Conformational responses of an arachidonate- and U46619-binding haemoprotein in relation to platelet activation

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Antibodies were raised, in rabbits, against an arachidonate- and U46619-binding protein purified from calf platelets. Spectral measurements and immunodiffusion experiments were employed to follow conformational responses of the protein in relation to platelet activation. Upon treatment with the platelet agonists, arachidonate and PGH_2, as well as the common haem ligands, imidazole and CN^-, the purified protein had its Soret band red-shifted, with hypochromicity, but the protein saturated with the agonists, not with the haem ligands, showed altered antigenic properties in immunodiffusion experiments. In an analogous manner activation of gel-filtered calf platelets with high concentrations of ADP and A23187, as well as by cold, had Soret bands of extracts of sonicated platelets red-shifted, with hypochromicity; concomitantly, antigenically different conformations of the protein appeared in Triton X-100 extracts of the activated platelets. A protein immunologically related to the platelet protein was detected in Triton X-100 extracts of calf neutrophils. It is suggested that conformational changes of the protein induced by arachidonate or prostaglandin endoperoxides or H_2O_2 formed in different compartments during platelet activation by different stimuli may be a biochemical mechanism of stimulus-response coupling and that similar mechanisms might operate in other cell types.

Platelet activation; Hemoprotein; Antibody; Conformational change; ADP; Ionophore A23187; Cold activation

1. INTRODUCTION

Blood platelets can be activated to varying degrees and stages by numerous stimuli, depending on their nature, dose, and duration of action. Some stimuli such as physiological ADP and thrombin act through their respective, specific, cell-surface receptors [1,2]. In contrast, some other stimuli such as the synthetic calcium ionophore, A23187, or physical agents like prosthetic surfaces [3,4] or low temperatures ([5] and references

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* This work will form part of a PhD dissertation

therein) activate independently of specific cell-surface receptors.

The mechanisms of stimulus-response coupling in platelets are not well understood at present [6,7]. However, a funnel-like channelling of different biochemical effects of diverse stimuli into a few response pathways appears to be discernible [6,8,9].

Nothing is known about the mechanisms of channelling biochemical signals towards response.

Recently we reported the purification, from calf platelets, of a haemoprotein that could bind both arachidonate and the PGH_2/thromboxane A_2 (TxA₂) analogue, U-46619 [10]. The half-saturation concentration of U-46619 was in the range that induces platelet aggregation [11,12]. This lent credence to the view [10] that the protein

could be a prostaglandin endoperoxide receptor. Consistent with this view and reports by others [11,13] that U-46619 can function as a platelet agonist independently of a TxA_2 receptor we have found that pinane-TxA_2, a specific TxA_2 receptor antagonist [14], is devoid of significant binding interaction with the haemoprotein and fails to inhibit U-46619-induced activation of gel-filtered calf platelets (unpublished). To define further the role of the protein in platelet activation we have now raised antibodies against it in rabbits. The results of spectral measurements and immunodiffusion experiments, reported here, suggest that conformational changes of the protein might form a mechanism of channelling biochemical signals of activation of calf platelets by ADP, A23187, and low temperatures.

2. MATERIALS AND METHODS

2.1. Materials

All unsaturated fatty acids, agar, Ficoll (type 400), imidazole, sodium diatrizoate (Hypaque), Triton X-100, and Tris base were from Sigma (USA). H_2O_2 was a product of Glaxo Laboratories, India. Its concentration in experimental systems was estimated spectrophotometrically using E_M^{240} 40 $M^{-1} \cdot cm^{-1}$ at pH 7.4. PGH_2 was prepared as in [15] but employing microsomes prepared from calf platelets. All other chemicals were of the highest purity available commercially. Deionized double quartz-distilled water was employed. All buffer solutions were filtered through a Millipore filter (0.45 μm). All operations were carried out at 30 $\pm$ 1°C (ambient temperature) unless otherwise stated. Collection, storage and centrifugation of blood and platelet preparations were in polypropylene tubes. Pipetting of platelet preparations was done using automatic pipettes and polypropylene tips.

2.2. Preparation of gel-filtered platelets (GFP)

Calf (male) blood collected by jugular venic puncture into 1/10 vol. acid-citrate-dextrose solution was centrifuged at 86 $\times$ g (r_{av} 7.7 cm) for 15 min. The platelet-rich plasma (PRP) was recovered and centrifuged again at 24 $\times$ g (r_{av} 8.5 cm) for 20 min to remove residual erythrocytes and leukocytes. GFP were obtained according to Tangen and Berman [16] using their noncolloidal

buffer, at pH 7.4. Only those preparations in which at least 90% appeared as single platelets, under the microscope, were used for studying the spectral and immunological effects upon activation.

2.3. Purification of calf neutrophils

The buffy coat obtained after removing PRP (section 2.2) was diluted 10 times with 0.13 M NaCl. Lymphocytes were removed from the suspension by the method of Boyum [17] and erythrocytes by the method of Boyle [18] except for the removal of debri of lysed red cells which was achieved by centrifugation at 3.8 $\times$ g (r_{av} 8.5 cm) for 10 min. The neutrophil preparations were washed 3 times with 0.12 M NaCl by centrifugation at 24 $\times$ g (r_{av} 8.5 cm) for 15 min. The purity of the final preparation was checked by examining its smear stained with Lieshman's stain under the microscope.

2.4. Purification of protein

The PRP obtained as in section 2.2 from 500 ml blood was centrifuged again at 345 $\times$ g (r_{av} 7.7 cm) for 15 min and the platelet-poor plasma (PPP) and platelet button were recovered separately. Platelets were reconstituted with 30 ml PPP and gel-filtered as in section 2.2, loading 10 ml at a time. GFP were made up to 100 ml and broken ultrasonically at 20°C, in 20 ml batches [10]. Broken platelets were centrifuged at 54000 $\times$ g (r_{av} 7.7 cm) for 30 min at 4°C and the supernatants (crude extracts) were processed as in [10] up to Sephadex G-200 column chromatography. Fractions having A_{405} > 0.05 were then loaded onto a DEAE-Sephadex A-50 column, in the order in which they emerged from the Sephadex G-200 column, and eluted as in [10], in a cold room (4°C).

2.5. Immunological techniques

Antibodies against the purified protein were raised in rabbits as in [19]. The purified haemoprotein (150 μg) emulsified in Freund's adjuvant was used for primary injection. Two booster injections of 100 μg protein each time were given on the 15th and 28th days. The antiserum collected on the 35th day was clarified by centrifugation at 54000 $\times$ g (r_{av} 7.7 cm) and stored at −20°C in small aliquots.

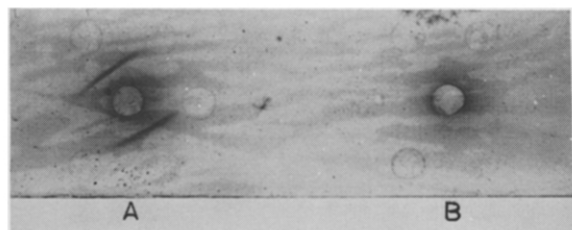Ouchterlony immunodiffusion experiments were carried out in agar gels as described [20], and
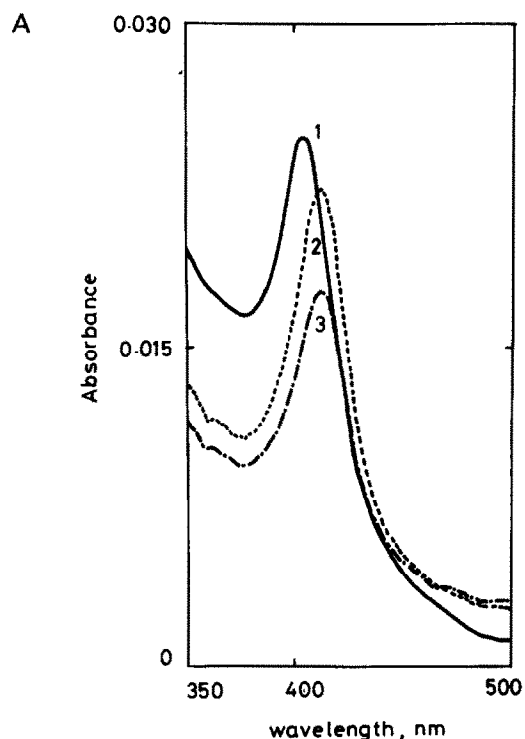
Fig.1. Results of Ouchterlony immunodiffusion experiments using rabbit antiserum (A) and normal rabbit serum (B). The antiserum (50 μl, 1.2 mg protein) taken in the central well was allowed to react with 1.8 μg antigen in the peripheral wells. Other procedures were as given in the text.
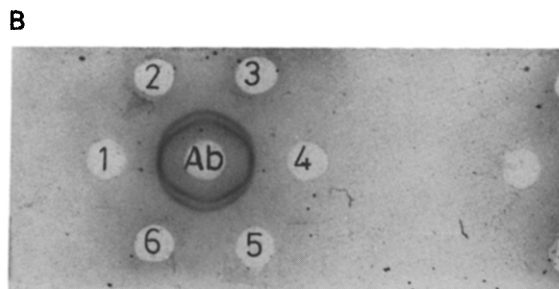
precipitin lines were allowed to develop in a cold room (4°C) for 48 h. The gels were stained and destained according to [20]. The stained precipitin lines were photographed.

3. RESULTS

The modified purification procedure yielded sufficient pure haemoprotein to raise antibodies against it, in rabbits. Immunodiffusion, in agar gels, showed the formation of a single precipitin line (fig.1A) when rabbit antiserum was tested against the purified protein, in contrast to the absence of any precipitin reaction with normal rabbit serum (fig.1B).

We reported [10] a batch-to-batch variability of the Soret band maximum of crude extracts of the protein. We suspected this to be due to varying degrees of activation of the platelets in vivo, or during handling, and the consequent binding of low-M_r ligands to the protein because binding of platelet-stimulatory ligands to the purified protein has been found to bring about such spectral effects [10]. To test this possibility, GFP, of which the Soret band maximum of crude extracts had previously been determined to occur at 405 nm, were

Fig.2. (A) Spectral properties of crude extracts of gel-filtered calf platelets before and after activation. GFP (2 ml, 6 × 10^8 platelets/ml) were activated with ADP (10 μM) or A23187 (5 nM) for 10 min at 32°C. Crude extracts of the platelets were prepared as described in the text and their absorption spectra recorded at 25°C employing a Shimadzu UV-240 recording spectrophotometer using the gel-filtration column buffer as reference. The spectra shown are those of crude extracts of control platelets (1), ADP-activated platelets (2), and A23187-activated platelets (3). (B) Typical immunodiffusion patterns of Triton X-100 extracts of activated calf platelets. GFP (1 ml, 6 × 10^8 platelets) were activated by lowering the temperature (1 h in melting ice), ADP and A23187 (as in A). The activated platelets were treated with Triton X-100 added to a final concentration of 1% (v/v) and mixed. The Triton X-100 extracts (30 μl) were then taken in the peripheral wells 2, 5 and 6, respectively. Similarly prepared Triton X-100 extracts of control platelets and platelets rewarmed at 32°C for 1 h after cold activation were taken in wells 1 and 2, respectively. The purified protein (2.2 μg) was taken in well 4. The antibody was present in the central well. Other details were as described in the text.

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activated with ADP (10 μM) and A23187 (5 nM), as being representative of stimuli acting through cell-surface receptor-dependent and -independent mechanisms. These agents by themselves did not affect the Soret band of the purified protein. The Soret bands of crude extracts of the activated platelets were found to be red-shifted with hypo-

chromicity compared to those of the control platelets (fig.2A). Similar but milder red shifts occurred with platelets activated by cooling at 0–4°C for 1 h (not shown).

Immunodiffusion experiments showed two precipitin lines when Triton X-100 extracts of the activated platelets were used whereas only one line could be detected in extracts of control platelets (fig.2B). The double precipitin lines did not disappear on warming the cold-activated platelets, for 1 h, at ambient temperature (figs 2B,3).

Spectral and immunological methods were also employed to test whether similar conformational changes could be induced by ligand binding to the purified protein.

The saturation of the protein by different ligands was followed by difference spectral methods [10] and the protein treated with a saturating concentration of each ligand (fig.3) was taken in peripheral wells, alternated with the purified protein, in immunodiffusion experiments. The results (fig.4A) showed that when arachidonate, or 5,8,11-eicosatrienoic acid, or H_2O_2 was bound only faint precipitin lines were formed which contrasted with those formed with the authentic protein in shape and position. In the case of PGH_2 only a slight shift in position of the precipitin line towards the peripheral well occurred (figs 2,4B). The protein was, apparently, not saturated with PGH_2 as may be judged from fig.3B because of limitations of the volume of the solvent
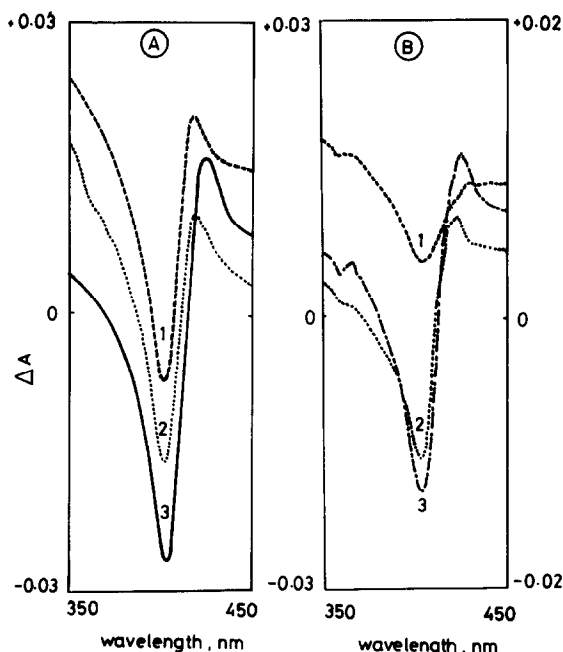

Fig.3. Difference spectral patterns obtained on saturating the calf platelet haemoprotein with various ligands. The purified protein sample (1 ml, 45 μg protein, $A_{405} = 0.088$) was taken in each of a pair of matched spectrophotometric cuvettes (path length 10 mm) and placed in the sample and reference positions of a Shimadzu UV-240 recording spectrophotometer provided with a thermostatted cuvette holder, maintained at 25°C. The baseline was corrected automatically. Increasing concentrations of each ligand (1 μl volume increments) were added to the sample cuvette and an equal volume of the diluent to the reference cuvette. Difference spectra were recorded 2 min after each addition and mixing, until no further spectral change was detected. The spectra shown in A are those obtained with 33.8 μM 5,8,11-eicosatrienoic acid (1), 39 μM arachidonic acid (2), added as their ethanolic solutions, and 25 M NaCN (aqueous solution adjusted to pH 7.4 with dilute HCl) (3). Spectra in B are those obtained with 6 μl of an acetone solution of PGH_2 (~5 μM) (1), 8.5 mM imidazole (aqueous solution, adjusted to pH 7.4) (2), and 56 μM H_2O_2 (3).
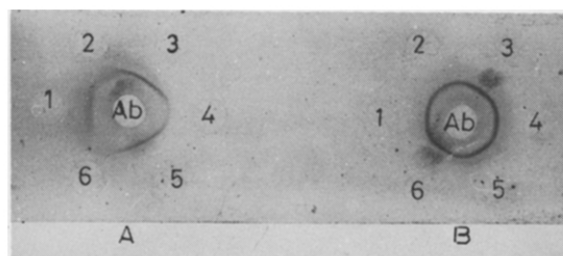

Fig.4. Immunodiffusion patterns obtained using protein saturated with various ligands as antigen. The protein samples (30 μl) from the sample cuvettes of experiments in fig.3 were taken in peripheral wells alternated with the same volume of the contents of their respective reference cuvettes. Wells 2, 4 and 6 contained protein saturated with H_2O_2, arachidonic acid and 5,8,11-eicosatrienoic acid, respectively in A, and PGH_2, imidazole and CN^-, respectively, in B. Other details were as given in the text.
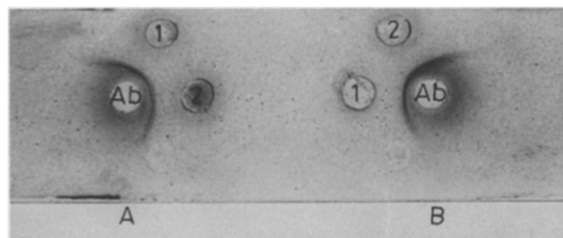
Fig.5. Immunological detection of a protein antigenically related to the platelet protein, in calf neutrophils. (A) Purified calf platelet protein (2 μg) (1) and Triton X-100 extract (30 μl) of calf neutrophils (2) prepared as in the text. (B) Triton X-100 extract of calf platelets prepared as described in the text (1) and Triton X-100 extract of calf neutrophils (2).

(acetone) that could be added to the protein and instability of the compound.

The precipitin lines formed in the presence of saturating concentrations of CN^- and imidazole seemed indistinguishable from those of the authentic protein (fig.4B).

Arachidonate can activate platelets without having recourse to metabolic conversion to prostaglandin endoperoxides and thromboxane A_2 ([21]; unpublished). Arachidonate and other fatty acids also activate neutrophils [22]. These observations prompted us to examine whether calf neutrophils possessed a similar protein and the results (fig.5) showed the presence of an antigenically related protein in Triton X-100 extracts of calf neutrophils.

4. DISCUSSION

Pooling of protein fractions eluted from the Sephadex G-200 column, $(NH_4)_2SO_4$ precipitation and the subsequent dialysis employed in the previous method [10] resulted in haem loss and conformational heterogeneity of the protein making it unsuitable for raising antibodies. This was rectified in the present method of loading the peak fractions of the Sephadex G-200 column to the DEAE-Sephadex A-50 column, in the order in which they emerged, without mixing. This brought forth peak fractions from the DEAE-Sephadex column each of which was more concentrated haemoprotein than the whole protein recovered in the original method.

Crude extracts of platelets that had not been activated had their Soret band centred on 405 nm (fig.2A) as had the purified protein [10]. The binding of low-M_r physiological agonists like arachidonate, PGH_2 and H_2O_2 as well as common haem ligands such as imidazole or CN^- induced a red shift of the Soret band of the purified protein with hypochromicity. However, binding of the former group of compounds alone could change the conformation of the protein to form precipitin lines closer to the antigen wells in immunodiffusion experiments (fig.4). The location of the precipitin lines indicated an increase in the apparent M_r of the antigen. Arachidonate is known to oligomerize the protein [10]. The other agonists might also do so.

H_2O_2 is a platelet agonist which seems to play a role in the aggregation of platelets by certain other agonists [23], probably by acting as an endoperoxide analogue.

When gel-filtered platelets were activated by ADP, A23187 or by chilling the Soret bands of their crude extracts were red-shifted with hypochromicity relative to those of the control platelets, mimicking the effect of binding of low-M_r ligands to the protein. Furthermore, Triton X-100 extracts of activated platelets showed the appearance of two precipitin lines, one of which was closer to the antigen wells, as in the case of ligand-treated protein.

But intensity differences were apparent between precipitin lines of activated platelets (fig.2B) and ligand-saturated protein (fig.4A). A plausible explanation for this was discerned from the difference spectra of saturation of the protein by increasing concentrations of the ligands. These spectra generally lacked isosbestic points (not shown) indicating formation of more than one type of complex. The immunological properties of variously saturated protein might then differ as exemplified by the precipitin line formed in the case of PGH_2 (fig.2,4B) and might form the basis for the intensity differences of precipitin lines.

An explanation for the presence of two precipitin lines after platelet activation (fig.2B) could be that agonist ligands are liberated or formed in response to specific stimuli at specific platelet compartments and the protein at the site of agonist availability only might respond.

The implied role of this protein in cold-induced

activation of platelets is intriguing. Enzymatic reactions undoubtedly occur during cold activation as evidenced by the phosphorylation of myosin light chain [5,24], a 47 kDa protein, and a 36 kDa protein of unknown function [5]. Arachidonate liberation could also occur since phospholipase A_2 might act at low temperatures (e.g. [25]). But metabolic conversion of arachidonate did not occur as we could not detect any significant difference in malondialdehyde formation by the thiobarbituric acid reaction [26,27] between cold-activated and control platelets (unpublished). Three possible explanations can be offered to account for these results: (i) cooling strengthened the interaction of the prostaglandin endoperoxides with the protein without a change in their levels; (ii) covalent modification is known to increase the sensitivity of biological response reactions [28,29] and the endoperoxides, at the same level, might have triggered protein conformational change in platelets which have undergone cold-induced protein phosphorylation (assuming priority of protein phosphorylation); and (iii) arachidonate which might be released during cold activation could bind directly to the protein triggering the activation cascade.

The third possibility is more interesting for the following reasons. Arachidonate does indeed activate platelets without having recourse to metabolic conversion ([21]; and unpublished). Initial rate kinetics of arachidonate-induced aggregation of gel-filtered calf platelets exhibited apparent positive cooperativity with $h = 3.8 \pm 0.3$ (mean $\pm$ SE, $n = 9$; unpublished). The binding of arachidonate to the purified protein also showed apparent positive cooperativity [10] and its half-saturation concentration was in the range required to induce aggregation of gel-filtered platelets (unpublished). Furthermore, arachidonate binding to the protein is accompanied by its irreversible modification ([10]; and unpublished) and the immunologically detected conformational change following cold activation was not reversed by rewarming the activated platelets (figs 2B,3). It may be noted, in this connection, that Pribluda and Rotman [9] found that cycles of temperature-mediated activation-deactivation of actin polymerization in platelets became refractory after a few cycles.

Direct activation of platelets by unsaturated fat-ty acids has previously been reported and interpreted in terms of membrane fluidity changes [30]. In neutrophils arachidonate and other unsaturated fatty acids may function directly by an effect on protein kinase C [22]. It is intriguing that a protein immunologically similar to the platelet haemoprotein has been detected in calf neutrophils.

In summary we have found a parallelism between the behaviour of the purified calf platelet haemoprotein towards platelet-agonist ligands and the behaviour of the same protein during platelet activation, by spectral measurements and immunodiffusion experiments. Although the possibility that some other ligands or effects or both might operate to change the conformation of the protein in vivo has not been ruled out, it is tempting to speculate that conformational changes induced by arachidonate, PGG_2/PGH_2 or possibly H_2O_2 formed in apparently different compartments of platelets during activation by various stimuli may form a biochemical mechanism of stimulus-response coupling in platelets and that similar mechanisms might operate in other cell types.

ACKNOWLEDGEMENT

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