Volume 214, number 1, 176–180 FEB 04582 April 1987

Stimulus-response coupling in a cell-free platelet membrane system

GTP-dependent release of Ca^{2+} by thrombin, and inhibition by pertussis toxin and a monoclonal antibody that blocks calcium release by IP_3

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Received 3 February 1987

The Ca^{2+}-mobilizing action of thrombin was demonstrated in a cell-free platelet membrane system consisting of open sheets of plasma membrane plus sealed membrane vesicles that accumulate Ca^{2+} and release Ca^{2+} in response to IP_3. Thrombin plus GTP, acting on plasma membrane (not vesicles), produced a soluble factor (destroyed by alkaline phosphatase) that released Ca^{2+} from the vesicles. This effect of thrombin/GTP was blocked by a monoclonal antibody that binds to vesicles and prevents Ca^{2+} release by IP_3. Pertussis toxin plus NAD ADP-ribosylated plasma membrane polypeptides of 39 and 41 kDa and blocked Ca^{2+} release by thrombin/GTP, but not by IP_3.

Platelet; Ca^{2+}; Pertussis toxin; Thrombin; GTP

1. INTRODUCTION

Mobilization of intracellular Ca^{2+} in platelets may be mediated by the hydrolysis of plasma membrane PIP_2 to form IP_3 [1] or cIP_3 [2], which can diffuse to the dense tubule system (smooth endoplasmic reticulum) to release Ca^{2+} [3–5]. Thrombin receptors may be coupled to the enzyme that hydrolyzes PIP_2 by some type of GTP-binding protein (G-protein) [6], which in some cells (e.g. [7–9]), but not others (e.g. [10–12]) is inactivated by ADP-ribosylation catalyzed by pertussis toxin. Although thrombin causes dissociation of G-protein oligomers [13], intact platelets are not affected by pertussis toxin presumably because they lack receptors for the internalization of its active component [14]. Therefore, we have devised a novel system consisting of open plasma membrane sheets (accessible to the medium at both surfaces) and Ca^{2+}-accumulating vesicles, which when combined together reconstitutes a GTP-dependent Ca^{2+}-mobilizing action of thrombin that is inactivated by pertussis toxin. This represents, to our knowledge, the first demonstration of the Ca^{2+}-releasing action of an agonist in a totally cell-free system.

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2. MATERIALS AND METHODS

2.1. *Isolation of vesicle and plasma membrane fractions*

Membrane vesicles derived from the platelet's dense tubule system [3–5] were isolated on a 40% Percoll gradient as described by O'Rourke et al. [3]. The sealed membrane vesicles, containing the

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00145793/87/$3.50

highest ATP-dependent Ca^{2+}-sequestering activity and response to IP_3 were used as the source of the releasable calcium pool (i.e. fraction 4, fig.1C). The plasma membrane-enriched fraction was isolated by homogenizing washed platelets in a hypotonic 'swelling' buffer as described by Halenda et al. [13], which produces open sheets of plasma membranes [15] having both external and cytoplasmic faces accessible to the contents of the medium. After centrifugation of the homogenate at 7000 × *g* for 10 min, 3.0 ml aliquots of supernatant were layered over 8 ml of 25% Percoll in buffer A (100 mM KCl, 20 mM Hepes, 5 mM $MgCl_2$ at pH 7.1) and centrifuged for 15 min at 17000 × *g* in a Sorvall centrifuge with SS-34 rotor. Fractions (1 ml) from the top of the gradient downwards were characterized as described below (sections 2.2 and 2.3).

2.2. *Characterization of vesicle and plasma membrane fractions*

Vesicle and membrane fractions were assayed for NADPH-cytochrome *c* reductase, succinate dehydrogenase, ATP-dependent Ca^{2+} uptake and Ca^{2+} release by IP_3, as described by O'Rourke et al. [3]. The binding of a monoclonal antibody to plasma membrane glycoprotein complex GpIIb/IIIa (HP1a-n1, Accurate Chemical and Scientific Corp., NY), and a monoclonal antibody mAb 213/21 (produced in our laboratory, unpublished) that blocks Ca^{2+} release from vesicles by IP_3, was determined by ELISAs (O'Rourke et al., unpublished), using as second antibodies affinity-purified peroxidase-labeled goat anti-mouse IgG (Zymed Laboratories, San Francisco, CA) or anti-mouse IgM (Boehringer Mannheim Biochemicals, Indianapolis, IN). Peroxidase activity was measured at 414 nm using 2,2′-azinobis-(3-ethylbenzthiazolinesulfonic acid) as substrate; endogenous peroxidase activity of membrane fractions was negligible.

2.3. *Calcium-releasing action of thrombin*

The vesicle fraction was loaded with calcium by incubation for 60 min in 100 mM KCl, 20 mM Hepes (free acid), 1.9 mM ATP, 5 mM $MgCl_2$, 10 mM KH_2PO_4 at pH 7.1, 0.4 mM $CaCl_2$ (4 μCi/ml $^{45}Ca^{2+}$) and 0.568 mM EGTA; free $[Ca^{2+}]$ = 1.1 μM [3]. These vesicles served as the target system for potential Ca^{2+}-releasing factors from plasma membrane. The Ca^{2+}-releasing action of thrombin was tested as follows: 2 U/ml thrombin was added for 45 s at 23°C to 100-μl aliquots of membranes (70–140 μg protein), then transferred to 0.6 ml $^{45}Ca^{2+}$-loaded vesicles (70 μg protein). At specified times 100-μl aliquots of the membrane-vesicle mixture were added to a 'stopping' solution of 25 μl of 0.63 M formalin in 50 mM K-EDTA at pH 7.0, which immediately terminated release of calcium by IP_3, and prevented calcium uptake, without discharging any Ca^{2+} from the vesicles. After centrifugation for 2.5 min at 100000 × *g* in a Beckman airfuge, supernatants were removed by aspiration and the pellets extracted with 10% trichloroacetic acid to measure the $^{45}Ca^{2+}$ by liquid scintillation spectrophotometry.

2.4. *ADP-ribosylation of membranes by pertussis toxin*

ADP-ribosylation was carried out as described by Halenda et al. [13], in buffer A containing 1 mM each of: DTT, thymidine, GTP, ATP, EDTA, NAD and [^{32}P]NAD (2 Ci/mol). Optimum ADP-ribosylation occurred with 50 μg/ml pertussis toxin; half-maximal reaction took 8–10 min, and was 90% completed at 30 min. The membrane proteins were separated by SDS-PAGE (11% polyacrylamide gels) and ^{32}P-labeled G-proteins measured by radioautography [13].

2.5. *Materials*

Inositol 1,4,5-trisphosphate (IP_3) was prepared from red blood cells [16], or purchased from Amersham. Pertussis toxin was obtained from Drs J. Munoz (NIH, Rocky Mountain Laboratory, Hamilton, MT) and E.L. Becker (University of Connecticut Health Center), and from List Biochemicals (Campbell, CA). These toxin preparations did not differ in their effects.

3. RESULTS AND DISCUSSION

The plasma membrane and vesicle fractions are characterized in fig.1. The vesicle fraction (fraction 4 from the 40% Percoll gradient) showed relatively low binding of a monoclonal antibody to the plasma membrane glycoprotein complex GpIIb/IIIa, compared to lighter fractions 1–3, and high binding of mAb 213/21 (fig.1A,B). Frac-

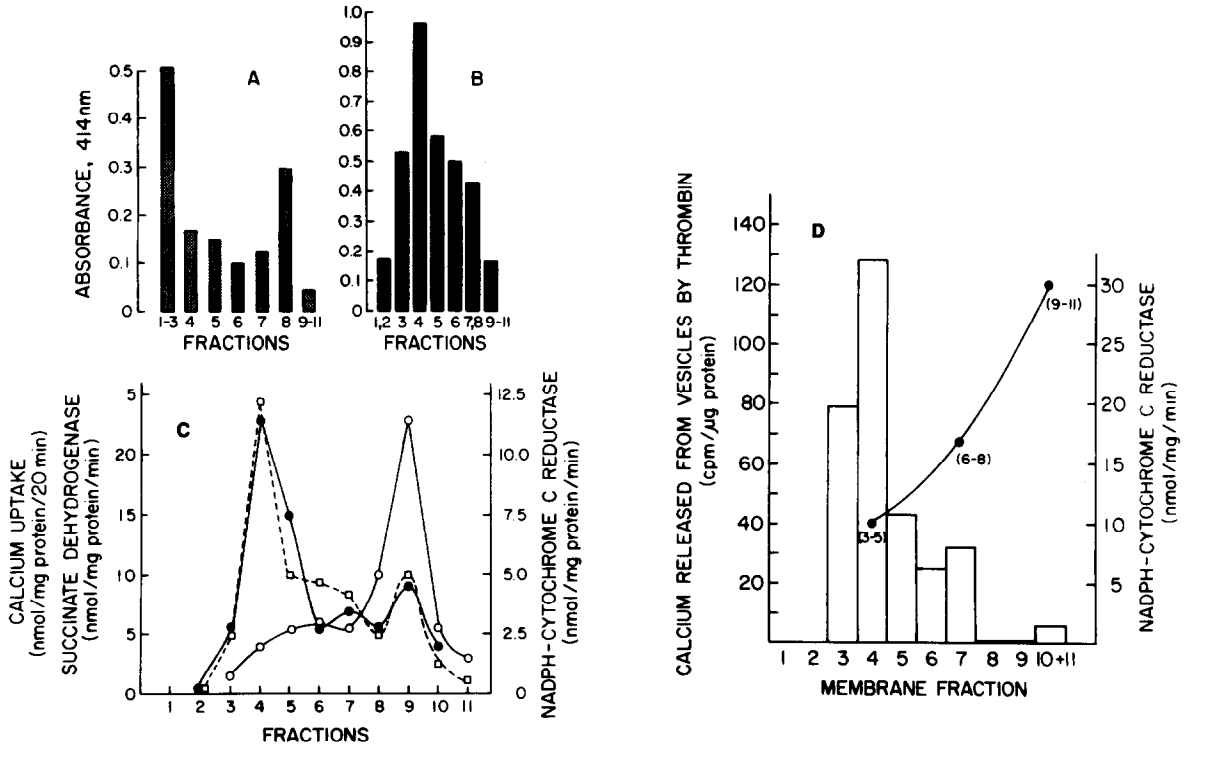

Fig.1. Characterization of vesicle and membrane fractions. Vesicle fraction: (A) binding of monoclonal antibody HP1a-n1 (vs Gp IIb/IIIa); (B) binding of antibody 213/21 (see section 2.3); (C) distribution of Ca^{2+} uptake (●), NADPH-cytochrome *c* reductase (□) and succinate dehydrogenase (○). Membrane fraction: (D) NADPH-cytochrome *c* reductase (●) in pooled fractions, and response to thrombin; i.e. Ca^{2+}-releasing activity when added to vesicles (bars).

tion 4 contained the peak activities for Ca^{2+} uptake and NADPH-cytochrome *c* reductase (fig.1C); as well as the peak activities for other dense tubule enzymes (i.e. glucose-6-phosphatase, NADH-cytochrome *c* reductase), and the greatest response to IP_3 [3]. Half-maximal response was obtained at 0.2–0.3 μM IP_3 (fig.1A). The plasma membranes used in these experiments (fractions 3–4 from the 25% Percoll gradient) did not accumulate Ca^{2+}, were lowest in the dense tubule marker NADPH-cytochrome *c* reductase and had the greatest Ca^{2+}-releasing response to thrombin (fig.1D). Binding of antibodies was similar to fractions 1–2 from the 40% Percoll gradient.

Membrane sheets preincubated with human thrombin (>1000 U/mg; US Biochemicals) plus GTP for 45–60 s and then added to the vesicles released 66% of the Ca^{2+} discharged from the same vesicles by 5 μM IP_3 (table 1). Neither throm-

Table 1

Effect of thrombin and GTP on Ca^{2+} release from vesicles in the presence or absence of plasma membranes

	Ca^{2+} released (nmol/mg vesicle protein ± SE)
Vesicles + GTP + thrombin	1.55 ± 1.55 (4)
Vesicles + IP_3	27.50 ± 4.80 (6)
Membranes plus vesicles	0.93 ± 0.93 (12)
+ GTP	1.84 ± 0.94 (6)
− GTP + thrombin	4.59 ± 2.21 (5)
+ GTP + thrombin	18.10 ± 1.41 (12)
+ IP_3	24.60 ± 2.45 (6)

Vesicles took up 88.4 nmol Ca^{2+}/mg protein. Release of vesicle Ca^{2+} was measured as described in section 2.3. Concentrations: thrombin, 2 U/ml; GTP, 1 mM; IP_3, 5 μM. Number of experiments in parentheses

bin nor GTP directly released Ca^{2+} from vesicles. Without added GTP, thrombin-treated membranes released a small amount of Ca^{2+}, but with GTP release of Ca^{2+} increased 4-fold (table 1). Pertussis toxin plus NAD abolished the plasma membrane response to thrombin/GTP, but not the direct release of Ca^{2+} caused by IP_3 (table 2). Treatment of the vesicles alone with toxin plus NAD did not alter their response to normal thrombin/GTP-stimulated membranes. The time course of the release of Ca^{2+} by thrombin/GTP is shown in fig.2; response was abolished only by treatment of the membranes with both activated toxin and NAD, and was associated with the ADP-ribosylation of two membrane polypeptides of M_r 41000 (minor band) and M_r 39000 (major band) (fig.2B, inset).

Release of Ca^{2+} was entirely due to a soluble factor (100000 × *g* supernatant) appearing in the medium from thrombin/GTP-treated membranes (fig.2D). This activity, like that of IP_3, was abolished by incubation with 1 U/ml alkaline phosphatase (1100 U/mg, Sigma P-0200) for 10 min at 23°C (not shown), suggesting that the Ca^{2+}-releasing substance could be IP_3 or cIP_3 [2].

Table 2

Inhibition of thrombin-induced, but not IP_3-induced, release of membrane vesicle calcium by pertussis toxin plus NAD

	Ca^{2+} released (nmol/mg vesicle protein ± SE)
Vesicles alone	
+ IP_3	26.90 ± 3.44 (8)
+ PT (+ NAD), then IP_3	20.40[a] ± 1.81 (4)
Membranes plus vesicles	
Controls, no treatment	1.33 ± 0.90 (9)
+ PT (+ NAD)	1.81 ± 1.81 (11)
+ PT (− NAD), then thrombin/GTP	21.30 ± 3.89 (5)
+ PT (+ NAD), then thrombin/GTP	2.70 ± 0.79 (9)

[a] Difference from control response to IP_3 is not statistically significant ($p > 0.05$)

Vesicles took up 84.3 nmol Ca^{2+}/mg protein. IP_3, 5 μM; thrombin, 2 U/ml; GTP, 1 mM; PT, 50 μg/ml

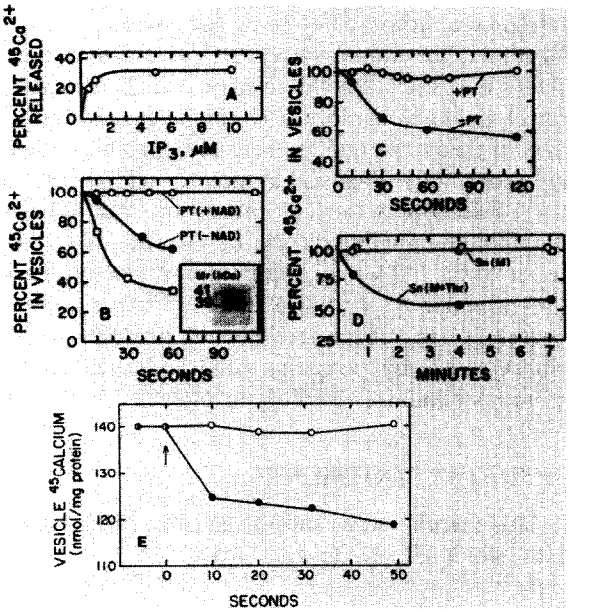

Fig.2. (A) Dose response for release of $^{45}Ca^{2+}$ from vesicles by IP_3. (B) Inhibition of thrombin-induced $^{45}Ca^{2+}$ release by pertussis toxin (PT): (○) membranes plus PT (50 μg/ml) and NAD for 30 min, then thrombin (2 U/ml) added for 45 s and membrane fraction transferred to $^{45}Ca^{2+}$-loaded vesicles; (●) same as above minus NAD; (□) $^{45}Ca^{2+}$ release by 5 μM IP_3. (Inset) Radioautograph of 41 kDa and 39 kDa [^{32}P]ADP-ribosylated polypeptides of the membrane fraction. (C) Same as (B) but membranes preincubated with NAD plus pertussis toxin (○), or without toxin (●). (D) (○) Supernatant, Sn(M), from membrane fraction added to $^{45}Ca^{2+}$-loaded vesicles; (●) supernatant, Sn(M + Thr), from membranes treated with 2.0 U/ml thrombin/1 mM GTP for 45 s added to $^{45}Ca^{2+}$-loaded vesicles. (□) Thrombin added to $^{45}Ca^{2+}$-loaded vesicles. (E) Thrombin/GTP-stimulated membranes added at arrow to control vesicles (●), or vesicles preincubated with 75 nM mAb 213/21 (○).

Furthermore, release of Ca^{2+} by thrombin/GTP-treated membranes was also blocked by treatment of vesicles (but not membranes) with mAb 213/21, an antibody that blocks the action of IP_3 (fig.2E). These findings are consistent with the report that thrombin/GTP stimulates IP_3 formation by isolated platelet membranes [17].

In saponin-permeabilized platelets Lapetina [18] reported that pertussis toxin (5 μg/ml, 30 min) enhanced IP_3 formation due to thrombin, but Brass et al. [19] found that thrombin-induced Ca^{2+}

release was inhibited 45% by the toxin (19 μg/ml, 30 min). Our model system of plasma membrane sheets and Ca^{2+}-loaded vesicles, which provides a novel means to explore both the mechanism of action of thrombin and the role of G-proteins, clearly shows: (i) that GTP greatly enhances the Ca^{2+}-releasing action of thrombin, and (ii) that maximal ADP-ribosylation of G-proteins by pertussis toxin (50 μg/ml, 30 min) completely blocks the response to thrombin/GTP. Further work is necessary to characterize the proteins ADP-ribosylatable by pertussis toxin and their role in stimulus-response coupling in platelets.

ACKNOWLEDGEMENTS

This work was supported by NIH grants HL 18937, HL 37422 and GM 17536.

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