an increase in light transmission both in the presence of 2 mM $CaCl_2$ and EGTA (45·8 ± 2·8 and 50·4 ± 0·8% respectively) no changes in impedance were detected. Lysis caused by this concentration of arachidonic acid was very high at all the concentrations of calcium used (mean of 81·3%). In addition, the turbidimetric response induced by 0·5 mM arachidonic acid implied an initial decrease in light transmission but did not correlate with a real shape change. Forty micromolar arachidonic acid induced a calcium-dependent aggregation measured both by aggregometry and impedance. Morphology of aggregates induced by both concentrations was also studied.

These results suggest that the optimal concentration for studying rat platelet activation by arachidonic acid is 40 μM; high concentrations (0·5 mM) cause aspecific effects not correlated to a physiological activation response.

Activation of platelets by arachidonic acid causes the platelets to aggregate and discharge their storage granules (Linder et al 1979; Hashimoto et al 1985). While the biochemistry and responses of human platelets exposed to exogenous arachidonic acid have been studied in detail (Hashimoto et al 1985; Nishikawa et al 1988; Siess 1989; Alonso et al 1990), relatively little information exists concerning the responses of rat platelets after arachidonic acid activation.

It has been reported that fibrinogen and calcium are important cofactors in the aggregation of platelets (Siess 1989). The extracellular requirements of both depends on the stimuli and species of platelet used, i.e. aggregation induced by thrombin in rat platelets depends on the presence of extracellular calcium more than in human platelets (Heemskerk et al 1991), and ADP only causes aggregation of human platelets if the medium contains fibrinogen, while rabbit platelets do not need it for aggregation (Harfenist et al 1988).

In the present study we have investigated the role of extracellular calcium and fibrinogen on the responses induced by arachidonic acid in rat washed platelets, one of the systems most used in experimental pharmacological investigations (including antiplatelet drugs). We have established the conditions under which arachidonic acid acts as an inducer of aggregation.

Materials and Methods

Materials

Fatty acids, fibrinogens, colchicine, vincristine and cytochalasin B were from Sigma Chemical Co. (St. Louis, USA). [^{3}H]5-HT was purchased from Du Pont, New England Nuclear (Herts, UK). All other reagents were of analytical grade.

Preparation of platelet-rich plasma

Blood was collected by cardiac puncture from sodium pentobarbitone-anaesthetized Sprague-Dawley rats and anticoagulated with 3·13% sodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation (400 g × 25 min) of the anticoagulated blood. Platelets were suspended to a cell density of 3×10^8 cells mL^{-1} by diluting the PRP with autologous platelet-poor plasma (2700 g × 10 min).

Preparation of washed platelets

Blood was collected on acid citrate dextrose (85 mM trisodium citrate, 65 mM citric acid and 111 mM glucose). Platelets were pelleted from PRP (1000 g × 18 min), washed in a Ca^{2+}-free modified Tyrode–HEPES solution (107 mM NaCl; 2·68 mM KCl; 1 mM $MgCl_2$; 3·8 mM $NaPO_4H_2$; 20 mM HEPES; 5·5 mM glucose; 0·35% (w/w) bovine serum albumin; and 1 : 6 acid citrate dextrose, pH 7·4), and finally resuspended at an appropriate density in modified buffer where albumin and acid citrate dextrose were omitted and NaCl was increased up to 132·5 mM to maintain osmolarity (300 ± 10 mOsm kg^{-1}). One hour prior to the experiments $CaCl_2$ (0·5–2 mM), buffer or 2mM EGTA was added.

Measurement of platelet aggregation and shape change

Aggregation was measured as percentage of the maximum change in light transmission in a dual channel aggregometer Chrono-log (Havertown, PA, USA). All experiments were

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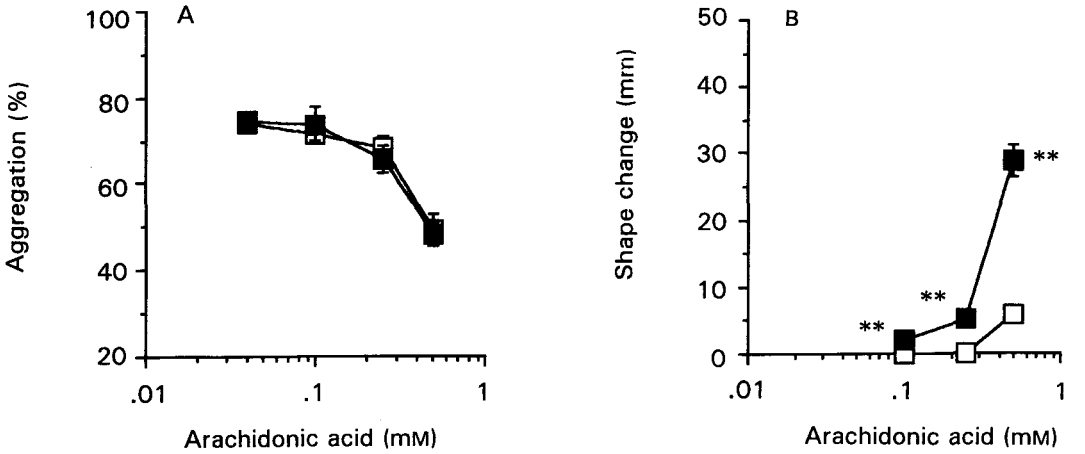

FIG. 1. Aggregation and shape change responses to arachidonic acid. Platelet aggregation (A) and shape change (B) induced by different concentrations of arachidonic acid in rat washed platelets in the presence of 2 mM $CaCl_2$ were measured in an aggregometer as change in light transmission. Both responses are shown in the presence of rat fibrinogen (0·25 mg mL^{-1}, ■) and its absence (□). $^{**}P < 0{\cdot}01$.

performed at 37°C under constant stirring. Platelet suspensions (0·45 mL) were prewarmed before the addition of compounds and incubated for 5 min before addition of the stimulus, arachidonic acid dissolved in ethanol and 25 mM Na_2CO_3 as described by Vane (1971). Platelet aggregation was also measured by the electric impedance method as change in resistance between two electrodes when the platelets aggregate, as described previously (Cardinal & Flower 1980). Shape change was measured in millimetres as the initial decrease in light transmission that precedes aggregation.

Measurement of secretion

For secretion studies, washed platelets were incubated with 1 μCi mL^{-1} of [^{3}H]5-HT (24·7 Ci $mmol^{-1}$) at room temperature (21°C) for 20 min. Experiments were stopped by transfer of samples to 1% (final concentration) formaldehyde at 0–4°C. Samples were centrifuged at 2000 *g* for 5 min and secretion was quantified in the supernatant as a percentage of the total platelet [^{3}H]5-HT.

Platelet lysis

Cell lysis was quantified measuring the lactate dehydrogenase activity present in the supernatant and expressed as a percentage of the total activity measured in platelet lysates. Lactate dehydrogenase activity was assayed using a commercial enzymatic test from Spinreact (Spinreact, Spain).

Microscopy

The presence of aggregates was confirmed by light microscopy. Samples for light microscopy were fixed in 1·5% (final concentration) formaldehyde at room temperature (21°C) and examined using a phase contrast optical microscope. Samples for scanning electron microscopy were processed immediately after fixation in 1·5% (final concentration) glutaraldehyde. Platelets were collected on a 0·22-μ pore filter and lyophilized. The dried filters were coated with gold and palladium to approximately 400-Å thickness in a Polaron apparatus (model E5000, Polaron Inst. Inc., PA, USA) and examined in a SS60 ISI scanning electron microscope (International Scientific Instruments, CA, USA).

Statistical analysis

Results are expressed as mean ± standard deviation of at least four experiments run in duplicate. Statistical significance was indicated using Student's *t*-test.

Results

Responses induced by arachidonic acid measured by the turbidimetric method

Aggregation was maximal at 40 μM arachidonic acid, less than this concentration did not stimulate rat platelets. At concentrations between 40 μM and 0·5 mM, arachidonic acid induced a dose-dependent decrease of platelet aggregation (Fig. 1A). This pattern of aggregation is similar to those exhibited by rabbit platelets (Harfenist et al 1982). Exogenous rat fibrinogen had no effect on this response.

It was also found that arachidonic acid induced a decrease of light transmission, generally considered to represent a shape change, between 0·1 and 0·5 mM. This response was increased ($P < 0{\cdot}01$) by addition of rat fibrinogen (0·25 mg mL^{-1}) (Fig. 1B).

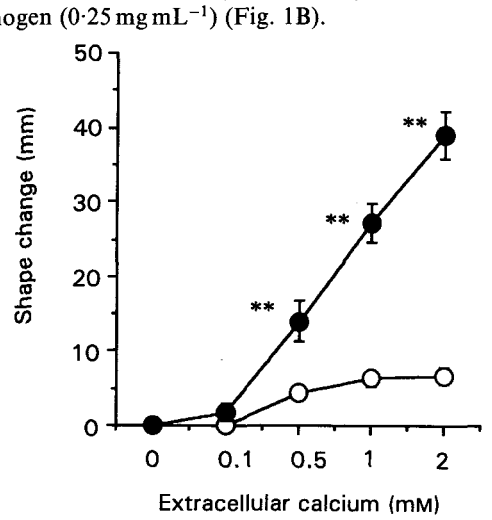

FIG. 2. Effect of extracellular calcium and fibrinogen on 0·5 mM arachidonic acid-induced shape change-like response in the presence (●) or absence (○) of rat fibrinogen (0·25 mg mL^{-1}). $^{**}P < 0{\cdot}01$.

Table 1. Effect of shape change inhibitors and fibrinogen on the shape change-like response (measured in millimetres as decrease in light transmission) induced by 0·5 mM arachidonic acid in rat washed platelets in the presence of 2 mM $CaCl_2$. Experiments were carried out in the aggregometer.

	Solvent[b]	Colchicine (0·5 mM)	Vincristine (50 μM)	Cytochalasin B (50 μM)
Solvent[a]	11·5 ± 0·8	10·0 ± 1·4	9·2 ± 1·1	12·5 ± 1·7
Rat fibrinogen (0·25 mg mL^{-1})	28·8 ± 1·7	26·7 ± 2·9	27·0 ± 1·7	25·5 ± 1·7

[a]Modified Tyrode buffer. [b]Modified Tyrode buffer for colchicine, DMSO for vincristine and cytochalasin B.

These experiments suggested two concentrations of arachidonic acid to use for further investigations: 0·5 mM which induced a shape change-like response followed by a mild and gradual aggregation and 40 μM which induced an intense and rapid trace of aggregation.

Study of shape change induced by 0·5mM arachidonic acid

Shape change-like response was clearly dependent on calcium and addition of rat fibrinogen ($P < 0·01$) (Fig. 2). This last effect was unique to this species since fibrinogen from other species like man, sheep and bovine fibrinogen, had no effect on the shape change caused by 0·5 mM arachidonic acid (results not shown).

To confirm that this shape change-like response was a real morphological transformation, we carried out pharmacological and microscopical studies.

Pharmacology. Three inhibitors of shape change were used: colchicine and vincristine by depolymerization of microtubules, and cytochalasin B which inhibits actin polymerization. At maximally effective concentrations (0·5 mM colchicine, 50 μM vincristine, 50 μM cytochalasin B) given by previous studies in ADP-stimulated PRP, these inhibitors did not inhibit the decrease in light transmission induced by 0·5 mM arachidonic acid, either in the absence or in the presence of fibrinogen (Table 1).

Microscopy. The samples for scanning electron microscopy taken at the maximum decrease of light transmission induced by arachidonic acid did not show the typical pseudopodia of shape-change platelets. There were no morphological differences between platelets in the presence or absence of rat fibrinogen (results not shown).

Platelet aggregation measured as light transmission induced by 0·5 mM and 40 μM arachidonic acid

The role of external calcium on aggregation in arachidonic acid-stimulated rat platelets was also studied. When washed platelets were challenged with 0·5 mM arachidonic acid, light transmission, indicated as aggregation, was almost of the same magnitude at all the concentrations of calcium used. Aggregation induced by 40 μM arachidonic acid was clearly dependent on calcium concentration (Table 2).

Although turbidimetric analysis indicated that this activation was still evident in buffer with no added calcium and in the presence of 2 mM EGTA, light microscopy showed that under these conditions there were no aggregates (not shown). This phenomenon was presumably caused by a decrease in cell volume induced by degranulation or by a loss of cellular density in the samples caused by lysis.

Table 2. Effect of extracellular calcium on aggregation, measured as change in light transmission, caused by 0·5 mM and 40 μM arachidonic acid.

Calcium (mM)	Aggregation (%)	
	Arachidonic acid (0·5 mM)	Arachidonic acid (40 μM)
2·0	45·8 ± 2·8	70·5 ± 1·3
1·0	58·9 ± 2.5*	66·1 ± 4·3**
0·5	60·1 ± 0·6**	53·0 ± 3·4**
0·25	53·5 ± 1·5*	46·7 ± 4·6**
0·1	50·8 ± 0·7	33·7 ± 3·3**
Buffer	46·8 ± 1·6	38·6 ± 5·3**
0·5 mM EGTA	49·5 ± 1·2	37·8 ± 9·1**
2·0 mM EGTA	50·4 ± 0·8	33·5 ± 4·2**

Mean ± s.d. *$P < 0·05$, **$P < 0·01$, compared with the response in the presence of 2 mM $CaCl_2$ using Student's *t*-test.

Table 3. Effect of extracellular calcium on the increase in light transmission (indicated as aggregation), 5-HT release (indicated as secretion) and cell lysis stimulated by A. 0·5 mM and B. 40 μM arachidonic acid.

A. Calcium (mM)	Aggregation (%)	Secretion (%)	Lysis (%)
2·0	34·6 ± 2·6	96·0 ± 3·5	81·4 ± 2·8
1·0	48·3 ± 3·1*	94·2 ± 5·1	86·1 ± 2·7
0·5	52·8 ± 1·3**	93·0 ± 1·2	82·9 ± 2·9
0·0	45·3 ± 0·7*	92·5 ± 1·6	81·7 ± 5·7
2 mM EGTA	43·3 ± 1·3*	96·6 ± 3·7	74·5 ± 5·6
B. Calcium (mM)	**Aggregation (%)**	**Secretion (%)**	**Lysis (%)**
2·0	75·2 ± 2·2	85·6 ± 2·5	33·2 ± 5·1
1·0	75·0 ± 1·6	95·7 ± 3·9	45·6 ± 7·9
0·5	47·2 ± 8·3*	92·6 ± 1·1	42·6 ± 14·9
0·0	39·7 ± 5·9**	81·5 ± 2·1	9·1 ± 0·2**
2·0 mM EGTA	38·5 ± 0·8**	84·4 ± 4·4	7·8 ± 2·9**

Mean ± s.d. *$P < 0·05$, **$P < 0·01$, compared with the response in the presence of 2 mM $CaCl_2$ using Student's *t*-test.

5-HT release and cellular lysis induced by arachidonic acid

5-HT release, indicated as secretion, and lysis induced by

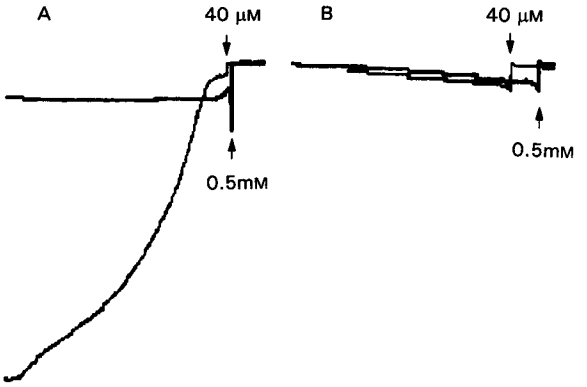

FIG. 3. Platelet aggregation induced by 0·5 mM and 40 μM arachidonic acid in rat platelets measured by electric impedance in the presence of 2 mM $CaCl_2$ (A) and 2 mM EGTA (B). Traces are representative of at least four experiments.

0·5 mM arachidonic acid were clearly independent of added calcium. The percentage of platelet lysis induced by this concentration of arachidonic acid was of the same order of magnitude as the percentage of secretion for all the concentrations of calcium (Table 3). This extent of lysis is too high (mean of 81·3%) to be considered as a normal platelet response. Although membrane-soluble stimuli, such as arachidonic acid and the ionophore A23187, are much more potent lytic substances than other physiologic stimuli (extent of lysis <5%) they do not usually exceed 20% (Lages 1986). Our results suggest that 0·5 mM arachidonic acid has aspecific effects on platelets, since it causes lysis or the non-selective permeabilization of the membrane.

On the other hand, we found that the loss of lactate dehydrogenase induced by 40 μM arachidonic acid followed the same pattern of behaviour as aggregation, showing it to be calcium-dependent, while secretion was independent (Table 3).

Platelet aggregation was also measured by the impedance method to eliminate the potentially interfering effects of lysis and secretion in the response obtained by the turbidimetric method. The addition of both concentrations of arachidonic acid in the presence of 2 mM EGTA did not cause changes in the electric impedance, and this finding corroborates the absence of aggregates. Platelets challenged with 40 μM arachidonic acid in a 2 mM calcium-containing buffer responded with aggregation. No changes in impedance were observed when 0·5 mM arachidonic acid was used as stimulus (Fig. 3).

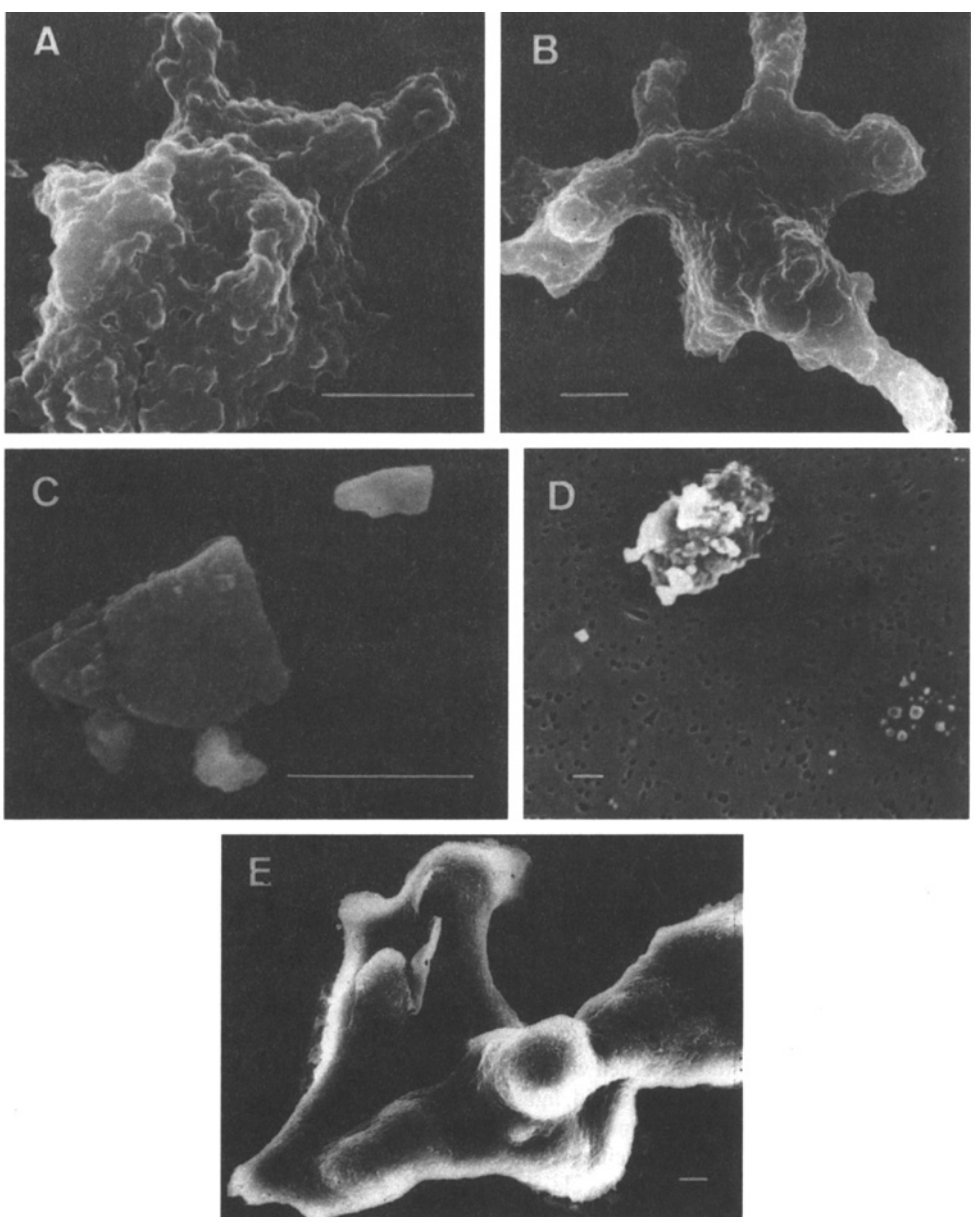

FIG. 4. Scanning electron micrographs of activated platelets: A and B PRP stimulated by 2 μM ADP, C and D platelets challenged with 0·5 mM arachidonic acid, and E aggregates from 40 μM arachidonic acid-stimulated platelets. White bar represents in all cases 10 μM except in D 1 μM.

Morphology of platelet aggregates assessed by scanning electron microscopy

Although light microscopy indicated the presence of aggregates when rat platelets were activated by 0·5 mM arachidonic acid in the presence of 2 mM calcium, electric impedance showed no aggregation under these conditions. We studied the morphology of these aggregates by scanning electron microscopy; they were found to be compact masses of platelets without the morphological appearance of real aggregates (Fig. 4C,D). Real aggregates were those obtained from PRP stimulated with 2 μM ADP or those from washed platelets stimulated with 40 μM arachidonic acid (Fig. 4A,B,E, respectively).

Discussion

Platelet functional responses to the main physiological stimuli are shape change, aggregation and secretion. The most common method used for measuring platelet activation is aggregometry which was introduced by Born (1962). In this technique the initial decrease in light transmission has been correlated with shape change. It has been reported that shape change in human platelets is independent of extracellular calcium (Zucker & Grant 1978) and fibrinogen receptor exposure (Peerschke et al 1980).

Using this technique it was found that 0·5 mM arachidonic acid induced in washed rat platelets a shape change-like response dependent on calcium and the extracellular presence of fibrinogen (Fig. 2). This result was not expected and it suggested that the response in the rat was different from other species. Confirming the authenticity of the observed response became a matter of great importance: three known inhibitors of the shape change had no effect on the decrease in light transmission induced by arachidonic acid. These preliminary results suggested that this response was not a shape change. In addition, a microscopical study was carried out.

Since emission of pseudopodia and spheration of platelets are near synchronous events (separated by only a few seconds) (Gear 1984), light scattering theory (Latimer et al 1977) predicts that a decrease in light transmission caused by shape change would lead to the observation, using the electron microscope, of spherical platelets possessing long pseudopodia. In electron micrographs of platelets challenged with 0·5 mM arachidonic acid, pseudopodia were not found. This non-pseudopodia pattern of shape change, as similarly observed in the case of ionophore A23187 (Gear 1984), could be caused by the fact that both agents are membrane-soluble stimuli. The turbidimetric differences found between platelets stimulated with 0·5 mM arachidonic acid in the presence of fibrinogen and in its absence, could not be explained in morphological terms since any morphological difference could be seen in the micrographs between platelets fixed at the maximum decrease of light transmission induced by arachidonic acid in both conditions. These results lead us to suggest that this phenomenon is not a real shape change, and could be an artefact resulting from our experimental conditions; at the highest arachidonic acid concentrations, this fatty acid could precipitate and create a turbidimetric shape change-like response. This could be influenced by saline concentration and structure of fibrinogen used, since the response depended on extracellular calcium and species of fibrinogen, respectively. In conclusion, when aggregometry is used to study shape change, it is also necessary to carry out a pharmacological or microscopical study to confirm authenticity of the response.

Aggregation induced by arachidonic acid was independent of extracellular fibrinogen at all concentrations tested. This protein is thought to be supplied by the secretion of α-granules that occurs in parallel with secretion of dense bodies (Lages 1986).

Although turbidimetric analysis indicated an aggregation response, we concluded from impedance and microscopical studies that a high concentration of arachidonic acid (0·5 mM) did not induce aggregation of rat platelets even in the presence of 2 mM $CaCl_2$. Arachidonic acid 0·5 mM causes lysis or non-selective permeabilization of the platelet membrane. This effect is not specific for arachidonic acid since other unsaturated fatty acids (oleic, eicosatrienoic and eicosapentaenoic acid) induce lysis to the same extent as arachidonic acid (results not shown). It seems that rat platelets are more susceptible to cellular destruction than those of other species since 0·5 mM oleic acid does not cause lysis in washed rabbit platelets (Nuñez et al 1990).

On the contrary, 40 μM arachidonic acid induces a real aggregation in rat platelets corroborated by aggregometry, impedance and scanning electron microscopy. Aggregation was dependent on extracellular calcium. According to the turbidimetric method, aggregation was present even at 2 mM EGTA (33·5 ± 4·2%) although under these conditions there were no aggregates; lysis and secretion are the phenomena that interfere in the measuring of aggregation by this method. These effects can be circumvented using the impedance method. Detwiler & Huang (1980) classified agonists as strong or weak based on the relationship between aggregation and secretion. In this regard, arachidonic acid acts as a strong agonist because it stimulates secretion without requiring aggregation and independently of extracellular calcium.

In conclusion, the present study has shown that 40 μM arachidonic acid is the optimal concentration to use in the study of the activation of rat platelets to arachidonic acid. We have established an in-vitro model for testing antiplatelet drugs as possible inhibitors of arachidonic acid platelet activation.

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