Calpain I activation is not correlated with aggregation in human platelets

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Calpain-catalysed hydrolysis of platelet substrates such as cytoskeletal and calmodulin-binding proteins, and of protein kinase C, is assumed to contribute to platelet aggregation. We have measured calpain I activation by immunoblotting, and $[Ca^{2+}]_i$ (cytoplasmic Ca^{2+} concn.) by fura-2 fluorescence, in parallel with measurement of aggregation, in stirred human platelets treated at different $[Ca^{2+}]_{ext}$ (external Ca^{2+} concns.) with A23187, leupeptin, phorbol ester and thrombin. Hydrolysis of actin-binding protein, and $[^3H]$ 5-hydroxytryptamine release, were also measured in some cases. A rise in $[Ca^{2+}]_i$, platelet aggregation and calpain activation often occurred together. With some combinations of agonists and $[Ca^{2+}]_{ext}$, however, this correlation was clearly not maintained. It was shown: (a) that activation of calpain and its hydrolysis of platelet substrates were not strictly necessary conditions for platelet secretion and aggregation; (b) conversely, that calpain activation could occur without aggregation.

INTRODUCTION

A physiological role for the calpains has not yet been clearly identified in any cell [1,2], but there is some evidence that hydrolysis by calpain of platelet substrates may contribute to platelet aggregation [3]. It is known that: (a) calpain I is a major neutral protease of platelets [4,5]; (b) calpain is activated *in vitro* by Ca^{2+} , phospholipids and membranes [1]; (c) many agonists increase $[Ca^{2+}]_i$ in platelets [6,7]; (d) hydrolysis of various assumed calpain substrates (ABP, PKC and others) accompanies platelet aggregation [8–11].

Although it therefore seems likely that calpain is involved in platelet aggregation, some uncertainties remain. The fact that the calpain inhibitor leupeptin inhibits thrombin-induced aggregation of platelets had been taken as evidence for a role of calpain in aggregation [12,13], but it has now been shown that leupeptin inhibits thrombin directly [14–16]. In addition, an enormous range of calpain substrates has been proposed, and it is not obvious that hydrolysis of all of these can be necessary for the very rapid events of the platelet response.

The action of calpain is frequently inferred from the appearance of breakdown products of its supposed substrates, although this very strictly does not prove that calpain is responsible. However, the presence of proteolytically active calpain I can be observed directly by immunoblot detection of the 76 kDa- and 78 kDa-subunit active forms [17]. We have therefore applied this method to platelets treated with several agonists, at nanomolar to millimolar $[Ca^{2+}]_{ext}$, in order to relate calpain activation to the response of platelets as measured by 5-hydroxytryptamine (serotonin) secretion, fura-2 fluorescence and aggregation.

The results indicated that, although some calpain activation probably occurs *in vivo* at about the same time as platelet aggregation, there is no strict correlation

between the two events, so that a uniquely causal relationship cannot exist.

MATERIALS AND METHODS

Materials

Bovine thrombin, ionophore A23187, fura-2AM, indomethacin, leupeptin and PMA were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. 5-Hydroxy- $[1,2-^3H]$ tryptamine (30 mCi/mmol) was from Dupont Canada Inc., Mississauga, Ont., Canada. The monoclonal antibody to the 80 kDa subunit of human calpain I has been described [17]. A monoclonal antibody to the calpain 30 kDa subunit was kindly provided by Dr. R. D. Lane, Medical College of Ohio, Toledo, OH, U.S.A., and a polyclonal antibody to ABP was kindly provided by Dr. J. E. B. Fox, UCSF, San Francisco, CA, U.S.A..

Platelet preparation

Washed human platelets were prepared as described [17], in 136 mM-NaCl/2.7 mM-KCl/0.42 mM- NaH_2PO_4 /12 mM- $NaHCO_3$ /2 mM- $MgCl_2$ /5.56 mM-glucose/22 mM-trisodium citrate, pH 6.5 (buffer A). Bovine serum albumin could not be used since it interfered with the resolution of calpain forms on SDS/polyacrylamide gels. For activation, the platelets were finally resuspended in buffer A lacking citrate but containing 20 mM-Hepes, pH 7.4 (buffer B), at a concentration of $(2-8) \times 10^7$ platelets/ml.

Loading and release of $[^3H]$ 5-hydroxytryptamine

Washed platelets were incubated in buffer A with 10 μ Ci of $[^3H]$ 5-hydroxytryptamine/ml for 30 min at 37 °C. The platelets were then washed three times in buffer A and resuspended in buffer B. After activation at 37 °C for 10 min, samples were fixed with an equal

Abbreviations used: ABP, actin-binding protein; $[Ca^{2+}]_i$, concentration of Ca^{2+} in the cytoplasm of platelets; $[Ca^{2+}]_{ext}$, concentration of Ca^{2+} in the external medium; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

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volume of 3.7% (w/v) formaldehyde in phosphate-buffered saline (0.15 M-NaCl/0.01 M-sodium phosphate, pH 7.4). Controls of unactivated platelets were also fixed at zero time and after 10 min of stirring. The fixed platelets were centrifuged at 3500 *g* for 10 min, and the radioactivity in the supernatant fluid was determined by liquid-scintillation counting.

Platelet activation

Platelet suspensions in buffer B were stirred at 37 °C with Ca^{2+} or EDTA (see below) and agonists and inhibitors in the following concentrations: thrombin, 0.2 N.I.H. unit/ml; A23187, 1 μM ; PMA, 0.2 μM ; leupeptin, 200 $\mu\text{g/ml}$ (0.42 mM); indomethacin, 20 μM . Measurements were made immediately upon addition of the reagents, without preincubation, either in a Payton aggregometer or in a Perkin-Elmer MPF 66 fluorimeter, or, when large numbers of replicate samples were assayed simultaneously, in 1.5 ml Eppendorf tubes on an Eppendorf shaker.

Ca^{2+} concentrations

(i) For measurement of $[\text{Ca}^{2+}]_i$, platelets in buffer A were incubated with 1 μM -fura-2AM at 37 °C for 30 min, followed by centrifugation and resuspension in buffer B at a concentration of 10^8 platelets/ml [18]. The platelets were stirred at 37 °C in the fluorimeter, and $[\text{Ca}^{2+}]_i$ was calculated from the fura-2 fluorescence as described [19].

(ii) The $[\text{Ca}^{2+}]_{\text{ext}}$ was: (a) 2 mM- Ca^{2+} when Ca^{2+} was added just before activation; (b) low, probably less than 20 μM [20], when Ca^{2+} was not specifically added, since the platelets had been washed several times in citrate buffer; (c) less than 0.1 μM if EDTA was added.

Determination of the calpain I 80 kDa to 76 + 78 kDa conversion

Platelet incubations were terminated by addition of excess EDTA, portions of the platelet suspensions were mixed immediately with 1 vol. of SDS-gel sample buffer preheated to 95 °C, and the mixtures were then heated at 95 °C for 5 min. Gel electrophoresis and immunoblotting were performed as described [17,21]. The extent of calpain activation was measured by laser densitometry of positive transparent prints of the blots on Litho film.

RESULTS AND DISCUSSION

The extent of calpain activation depended on the concentrations of agonists and inhibitors, and the times of incubation (results not shown), but within a given set of replicate experiments the s.d. of calpain activation was 10–15% of the mean. The data in Table 1 are representative of experiments repeated on at least 10 separate occasions with different blood donors, with fully consistent results.

To consider first the thrombin results: the controls showed that calpain activation did not occur without agonist at any value of $[\text{Ca}^{2+}]_{\text{ext}}$ (lines 1 and 2), and that the approximately physiological conditions of thrombin + 2 mM- $\text{Ca}^{2+}_{\text{ext}}$ caused a rise in $[\text{Ca}^{2+}]_i$, secretion, aggregation, and calpain activation (line 3). As reported previously [17,22], activation of calpain and ABP hydrolysis in platelets treated with thrombin and 2 mM- $\text{Ca}^{2+}_{\text{ext}}$ were dependent also on stirring.

In some other conditions, however, the expected correlation between aggregation and calpain activation

was not maintained. Platelets stirred with thrombin in the presence of low $[\text{Ca}^{2+}]_{\text{ext}}$, which permits aggregation (line 4), or of EDTA, which prevents it (line 5), both showed a rise in $[\text{Ca}^{2+}]_i$ to about 500 nM, derived from internal sources [23], but calpain was not activated. Further, although leupeptin inhibited thrombin-stimulated aggregation at both high and low $[\text{Ca}^{2+}]_{\text{ext}}$ [12–16], it did not prevent calpain activation in the presence of 2 mM- $\text{Ca}^{2+}_{\text{ext}}$ (line 7).

The results with both A23187 and PMA were consistent with those of thrombin treatment. A23187 caused a rise in $[\text{Ca}^{2+}]_i$, secretion and aggregation, at both low and high $[\text{Ca}^{2+}]_{\text{ext}}$, but calpain became activated only in the presence of high $\text{Ca}^{2+}_{\text{ext}}$ (lines 9 and 10).

PMA caused platelet aggregation while maintaining low $[\text{Ca}^{2+}]_i$ [24,25], and calpain activation was observed only at 2 mM- $\text{Ca}^{2+}_{\text{ext}}$ (lines 14 and 15). PMA is thought to cause platelet aggregation via activation of PKC, and the activation may also involve proteolysis of PKC by calpain [10,11]. Proteolysis of PKC appears to be unlikely, however, at low $[\text{Ca}^{2+}]_{\text{ext}}$, since calpain was not activated (lines 12 and 13). This observation is also consistent with the report that both A23187 and thrombin activated platelet PKC without proteolysis of PKC, although the value of $[\text{Ca}^{2+}]_{\text{ext}}$ was not given [26].

As in the thrombin work, leupeptin did not affect calpain activation in the presence of A23187, in agreement with a recent report [27], or with PMA, showing that leupeptin did not enter the platelets in our reaction conditions in amounts sufficient to inhibit calpain.

ABP hydrolysis was not detectable by immunoblotting in these 10 min incubations even when calpain was about 20–30% activated. However, in agreement with previous work [3,8,9], ABP hydrolysis was observed, together with much greater calpain activation, in platelets stirred for 20–30 min with 2 mM- $\text{Ca}^{2+}_{\text{ext}}$ and A23187 or thrombin (result not shown). No change in the 30 kDa subunit of calpain [28] was observed by immunoblotting when the 80 kDa subunit was extensively activated, although it has been suggested that proteolysis of the 30 kDa subunit is of regulatory significance [1].

Indomethacin (20 μM), which is used to inhibit thromboxane synthesis, had no detectable effect on aggregation or calpain activation in the experiments described.

Calpain I activation therefore was not correlated with aggregation, nor was it correlated, surprisingly, with the rise in $[\text{Ca}^{2+}]_i$ as detected by fura-2. It was correlated only with stirring of platelets in the presence of both 2 mM- $\text{Ca}^{2+}_{\text{ext}}$ and an agonist. Aside from various conceivable indirect mechanisms, one possibility is that 2 mM- $\text{Ca}^{2+}_{\text{ext}}$ and an agonist create a high local plasma-membrane $[\text{Ca}^{2+}]$ which is not detected by cytoplasmic fura-2, but can assist in activation of membrane-bound calpain. Calpain activation *in vivo* is normally assumed to involve membrane binding [1], although we have shown (in Ca^{2+} -loaded erythrocytes) that calpain I did not become membrane-bound, except perhaps transiently [29], so that this point remains to be clarified.

If calpain does not have an obligatory role in platelet activation, it remains possible that calpain facilitates some steps in the platelet response, or has other as yet unidentified functions. In this context, two roles have been proposed for calpain in post-aggregation events. Both assume that hydrolysis of the cytoskeleton leads, via alterations in the platelet membrane, either to effects

Table 1. Calcium concentrations, 5-hydroxytryptamine release, aggregation, and calpain activation

5-Hydroxytryptamine release is expressed as the percentage release of the total [³H]5-hydroxytryptamine content of platelets, after subtraction of the untreated-platelet control values. Aggregation is expressed as complete (++) or zero (—), and calpain activation is expressed as percentage conversion of the 80 kDa subunit into the 76 + 78 kDa forms, as means ± s.d. (*n* = 3). All incubations were carried out for 10 min at 37 °C with stirring. Abbreviation: n.d., not determined.

Agonist	Leupeptin (0.42 μM)	[Ca ²⁺] _{ext}	[Ca ²⁺] _i (nM)	5-Hydroxytryptamine release	Aggregation	Calpain activation
1. —	—	Low	110	0	—	Zero*
2. —	—	2 mM	120	10	—	Zero
3. Thrombin	—	2 mM	700–900	64	++	39 ± 5
4. Thrombin	—	Low	500–600	73	++	Zero
5. Thrombin	—	EDTA	400–500	n.d.	—	Zero
6. Thrombin	++	Low	150	20	—	Zero
7. Thrombin	++	2 mM	250	28	—	30 ± 6
8. A23187	—	Low	500	81	++	Zero
9. A23187	—	2 mM	900–1400	75	++	64 ± 4
10. A23187	++	2 mM	n.d.	70	++	50 ± 5
11. PMA	—	(EDTA)	110	n.d.	—	Zero
12. PMA	—	Low	150	n.d.	++	Zero
13. PMA	++	Low	n.d.	n.d.	++	Zero
14. PMA	—	2 mM	200	n.d.	++	33 ± 2
15. PMA	++	2 mM	n.d.	n.d.	++	30 ± 4

* In fresh untreated platelets, and in all controls, a trace of activation (0–5%) was occasionally observed.

on clot retraction [22] or to promotion of the surface prothrombinase activity [30].

It is assumed that the pro-enzyme 80 kDa form of calpain I has no intrinsic activity, although that is difficult to prove. Also, the methods used cannot detect very low levels either of calpain activation or of hydrolysis of the proposed substrates. Given those reservations, the experimental results outlined in Table 1 demonstrate that detectable calpain activation is not essential to platelet aggregation and secretion, and they suggest that extensive hydrolysis of cytoskeletal protein is also not essential for these events; conversely, they demonstrate that extensive calpain activation can occur without aggregation.

The most important question which remains may be whether a very low percentage of these reactions is physiologically relevant in the very rapid events of platelet aggregation *in vivo*.

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