PAF-acether (1-*O*-hexadecyl/octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine)-induced fibrinogen binding to platelets depends on metabolic energy

Ed KLOPROGGE, Paula HASSELAAR and Jan-Willem N. AKKERMAN*
Department of Haematology, University Hospital Utrecht, P.O. Box 16250, 3500 CG Utrecht, The Netherlands

A combination of CN^- and 2-deoxy-D-glucose decreases the binding of fibrinogen to platelets stimulated with PAF-acether (1-*O*-hexadecyl/octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine). Decreased binding is found after pretreatment with metabolic inhibitors, thereby lowering the energy content before stimulation as well as at various stages after stimulation of undisturbed cells. Binding and ATP hydrolysis occur in parallel, suggesting tight coupling between both phenomena. Energy appears to be predominantly required for exposure and maintenance of accessible binding sites, whereas the interaction between fibrinogen and the exposed sites does not depend on metabolic energy.

INTRODUCTION

Upon proper stimulation, platelets change shape, aggregate and secrete the contents of dense, α- and lysosomal granules. Each of these processes requires metabolic energy, as illustrated by the increase in ATP-resynthesizing sequences during these functions (Akkerman & Holmsen, 1981) and their sensitivity to pretreatment with metabolic inhibitors (Verhoeven et al., 1984a). By using a quantitative technique for measurement of energy costs of separate platelet responses, the 'metabolic price' for each function can be assessed. The combined secretion of lysosomal and α-granule contents, which cannot be sufficiently separated, requires 5–7 μmol ATP equivalents ($ATP_{eq.}$) per 10^{11} platelets. Complete dense-granule secretion costs only 0.5–0.8 μmol (same units), whereas shape-change is not accompanied by a detectable increase in energy consumption, indicating that its metabolic cost is minor (Verhoeven et al., 1984b, 1985, 1986).

The energy requirement of aggregation is more difficult to quantify. Aggregation is the result of a sequence of events in which different processes participate, e.g. binding of an agonist to its receptors, signal processing via generation of second messengers, exposure of fibrinogen-binding sites and binding of fibrinogen to the exposed sites (Niewiarowski et al., 1983). If stirring permits the cells to collide, platelet–platelet bridges are formed, leading to the formation of aggregates that can be detected either optically in an aggregometer or electronically by particle sizing. Optical aggregation correlates poorly with alterations in energy consumption, but its sensitivity to sudden ATP depletion indicates a need for energy at any stage during this response (Verhoeven et al., 1984a). In contrast, electronic determination of aggregation, generally called 'single-platelet disappearance', varies closely with changes in energy consumption and appears to require about

2.5 μmol of $ATP_{eq.}$ per 10^{11} platelets to come to completion (Verhoeven et al., 1985).

The present paper describes a more detailed study of the role of metabolic energy in platelet aggregation, with special emphasis on a possible link between energy and binding of fibrinogen to the platelets, which is an essential step in the aggregation process.

MATERIALS AND METHODS

Chemicals

PAF-acether from Calbiochem–Behring Corp. (La Jolla, CA, U.S.A.) was dissolved in Ca^{2+}-free Tyrode's solution (Walsh, 1972) containing 2.5 mg of BSA (Organon Teknika, Oss, The Netherlands)/ml and stored at -20 °C. Fibrinogen (Kabi, Stockholm, Sweden) was made fibronectin-free by passage through a gelatin–Sepharose 4B column (Pharmacia, Uppsala, Sweden) (Engvall et al., 1978) in the presence of 10 mM-6-aminohexanoic acid (Baker Chemicals, Deventer, The Netherlands) and 1 mM-benzamidine hydrochloride (Sigma, St. Louis, MO, U.S.A.). After four repeated dialyses against 40 vol. of Tyrode's solution, the fibrinogen solution was concentrated against Sephadex G-200 (Pharmacia) and stored at -70 °C. The protein concentration of the final preparation was determined by the method of Lowry et al. (1951), with BSA as a standard. Contamination of Factor VIII–von Willebrand factor was less than 0.015% on molar basis, as measured by an enzyme immunoassay (Cejka, 1982).

Platelet isolation

Freshly drawn venous blood was collected from healthy human volunteers (with informed consent) into citrate (0.1 vol. of 129 mM-sodium citrate). The donors claimed not to have taken any medicine during the 10 days before blood collection. After preparation of the platelet-rich plasma by centrifugation (200 g, 10 min,

---

Abbreviations used: PAF-acether, platelet-activating factor (1-*O*-hexadecyl/octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; BSA, bovine serum albumin; AEC, adenylate energy charge.

* To whom correspondence and reprint requests should be sent.

room temperature) the platelets were isolated by gel filtration on a Sepharose 2B column (Pharmacia) (Tangen *et al.*, 1971) at room temperature in Ca^{2+}-free Tyrode's solution at pH 7.25, containing 0.2% BSA (Organon Teknika). In some experiments the metabolic pool of adenine nucleotides was radiolabelled by incubating the platelet-rich plasma with [^{14}C]adenine (Amersham International, Amersham, Bucks., U.K.; sp. radioactivity 286 Ci/mol) for 45 min at 37 °C before gel filtration. The concentration of the platelet suspension was adjusted to 2×10^8 cells/ml by dilution in gel-filtration buffer. All subsequent incubations were carried out after dividing the suspension into two parts. One part was used for analysis of fibrinogen binding, whereas the second part was used for analysis of metabolic parameters.

Binding studies with ^{125}I-fibrinogen

Fibrinogen was radiolabelled with $Na^{125}I$ (Amersham International) with an Iodogen-labelling procedure (Nieuwenhuizen *et al.*, 1980) and stored, after four repeated dialyses against 500 vol. of Tyrode's solution, at −80 °C. The specific radioactivity ranged between 36 and 222 Ci/mmol; clottability was > 99% [5 units of thrombin (Hoffman–La Roche, Basel, Switzerland)/ml; 5 min at 37 °C] and free ^{125}I was never more than 4.0%.

Binding studies were performed by incubating the platelets with 500 nM-PAF-acether in the presence of ^{125}I-fibrinogen (10–500 nM) at 22 °C. At different time points (see the Results section) three to six samples (200 μl each) were placed on 100 μl of 20% (w/v) sucrose in Tyrode's solution in polypropylene microsedimentation tubes (0.4 ml; Sarstedt, Vienna, Austria). Immediately thereafter the samples were centrifuged in a Beckman Microfuge (Beckman Instruments, Mijdrecht, The Netherlands; 12000 *g*, 22 °C) for 2 min and the tips of the tubes were cut off just above the pellet. The number of molecules of fibrinogen bound per platelet was calculated by comparing the specific radioactivity of the ^{125}I-labelled fibrinogen in the platelet fraction with the total radioactivity in the pellet plus supernatant. The amount of non-specific binding was measured by parallel incubation with ^{125}I-fibrinogen in the absence of PAF-acether. Control studies have shown that those cells bound as much ^{125}I-fibrinogen as did PAF-acether-treated platelets incubated with ^{125}I-fibrinogen in the presence of a 100-fold molar excess of non-radiolabelled fibrinogen (Kloprogge & Akkerman, 1984).

Analysis of metabolic parameters

In a parallel set of incubations [^{14}C]adenine-labelled platelets were incubated with PAF-acether in the presence of the same concentrations of non-radiolabelled fibrinogen as those used in the binding assay. At different times, samples of cell suspension were collected and mixed with 2 vol. of freshly prepared EDTA/ethanol [10 mM-EDTA in 86% (v/v) ethanol, pH 7.4, 0 °C]. The extract was centrifuged and the supernatant was analysed for ethanol-soluble ^{14}C-labelled adenine nucleotides and derivatives, as outlined elsewhere (Daniel *et al.*, 1980). Previous studies (Daniel *et al.*, 1980) have shown that, with this technique, metabolic ADP and ATP can be measured accurately without being disturbed by actin-bound ADP (which is insoluble in ethanol) or granule ATP–ADP (which remain unlabelled). The adenylate energy charge, given by the equation:

$$AEC = (ATP + \tfrac{1}{2}ADP)/(ATP + ADP + AMP)$$

(Atkinson, 1977) was calculated from the ^{14}C radioactivity in ATP, ADP and AMP.

Coefficients of variations for measurement of [^{14}C]ATP, [^{14}C]ADP and [^{14}C]AMP were 1%, 8% and 8% respectively (*n* = 6). The radioactivity data were recalculated into absolute amounts on the basis of a metabolic ATP content of 4.5 μmol/10^{11} platelets and the fact that, in normal [^{14}C]adenine-labelled platelets, 80% of the total radioactivity is found in ATP (Verhoeven *et al.*, 1984*b*; Daniel *et al.*, 1980). The energy stored in ATP and ADP was expressed in ATP equivalents (ATP_{eq}), which denote the energy yield of the conversion of ATP into ADP (Atkinson, 1977).

Metabolic inhibitors

ATP regeneration in the platelet suspension was inhibited by 30 mM-2-deoxy-D-glucose (Merck, Darmstadt, Germany), an inhibitor of glycolysis, and 1 mM-KCN (Baker Chemicals), an inhibitor of mitochondrial ATP resynthesis, added separately or in combination. These metabolic inhibitors were added before, or at different times after, initiation of fibrinogen binding. In some experiments, 10 mM-glucono-δ-lactone (Sigma), an inhibitor of glycogenolysis, was included in the inhibitor mixture.
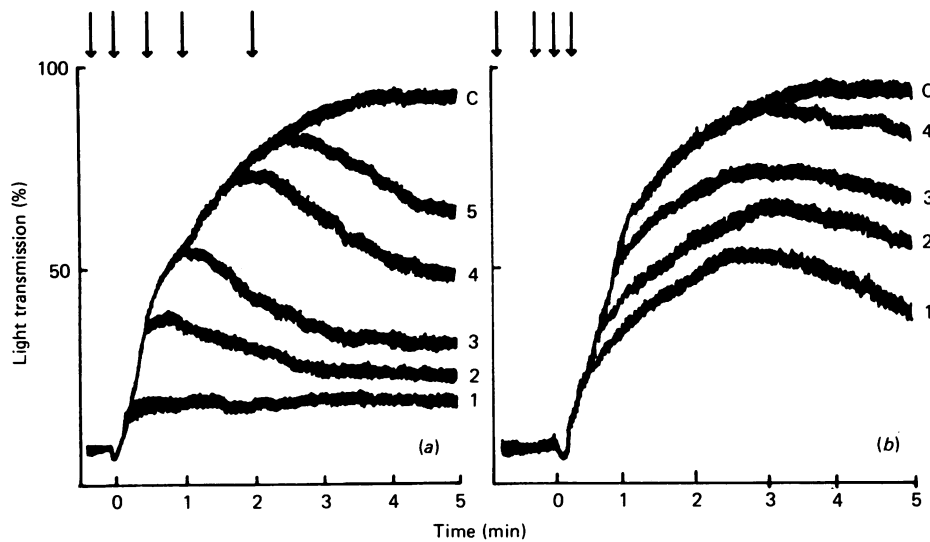
Platelet aggregation

The gel-filtered platelets were incubated in the presence of 500 nM-PAF-acether and 500 nM-fibrinogen and stirred at 900 rev./min. Aggregation was measured by two techniques: first, by monitoring the change in light transmission in a Payton (Buffalo, NY, U.S.A.) dual-channel aggregometer at 37 and 22 °C; second, by counting the disappearance of single platelets according to a modification of the method described by Frojmovic *et al.* (1983). For the last-mentioned procedure, samples were collected from the aggregating suspension and immediately mixed with 9 vol. of 0.5% glutaraldehyde (Fluka, Buchs, Switzerland) in 0.15 M-NaCl. Platelets were counted in a Platelet Analyzer 810 (Baker Instruments, Allentown, PA, U.S.A.) with the apertures set between 3.2 and 16 μm³. In an unstimulated suspension, 94±2% (mean±S.D.; *n* = 40) of total platelets fell within these settings.

RESULTS

Effect of metabolic inhibitors on PAF-acether-induced aggregation

As illustrated in Fig. 1(*a*), PAF-acether-induced aggregation was inhibited by addition of a mixture of deoxyglucose, gluconolactone and CN⁻, which is known to abolish ATP regeneration within 5 s (Akkerman *et al.*, 1983*a*). There was a lag time of about 30 s between addition of the inhibitors and the first deflection of the aggregation patterns, which is about the time required to decrease the platelet energy content to values where no further ATP hydrolysis is possible (Akkerman *et al.*, 1983*a*). Since aggregation is mediated via fibrinogen binding to sites that are exposed when the cells are activated, these findings suggested that the PAF-acether-induced fibrinogen binding to platelets was sensitive to metabolic blockade.

In order to evaluate the role of metabolic energy in fibrinogen binding in more detail, binding studies were performed with ^{125}I-labelled fibrinogen and PAF-acether-

Fig. 1. Effect of energy depletion on optical aggregation induced by PAF-acether

Platelets were stimulated with 500 nM-PAF-acether in the presence of 500 nM-fibrinogen at 37 °C (a) and 22 °C (b) at a stirring speed of 900 rev./min. At the times indicated by the arrows, a mixture of 2-deoxy-D-glucose, gluconolactone and CN^- was added at final concentrations of 30 mM, 10 mM and 1 mM respectively. Additions in (a) were at 20 s before stimulation (curve 1), and 1, 30, 60 and 120 s after stimulation (curves 2, 3, 4, 5 respectively). Additions in (b) were at 60 s and 20 s before stimulation (curves 1 and 2 respectively) and at 2 and 15 s after stimulation (curves 3 and 4 respectively). Curves indicated by 'C' are from uninhibited suspensions.

Table 1. PAF-acether-induced fibrinogen binding: effect of energy decrease before stimulation

Platelets were preincubated without and with 1 mM-CN^-, 30 mM-deoxyglucose (dGlc) or both for 30 min at 22 °C. At zero time, samples were taken for measurement of [^{14}C]ATP (expressed as a percentage of total ^{14}C radioactivity) and the AEC. Immediately thereafter, 500 nM-PAF-acether and 500 nM-^{125}I-fibrinogen were added. After another 40 min incubation the energy parameters were measured again and compared with the amount of specific fibrinogen binding (means ± s.D., $n = 6$). The addition of fibrinogen to unstimulated platelets did not affect the [^{14}C]ATP level or energy charge. The asterisk indicates a significant ($P < 0.05$) difference when compared with uninhibited suspensions.

| | Energy-parameter value at: | | | | |
| | Zero time | | 40 min | | Fibrinogen binding (40 min incubation) (% of control) |
Inhibitor	[^{14}C]ATP (% of total)	AEC	[^{14}C]ATP (% of total)	AEC	
None	80.7 ± 2.9	0.92 ± 0.02	76.6 ± 2.9	0.91 ± 0.01	(100)
CN^-	77.5 ± 3.5	0.91 ± 0.03	57.0 ± 5.0*	0.89 ± 0.02*	103 ± 10
dGlc	55.9 ± 4.1*	0.87 ± 0.04*	29.6 ± 1.2*	0.74 ± 0.06*	77 ± 15*
CN^- + dGlc	30.9 ± 3.6*	0.75 ± 0.03*	15.5 ± 0.9*	0.53 ± 0.03*	17 ± 6*

stimulated platelets. At 37 °C the amount of ^{125}I-fibrinogen that bound specifically to platelets was small and too low to study a possible further decrease by metabolic inhibitors (results not shown). An 8-fold increase in specific binding was obtained by lowering the temperature to 22 °C. At this temperature the metabolic inhibitors also interfered with optical aggregation, although the time before the inhibitors became effective increased to 1–3 min (Fig. 1b). This is consistent with the lower flux through the metabolic sequences at this temperature (Akkerman et al., 1983a). A further complication was that formation of large aggregates interfered with the binding studies. Hence, the effect of metabolic blockade on PAF-acether-induced fibrinogen binding was measured in unstirred suspensions kept at

22 °C. Although under these conditions no optical aggregation could be detected, a slight degree of single platelet disappearance occurred, amounting to 20 ± 4% at 10 min and 65 ± 3% after 1 h. Addition of inhibitors at 10 min incubation also inhibited this response, leading to 29 ± 2% single-platelet disappearance after 1 h.

PAF-acether-induced fibrinogen binding: effect of energy decrease before stimulation

Preincubation of platelets with 1 mM-CN^- for 30 min neither affected the level of [^{14}C]-ATP nor the AEC. After stimulation with PAF-acether, there was a slight decrease in both parameters, but concurrent binding of fibrinogen was unaffected (Table 1). In contrast, 30 mM-deoxyglucose decreased the energy parameters to
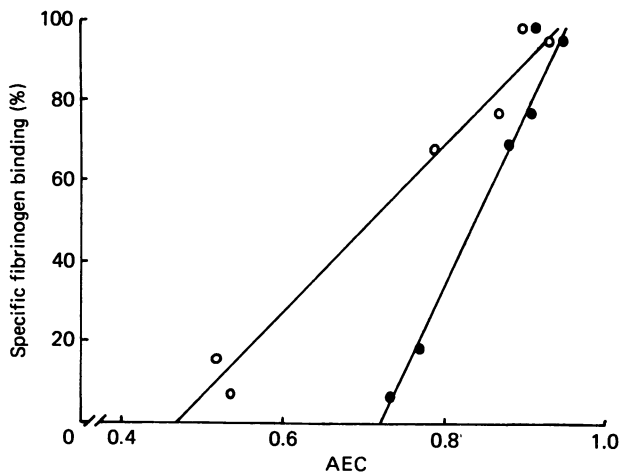
Fig. 2. Comparison between fibrinogen binding and AEC: effect of energy decrease before stimulation

Platelets were pretreated with 30 mM-2-deoxy-D-glucose and/or 1 mM-CN⁻ for 30 min before stimulation with 500 nM-PAF-acether (22 °C). ¹²⁵I-fibrinogen binding was measured 40 min later and compared with the AEC measured at the time of PAF-acether addition (●) and at the time of the fibrinogen measurement (○). Results are a representative example from five similar experiments.

a much greater extent before, as well as during, incubation with PAF-acether. These changes were accompanied by a 23% decrease in fibrinogen binding. The combined treatment with CN⁻ and deoxyglucose induced an even steeper decrease in the level of [¹⁴C]ATP and AEC before the cells were stimulated. At 40 min thereafter, these values had further declined to 20% and 55% of initial values respectively. This was accompanied by a more-than-80% decrease in fibrinogen binding. These data indicate that, for optimal fibrinogen binding, an optimal energy content is required when the cells are stimulated and during subsequent platelet–fibrinogen interaction. Fig. 2 shows the relationship between the fibrinogen binding at 40 min after stimulation and the AEC at the time when PAF-acether was added and 40 min thereafter. At both incubation times a linear correlation was obtained between fibrinogen binding and the AEC. The coupling between both parameters was not constant, indicating that other factors affected this relationship. Similar findings were obtained when the

level of [¹⁴C]ATP was compared with fibrinogen binding (results not shown).

PAF-acether-induced fibrinogen binding: effect of energy decrease after stimulation

As listed in Table 2, the addition of CN⁻, deoxyglucose or a combination of both inhibitors added 10 min after stimulation with PAF-acether induced effects similar to those observed when the inhibitors were added before stimulation of the platelets. Again, CN⁻ alone did not change fibrinogen binding at 60 min after stimulation, although it induced a slight decrease in energy parameters. Deoxyglucose showed a stronger effect, leading to a 50% decrease in fibrinogen binding after 60 min, which was accompanied by a fall in [¹⁴C]ATP and AEC of 55 and 15% respectively. The greatest inhibition was found when CN⁻ and deoxyglucose were added simultaneously: this decreased fibrinogen binding by 70%, with a concomitant decrease of 75% in [¹⁴C]ATP and a fall in the AEC from 0.94 to 0.57. Thus, when platelets were left undisturbed during stimulation with PAF-acether, subsequent fibrinogen binding was sensitive to metabolic blockade.

Fig. 3 illustrates the relation between fibrinogen binding and platelet energy content at various stages after stimulation with 500 nM-PAF-acether. Inhibition of ATP resynthesis by the CN⁻/deoxyglucose mixture induced a sharp fall in energy content to values of about 20% of those of uninhibited suspensions and thereafter no further ATP hydrolysis could be detected. These patterns were similar at various stages after stimulation with PAF-acether. In contrast, the effect of the inhibitors on fibrinogen binding greatly depended on the time of PAF-acether–platelet interaction. Addition of inhibitors at 2 s after stimulation inhibited binding by about 75%. The time during which the remaining binding took place fell within the period in which ATP hydrolysis was still detectable, suggesting that fibrinogen binding depended on simultaneous ATP hydrolysis. When the inhibitors were added at 10 min after stimulation, binding was again inhibited considerably, whereas metabolic blockade at a stage where fibrinogen binding had reached equilibrium induced a slight dissociation. The degree of inhibition varied between platelets from different donors, showing an average of 47±18% (n = 6) after 60 min incubation with PAF-acether and 50 min incubation with metabolic inhibitors. Hence these data suggest that initial binding of fibrinogen depends on concurrent

Table 2. PAF-acether-induced fibrinogen binding: effect of energy decrease after stimulation

Platelets were incubated with 500 nM-PAF-acether in the presence of 500 nM-¹²⁵I-fibrinogen (22 °C). After 10 min, 1 mM-CN⁻ and 30 mM-deoxyglucose (dGlc) were added (final concentrations). After another 50 min incubation, energy parameters {[¹⁴C]ATP (% of total ¹⁴C radioactivity) and AEC} were measured and compared with the amount of specific fibrinogen binding (means±S.D., n = 5). The asterisk indicates a significant (P < 0.05) difference compared with uninhibited suspensions.

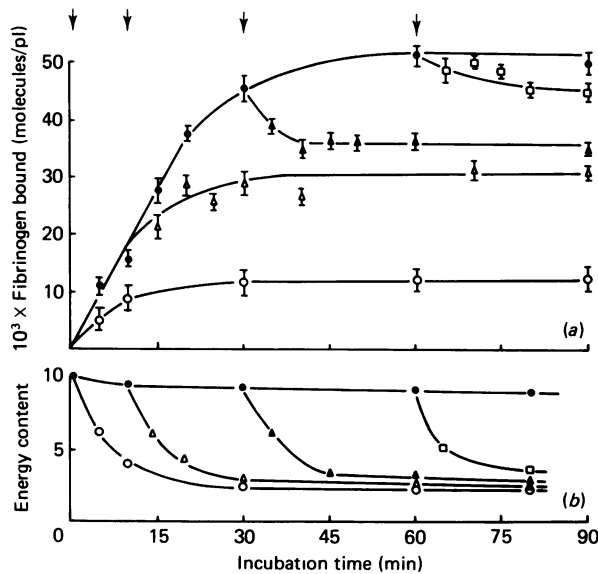| Inhibitor	[¹⁴C]ATP at 60 min (% of total)	AEC at 60 min	Fibrinogen binding at 60 min (% of control)
None	82.4±2.8	0.94±0.02	(100)
CN⁻	67.4±4.7*	0.92±0.02*	98±9
dGlc	35.1±3.0*	0.80±0.01*	51±4*
CN⁻+dGlc	15.8±2.7*	0.57±0.04*	31±7*

Fig. 3. Comparison between fibrinogen binding and energy content (μmol of $ATP_{eq.}/10^{11}$ platelets): effect of energy decrease after stimulation

Platelets were incubated with 500 nM-PAF-acether and 500 nM-^{125}I-fibrinogen (22 °C). Simultaneously with PAF-acether, or 10, 30 and 60 min thereafter, a mixture of 30 mM-2-deoxyglucose and 1 mM-CN$^-$ (final concentrations) was added as indicated by the arrows (a). In a parallel set of incubations the energy content was measured on the basis of determination of [^{14}C]adenine-labelled adenine nucleotides (b). Fibrinogen binding values are given as means ± s.D., $n = 3$. Results are a representative example from six similar experiments.

energy support. At later stages the correlation with energy weakens and at equilibrium most of the binding is irreversible and not affected by metabolic blockade. In none of these conditions did the inhibitors induce considerable dissociation of bound fibrinogen, suggesting that, once fibrinogen is coupled to the exposed sites, further energy support is not required.

Energy metabolism during PAF-acether stimulation under binding conditions

Normal unstimulated platelets preserved their energy content during 60 min incubation at room temperature (Fig. 4). Similarly, PAF-acether-stimulated platelets maintained a stable energy content, indicating complete ATP homoeostasis. The addition of the CN$^-$/deoxyglucose mixture at 10 min after stimulation induced a fall in energy content in concert with the data shown in Fig. 3. When the inhibitors were added to the stimulated platelets, this fall was slightly steeper than in unstimulated cells, resulting after 60 min in a [^{14}C]ATP level of 12.7 ± 0.5% compared with 18.0 ± 1% of total ^{14}C radioactivity in the unstimulated cells. These data indicate that the concurrent PAF-acether-induced responses increased the demand for metabolic energy. The difference between both patterns was small, however, and indicative of only a minor increase in energy need. These patterns were similar in both the absence and presence of 500 nM-fibrinogen. The successive addition of PAF-acether (500 nM) and inhibitors (10 min later)
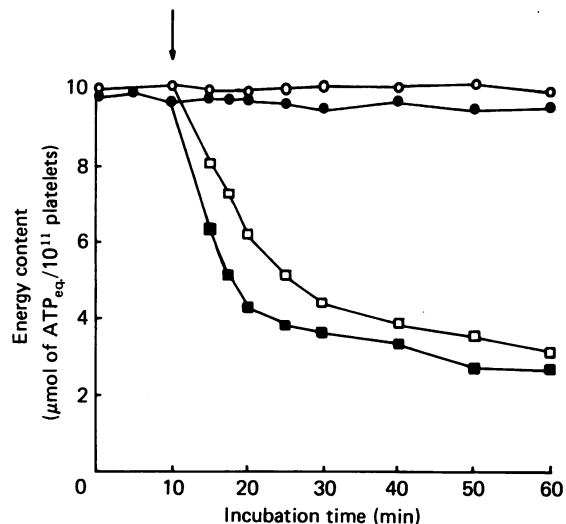

Fig. 4. Energy content in unstimulated platelets and platelets treated with PAF-acether

Platelets were incubated with (●, ■) and without (○, □) 500 nM-PAF-acether. After 10 min a mixture of 30 mM-2-deoxyglucose and 1 mM-CN$^-$ (final concentrations) was added and the energy content was determined in uninhibited suspensions (○, ●) and after addition (arrow) of the inhibitors (□, ■). Results are a representative example from three similar experiments.

resulted in a [^{14}C]ATP level of 12.7 ± 0.5 and 12.1 ± 0.6% of total ^{14}C radioactivity ($n = 4$; $P > 0.05$) after 60 min incubation without and with fibrinogen respectively. Hence, in terms of energy requirement, there was not much difference whether or not fibrinogen bound to the exposed binding sites.

DISCUSSION

The present study reveals a major role for metabolic energy in the binding of fibrinogen to platelets stimulated with PAF-acether, which is in accordance with previous studies on ADP-induced binding (Peerschke & Zucker, 1981). When the energy content is lowered by treatment with metabolic inhibitors, a sharp decrease in specific fibrinogen binding is found after subsequent stimulation. The decrease in binding is proportional to the decrease in energy parameters induced with separate inhibitors or combinations, making it unlikely that the inhibitors affect binding by mechanisms other than those lowering the cells' energy content. An exception is the glycogenolytic inhibitor gluconolactone, which decreased binding without affecting [^{14}C]ATP or energy charge. An explanation lies in the instability of gluconolactone at neutral pH (Akkerman et al., 1983a), where it rapidly hydrolyses, leading to a pH change from 7.3 to 6.8 in the course of the binding studies (40–60 min). Fibrinogen binding is extremely sensitive to pH (Marguerie & Plow, 1981), in contrast with optical aggregation (5 min incubations), which was not affected by this property (results not shown). Hence, subsequent studies were done without gluconolactone, thereby leaving glycogenolytic ATP resynthesis partly intact (Holmsen & Akkerman, 1980). Both the level of

[^{14}C]ATP and the AEC correlated linearly with fibrinogen binding, suggesting that metabolic energy and fibrinogen binding are closely coupled. The relation between binding measured after 40 min stimulation and the energetic parameters measured either at the same time or at the time when PAF-acether was added was different, indicating that, during binding, other factors affected this relationship. A better comparison can be made when binding and energy metabolism are measured simultaneously immediately after addition of PAF-acether. Under these conditions, however, binding was small and could not be measured accurately in the presence of metabolic inhibitors.

In order to gain more insight in the role of energy at different stages during fibrinogen binding, platelets were left undisturbed during stimulation and subsequently treated with a combination of CN$^-$ and deoxyglucose. Fibrinogen binding was sensitive to metabolic inhibitors at any stage during initial binding until equilibrium was reached. There was a lag time of 20–30 min between addition of the inhibitors and arrest of binding, which fell within the period during which the energy content declined to a range where no further ATP hydrolysis took place. Studies at 37 °C with a mixture that included the glycogenolytic inhibitor gluconolactone (Akkerman et al., 1983a; Verhoeven et al., 1984a,b), have shown that the fall in energy content after complete arrest of ATP resynthesis reflects the energy consumption of concurrent platelet responses in addition to the energy requirement of basic processes already apparent in the unstimulated cell. At 22 °C this fall is considerably slower, but the coupling with functional responses is maintained (Akkerman et al., 1983a). Our present findings suggest a similar coupling between fibrinogen binding and concurrent ATP hydrolysis. However, the fact that gluconolactone had to be excluded from the inhibitor mixture left glycogenolytic energy generation intact, making quantitative assessment of energy consumption impossible. Despite this uncertainty, there are indications that the energy requirement of fibrinogen binding is minor. Uninhibited platelets maintain complete ATP homoeostasis during stimulation with PAF-acether, which contrasts with the fall in metabolic ATP seen when responses that require much energy are carried out (Akkerman & Holmsen, 1981). Furthermore, the fall in metabolic ATP after addition of inhibitors is only slightly steeper in stimulated cells than in control suspensions. Since part of this difference is probably related to a slight secretion (less than 10% of maximal) that takes place even under the conditions of binding experiments (Kloprogge & Akkerman, 1985), the actual involvement of energy in fibrinogen binding is probably minor.

The slight but significantly higher energy need in the presence of PAF-acether may reflect a crucial property of PAF-acether-induced fibrinogen binding. As reported in preliminary form (Kloprogge & Akkerman, 1985), a combination of ADP scavengers and a thromboxane synthase inhibitor completely inhibits PAF-acether-induced fibrinogen binding, suggesting that secretion of ADP and formation of thromboxane A$_2$ are crucial steps in the exposure of binding sites. The extent to which each mediator contributes to binding-site exposure differs between platelets from different donors. This property, together with the fact that secretion requires more energy than does prostanoid synthesis (Verhoeven et al., 1986;

Rittenhouse-Simmons & Deykin, 1977), may explain why binding in some platelets is more susceptible to metabolic inhibitors than in others.

Our findings indicate a role for metabolic energy at various stages during platelet–fibrinogen interaction. First, energy is required during stimulation of the cells, as illustrated by the decreased binding after pretreatment with metabolic inhibitors. This accords with the concept that fibrinogen binding is the final result of PAF-acether binding to its receptors, generation of second messengers and exposure of binding sites. Control studies show that the binding of [^{3}H]PAF-acether is not affected by metabolic inhibitors (E. Kloprogge, unpublished work), but alterations in phosphatidylinositol metabolites and Ca^{2+} translocations are among the many steps in signal processing known to require ATP hydrolysis. In addition, exposure of binding sites, located at the glycoprotein IIb–IIIa complex, probably involves alterations in the platelet cytoskeleton (Cox et al., 1984; Tuszynski et al., 1984; Wheeler et al., 1984), microfilament polymerization (Peerschke, 1984) and rearrangements in the membrane bilayer (Peerschke, 1982; Loftus & Albrecht, 1984), which are known to depend on metabolic energy (Bourguignon, 1984). Hence, pretreatment with metabolic inhibitors probably interferes with signal processing and the mechanism by which binding sites are exposed. Second, energy is required after the binding sites have been exposed. This explains the sensitivity of optical aggregation to sudden ATP depletion (Fig. 1) and the strong correlation between AEC and aggregation velocity in platelets with diminished ATP turnover (Akkerman et al., 1983b). Under the conditions of our binding studies (22 °C, 500 nM-PAF-acether, 500 nM-fibrinogen), binding-site exposure is completed within 2–5 min (Kloprogge et al., 1986). Although addition of inhibitors during this period (Fig. 3) may interfere with the process of binding-site exposure, an effect at later stages is unexpected. A possible explanation lies in the fact that, in the absence of fibrinogen, exposed sites gradually disappear, suggesting a conversion of an accessible into an inaccessible state (Kloprogge et al., 1986). Preliminary studies show that this disappearance is enhanced by metabolic inhibitors, suggesting that energy is required to preserve the exposed state. Since with maximal stimulation with PAF-acether the actual binding of fibrinogen is much slower than the exposure of binding sites, exposed but unoccupied sites may disappear faster when ATP supply is diminished, leading to decreased fibrinogen binding at later stages of the incubation.

Finally, the binding of fibrinogen to the exposed sites appears not to require energy, since the changes in energy content in stimulated platelets are the same whether fibrinogen is present or not. In addition, the maintenance of the fibrinogen-binding-site complex appears energy-independent, since metabolic inhibition fails to induce the considerable dissociation of bound fibrinogen that can be achieved by excess unlabelled fibrinogen or agents that raise the cyclic AMP content (Graber & Hawiger, 1982). Thus metabolic energy appears to be predominantly required for mechanisms that regulate the exposure of the binding sites.

REFERENCES

Akkerman, J. W. N. & Holmsen, H. (1981) Blood **57**, 956–966

Akkerman, J. W. N., Gorter, G., Schrama, L. & Holmsen, H. (1983a) Biochem. J. **210**, 145–155

Akkerman, J. W. N., Gorter, G. & Holmsen, H. (1983b) Biochim. Biophys. Acta **760**, 34–41

Atkinson, D. E. (1977) Cellular Energy Metabolism and Its Regulation, pp. 88–107, Academic Press, New York

Bourguignon, L. Y. W. (1984) Cell Biol. Int. Rep. **8**, 19–26

Cejka, J. (1982) Clin. Chem. **28**, 1356–1358

Cox, A. C., Carroll, R. C., White, J. G. & Rao, G. H. R. (1984) J. Cell. Biol. **98**, 8–15

Daniel, J. L., Molish, I. R. & Holmsen, H. (1980) Biochim. Biophys. Acta **632**, 444–453

Engvall, E., Ruoshlathi, E. & Miller, E. J. (1978) J. Exp. Med. **174**, 1584–1595

Frojmovic, M. M., Milton, J. G. & Duchastel, A. (1983) J. Lab. Clin. Med. **101**, 964–976

Graber, S. E. & Hawiger, J. (1982) J. Biol. Chem. **257**, 14606–14609

Holmsen, H. & Akkerman, J. W. N. (1980) in The Regulation of Coagulation (Mann, K. G. & Taylor, F. B., eds.), pp. 409–417, Elsevier/North-Holland, New York

Kloprogge, E. & Akkerman, J. W. N. (1984) Biochem. J. **223**, 901–909

Kloprogge, E. & Akkerman, J. W. N. (1985) Thromb. Haemostasis **54**, 220

Kloprogge, E., Mommersteeg, M. E. & Akkerman, J. W. N. (1986) J. Biol. Chem., in the press

Loftus, J. C. & Albrecht, M. (1984) J. Cell. Biol. **99**, 822–829

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275

Marguerie, G. A. & Plow, E. F. (1981) Biochemistry **20**, 1074–1080

Nieuwenhuizen, W., Emeis, J. J., Vermond, A., Kurven, P. & van der Heide, D. (1980) Biochem. Biophys. Res. Commun. **97**, 49–55

Niewiarowski, S., Kornecki, E., Budzynski, A. Z., Morinelli, T. A. & Tuszynsky, G. P. (1983) Ann. N.Y. Acad. Sci. **408**, 536–555

Peerschke, E. I. B. (1982) Blood **60**, 973–978

Peerschke, E. I. B. (1984) Proc. Soc. Exp. Biol. Med. **175**, 109–115

Peerschke, E. I. B. & Zucker, M. B. (1981) Blood **57**, 663–669

Rittenhouse-Simmons, S. & Deykin, D. (1977) J. Clin. Invest. **60**, 495–498

Tangen, O., Berman, H. J. & Marfey, P. (1971) Thromb. Diath. Haemorrh. **25**, 268–278

Tuszynski, G. P., Kornecki, E., Cierniewski, C., Knight, L. C., Hoshy, A., Srivasta, S., Niewiarowski, S. & Walsh, P. N. (1984) J. Biol. Chem. **259**, 5247–5254

Verhoeven, A. J. M., Mommersteeg, M. E. & Akkerman, J. W. H. (1984a) Biochim. Biophys. Acta **800**, 242–250

Verhoeven, A. J. M., Mommersteeg, M. E. & Akkerman, J. W. N. (1984b) Biochem. J. **221**, 777–787

Verhoeven, A. J. M., Gorter, G., Mommersteeg, M. E. & Akkerman, J. W. N. (1985) Biochem. J. **228**, 451–462

Verhoeven, A. J. M., Mommersteeg, M. E. & Akkerman, J. W. N. (1986) Biochem. J. **236**, 879–887

Walsh, P. N. (1972) Br. J. Haematol. **22**, 205–217

Wheeler, M. E., Cox, A. C. & Carroll, R. C. (1984) J. Clin. Invest. **74**, 1080–1089