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Accumulation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in thrombin-stimulated platelets

Different sensitivities to Ca^{2+} or functional integrin

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Differences in regulation of the accumulation of PtdIns(3,4)P_2 versus that of PtdIns(3,4,5)P_3 were noted in thrombin-stimulated human platelets. The rapid (within 20 s) response of PtdIns(3,4,5)P_3 contrasted with a distinct lag in the accumulation of PtdIns(3,4)P_2 that was followed by a pronounced increase by 90 s. The presence of 2.5 mM-$CaCl_2$ further elevated PtdIns(3,4)P_2 by 50–120%, but only at a late stage (after 90 s). Tetrapeptide RGDS (Arg-Gly-Asp-Ser), which blocks the interaction of ligands such as fibrinogen with platelet integrin $\alpha_{IIb}\beta_3$ (GPIIb–IIIa), inhibited only the late-phase PtdIns(3,4)P_2 accumulation that was associated with added Ca^{2+}. Although stimulated tyrosine phosphorylation of platelet protein (total cell lysate) was altered by Ca^{2+} or RGDS, we could not identify any such proteins that were affected comparably to PtdIns(3,4)P_2. In contrast to the PtdIns(3,4)P_2 response, the accumulation of PtdIns(3,4,5)P_3 was unaffected by Ca^{2+} or RGDS at any time.

INTRODUCTION

The accumulation of 3-phosphorylated phosphoinositides, i.e. PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, in stimulated human platelets is now well documented [1–6]. These phospholipids may play an important part in cell mitogenesis and transformation [7], although their actual function remains obscure. Proposed modes of action include regulation of cytoskeletal organization [8], perhaps via direct interaction with cytoplasmic protein kinases [9].

Positive modulation of the activity of PtdIns 3-kinase (and thus 3-phosphorylated phosphoinositide formation) by tyrosine phosphorylation has been described for several cell types [7]. In the platelet, agonist-induced stimulation promotes tyrosine phosphorylation of numerous proteins [10–13]. Platelet integrin $\alpha_{IIb}\beta_3$ (the fibrinogen receptor) has been implicated in the regulation of tyrosine phosphorylation in platelets, in that a deficiency of $\alpha_{IIb}\beta_3$ or inhibition of fibrinogen binding to $\alpha_{IIb}\beta_3$ by RGDS (Arg-Gly-Asp-Ser) inhibits certain tyrosine phosphorylations induced by thrombin or by a combination of phorbol ester and Ca^{2+} ionophore [11,13]. Integrin $\alpha_{IIb}\beta_3$ has also been reported to contribute to intracellular Ca^{2+} homeostasis in the platelet, since the presence of RGDS impairs the net accumulation of cytosolic Ca^{2+} measured in stimulated platelets [14,15].

We have examined the effects of Ca^{2+} and $\alpha_{IIb}\beta_3$ function on the accumulation of 3-phosphorylated phosphoinositides and tyrosine phosphorylation over time. Our results imply a role for $\alpha_{IIb}\beta_3$ and Ca^{2+} in regulating the late accumulation of one species of 3-phosphorylated phosphoinositide, PtdIns(3,4)P_2, but not that of another, PtdIns(3,4,5)P_3.

EXPERIMENTAL

Materials

Except where otherwise indicated, sources of reagents used were as described previously [1]. α-Thrombin was donated by Dr. K.G. Mann (University of Vermont, Burlington, VT, U.S.A.). RGDS was purchased from Peninsula Laboratories (Belmont, CA, U.S.A.). The anti-phosphotyrosine monoclonal antibody 4G10 was kindly provided by Dr. Brian Druker (Dana Farber Cancer Institute, Boston, MA, U.S.A.). The enhanced chemiluminescence detection system and Hyperfilm-ECL were from Amersham. [^{3}H]Ins(1,4,5)P_3, [^{3}H]Ins(1,3,4)P_3 and [^{3}H]Ins(1,3,4,5)P_4 were from Du Pont–New England Nuclear (Boston, MA, U.S.A.). [^{3}H]GroIns(3,4)P_2, [^{3}H]GroIns(3,4,5)P_3 and [^{3}H]GroIns(4,5)P_2 (where Gro is glycero) were generously provided by Dr. C. Peter Downes (University of Dundee, Dundee, Scotland, U.K.).

Platelet preparation

Platelet-rich plasma (in the presence of 0.5 μM-prostaglandin E_1 and 1.0 mM-aspirin) and washed platelet suspensions were prepared as described [1] from blood donated by healthy volunteers. Labelling with [^{32}P]P_i and subsequent washing of platelets was as outlined before [1]. With this protocol, after 1.5 h at 37 °C, incorporation of radioactivity into the major phosphoinositide phosphates has reached a plateau, i.e. a steady state, as assessed by comparison at 115 min versus 75 min. As previously reported, the response to thrombin of [^{32}P]PtdIns(3,4)P_2 is mirrored by a similar rise in [^{3}H]PtdIns(3,4)P_2, which indicates a net accumulation of this latter phosphoinositide [1].

Incubations with intact platelets

Aggregation was monitored with a Payton Aggregation Module model 300. Platelets at 10^9/ml were equilibrated with $CaCl_2$ (2.0 mM) at 37 °C with or without RGDS (0.5 mM), and then thrombin was added (1 unit/ml). At this concentration, RGDS was 80% inhibitory for aggregation.

^{32}P-labelled platelets (10^9/ml; pH 7.3) were preincubated at 37 °C with or without added $CaCl_2$ (2.5 mM). RGDS (0.5 mM) was added simultaneously with or 15 s prior to thrombin addition (1 unit/ml). Incubations were terminated at various times by the addition of 3.75 vol. of ice-cold chloroform/methanol/1.0 M-HCl (4:10:1, by vol.). Phases were separated by the addition of

Abbreviations used: GroPIns, glycerophosphoinositol; PtdOH, phosphatidic acid.

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0.5 vol of 1.0 M-HCl and 1.5 vol. of chloroform. The chloroform phase was withdrawn and the aqueous upper phase was washed with 3.0 vol. of chloroform. The chloroform phases were then pooled and the lipids were dried in preparation for t.l.c.

Immunoblotting of phosphotyrosine

Unlabelled platelets (10^9/ml) were incubated under parallel conditions to those for the ^{32}P-labelled platelets indicated above. Incubations were terminated by the addition of 3 × concentrated Laemmli SDS reducing buffer at various times [2]. Proteins were separated by SDS/PAGE (7.5% gels), then transferred to nitrocellulose filters, and incubated with monoclonal antibody 4G10 (1 μg/ml) [2]. Detection was carried out with an enhanced chemiluminescence system as described in the kit protocol. Phosphotyrosine-containing protein bands detected by Hyperfilm-ECL were scanned densitometrically for the linear response range of the system.

Deacylation of lipids and h.p.l.c. of glycerophospholipids

Dried lipids from the above extractions were separated by t.l.c. on K5 Whatman plates [dipped in 1% potassium oxalate/2 mM-EDTA in 1:1 (v/v) ethanol/water and dried] developed with methanol/chloroform/ammonium hydroxide/water (20:14:3:5 by vol.). ^{32}P-labelled lipids were visualized by autoradiography and directly deacylated [1] without elution from silica. The resulting GroPIns polyphosphates and Gro*P* [deacylated form of phosphatidic acid (PtdOH)] were separated on a Whatman Partisphere 5 SAX 25 cm column as described [1], with the following modifications: the column was developed with a gradient of 0–1.25 M-$(NH_4)_2HPO_4$ (titrated to pH 3.2 with phosphoric acid) at 1 ml/min (pump A, water; pump B, 1.25 M-$(NH_4)_2HPO_4$, pH 3.2, as above). The elution gradient was altered in that the column was washed with 100% pump A for 10 min followed by a linear increase to 20% pump B over 50 min and then to 60% pump B over 60 min. The column was washed with 100% pump B for 10 min before returning to 100% pump A. Retention times for GroPIns polyphosphate species were determined in comparison with ^{3}H standards of GroPIns polyphosphates and inositol polyphosphates.

RESULTS

We determined the time course for and the effects of Ca^{2+} on the responses of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in thrombin-stimulated platelets (Fig. 1). The amounts of a third, previously described 3-phosphorylated phosphoinositide, [^{32}P]PtdIns3*P*, do not change significantly under these conditions [1,2]. In the absence of 2.5 mM-Ca^{2+}, PtdIns(3,4,5)P_3 reached its peak value within 20 s, and this was maintained until 300 s. In contrast, PtdIns(3,4)P_2 increased slightly by 20 s but rose 10-fold by 90 s, and remained unchanged until 300 s. Preincubation of platelets with 2.5 mM-Ca^{2+} had no clearly discernible effect, early or late, on thrombin-stimulated accumulation of PtdIns(3,4,5)P_3. However, in the case of PtdIns(3,4)P_2, the presence of 2.5 mM-Ca^{2+} resulted in a slightly enhanced accumulation at 90 s, followed by a pronounced increase by 300 s.

We compared these effects of Ca^{2+} on 3-phosphorylated phosphoinositides to those of RGDS. In platelets preincubated with Ca^{2+}, a definite inhibitory effect of RGDS on the accumulation of PtdIns(3,4)P_2 was observed that appeared only at 300 s (Fig. 2*a*). RGDS did not perturb the response of PtdIns(3,4,5)P_3 under the same conditions. Fig. 2(*b*) depicts the effects of Ca^{2+} on the accumulation of 3-phosphorylated PtdIns, with the data expressed as in Fig. 2(*a*) to facilitate direct comparison. The absence of added Ca^{2+} caused a decrease in the response of PtdIns(3,4)P_2 at 300 s, similar in magnitude to that caused by RGDS in the presence of 2.5 mM-Ca^{2+} (Fig. 2*a*).

Parallel studies on unlabelled thrombin-stimulated platelet preparations were performed to study the effects of Ca^{2+} and RGDS on tyrosine phosphorylation, since this was one plausible route through which the accumulation of 3-phosphorylated phosphoinositide might be altered (Fig. 3). Densitometric scanning of the protein bands was performed on the immunoblot exposures, and we compared the signal intensities at 20 s, 90 s and 300 s to the corresponding basal values for each band. Analysis of the data as fold increases over basal (results not shown) permitted comparison of two different immunoblot exposures, and verified that signal intensities remained in the linear range for each band that was quantified.

Significant but small increases were observed at 60 kDa, the

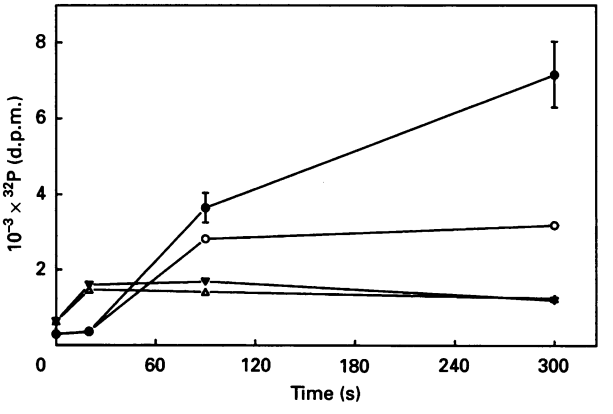

Fig. 1. Time course of accumulation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in thrombin-stimulated platelets with or without added Ca^{2+}

Human platelets were labelled with [^{32}P]P_i as described and stimulated with thrombin (1 unit/ml) in the presence (●, ▼) or absence (○, ▽) of 2.5 mM-$CaCl_2$. These data, from a single experiment, are expressed as averages ± ranges of duplicates. They are representative of five similar experiments. ●, ○, PtdIns(3,4)P_2; ▼, △, PtdIns(3,4,5)P_3.

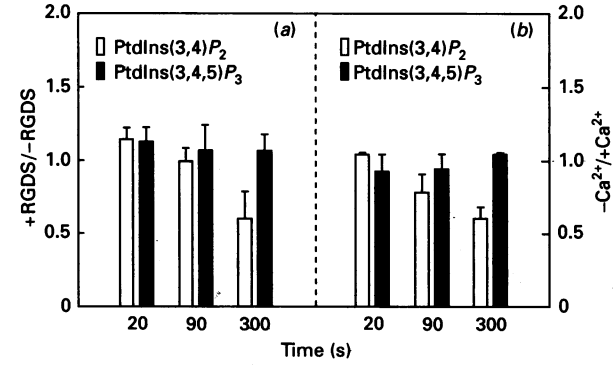

Fig. 2. Effects of Ca^{2+} or RGDS on the accumulation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 with time in thrombin-stimulated platelets

Human platelets were labelled with [^{32}P]P_i as described and stimulated with thrombin (1 unit/ml) under various conditions. (*a*) Data are expressed as values in the presence of RGDS divided by values in the absence of RGDS. All incubations contained 2.5 mM-$CaCl_2$. No difference was observed in + RGDS/ − RGDS in the absence of added Ca^{2+}. (*b*) Data are expressed as values in the absence of added Ca^{2+} divided by values in the presence of 2.5 mM-$CaCl_2$. RGDS was absent from these incubations.

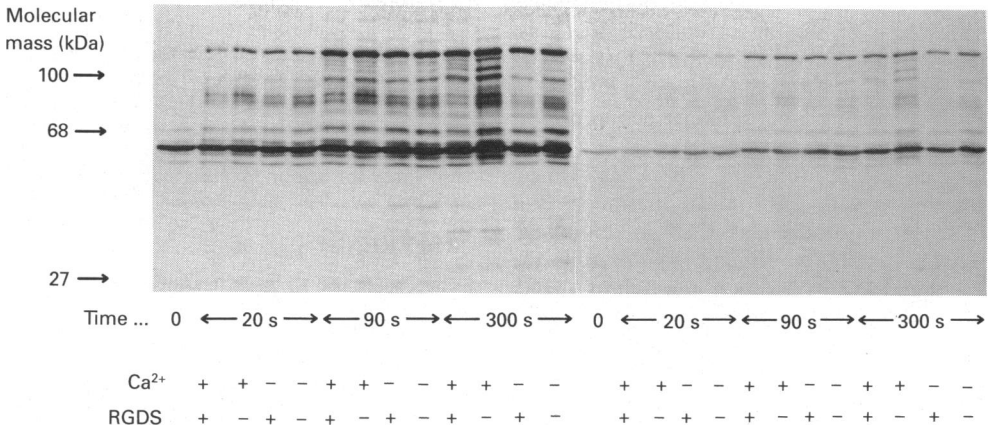

Fig. 3. Effects of Ca^{2+} or RGDS on tyrosine phosphorylation with time in thrombin-stimulated platelets

Human platelets were stimulated with thrombin (1 unit/ml) under various conditions as described. Immunodetection was performed using monoclonal antibody 4G10 and an enhanced chemiluminescence system. The X-ray film was exposed for two different times so that signal intensity of various protein bands remained in the linear range. This immunoblot, from a single experiment, is representative of two similar experiments. Densitometric scanning of the immunoblot exposures was performed in the linear range as described (results not shown).

region of $pp60^{c\text{-}src}$ migration. Other bands, in contrast, displayed decreases upon platelet activation. We observed a major and rapid increase in tyrosine phosphorylation (by 20 s), that levelled off somewhat after 90 s for protein bands of apparent molecular masses of 125, 109, 99, 89 and 84 kDa. Tyrosine phosphorylation of these proteins was affected by the presence of added Ca^{2+} or RGDS as early as 20 s, and these effects continued until 300 s. Unlike the accumulation of PtdIns(3,4)P_2, the tyrosine phosphorylation response of these protein bands was inhibited by RGDS in the absence as well as in the presence of added Ca^{2+}. No protein band could be identified whose tyrosine phosphorylation response matched that of the accumulation of PtdIns(3,4,5)P_3, i.e. occurring within 20 s and unaffected by Ca^{2+} or RGDS.

We did not detect any effect of RGDS on phospholipase C (PLC) activation, as monitored by the accumulation of PtdOH [16], in agreement with other reports on RGDS-treated or thrombasthenic (lacking $\alpha_{IIb}\beta_3$) platelets [17,18]. Added Ca^{2+} was similarly without effect (results not shown).

DISCUSSION

We conclude that there are distinct regulatory features that govern the accumulation of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 in platelets. Our data demonstrate that (1) Ca^{2+} increases the late-phase accumulation of PtdIns(3,4)P_2 only, and (2) RGDS, which blocks the interaction of platelet integrin $\alpha_{IIb}\beta_3$ with adhesive proteins such as fibrinogen, decreases the late-phase accumulation of PtdIns(3,4)P_2 that is associated with Ca^{2+}.

A recently discovered pathway in mitogenic signal transduction, the accumulation of 3-phosphorylated phosphoinositides [7], has been identified in the terminally differentiated platelet [1–6], suggesting other physiological roles for these phosphoinositides. The specific functions of the two such species that accumulate in the stimulated platelet, i.e. PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3, are not known. The definition of regulatory components of the response is one approach that may lead to an understanding of the ultimate function of these phosphoinositides. Accordingly, we have studied the effects of integrin $\alpha_{IIb}\beta_3$ function and Ca^{2+} on 3-phosphorylated phosphoinositide accumulation and tyrosine phosphorylation over time.

Platelet integrin $\alpha_{IIb}\beta_3$ is a receptor for several adhesive proteins (fibrinogen, fibronectin, von Willebrand factor and vitronectin) and is thereby involved in mediating aggregation and adhesion [19]. Other functions of this integrin include the regulation of Ca^{2+} influx and tyrosine phosphorylation. A role for $\alpha_{IIb}\beta_3$ in Ca^{2+} influx in stimulated platelets has been reported: RGDS or a specific monoclonal antibody which blocks binding of the above ligands to $\alpha_{IIb}\beta_3$ decreases the net accumulation of intracellular Ca^{2+} observed in stimulated platelets [14,15]. Moreover, purified $\alpha_{IIb}\beta_3$ acts as a Ca^{2+} channel in a phospholipid bilayer [20,21], and it remains subject to inhibition by RGDS in this setting [22]. Integrin $a_{IIb}\beta_3$ has also been implicated in regulation of stimulated platelet tyrosine phosphorylation, in that RGDS inhibits certain tyrosine phosphorylations, and thrombasthenic platelets, lacking $\alpha_{IIb}\beta_3$, have impaired tyrosine phosphorylation [11,13].

We have observed a prominent late-phase increase in PtdIns(3,4)P_2 in the presence of added Ca^{2+} that is blocked by RGDS, whereas no effects of Ca^{2+} are seen for the PtdIns(3,4,5)P_3 response (Figs. 1 and 2). The explanation for the specificity of these effects for PtdIns(3,4)P_2 is not evident. At present, a clear picture of the routes by which PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are formed and further metabolized has not yet emerged, and consequently the sites of action of regulatory factors are not defined. Conflicting reports suggest either a phosphorylation of PtdIns3P and subsequent phosphorylation of the resulting PtdIns(3,4)P_2 to PtdIns(3,4,5)P_3 [6], or a dephosphorylation of PtdIns(3,4,5)P_3, formed by phosphorylation of PtdIns(4,5)P_2, to yield PtdIns(3,4)P_2 [23]. The latter study has not, however, ruled out the possibility of additional formation of PtdIns(3,4)P_2 by a 3-kinase acting on PtdIns4P. The lag in the accumulation of PtdIns(3,4)P_2 versus the rapid response of PtdIns(3,4,5)P_3 that we have observed could be explained by a pathway in which PtdIns(3,4)P_2 is a product of PtdIns(3,4,5)P_3 and/or by a separate route of PtdIns(3,4)P_2 formation that is stimulated later than one leading to PtdIns(3,4,5)P_3 production.

The mechanism by which Ca^{2+} exerts its effect may be multifaceted. Ca^{2+} ionophore and phorbol ester only slightly stimulate the accumulation of PtdIns(3,4)P_2 [1]. Thus, although such second messenger mimics are themselves not sufficient to account for the response to thrombin, potentiation by Ca^{2+} is possible. A soluble phosphatase activity that releases [^{32}P]P_i from [3-^{32}P]PtdIns(3,4)P_2 and is activated by EDTA has been described [23]. It is therefore possible that Ca^{2+} serves to inhibit phosphatase action on PtdIns(3,4)P_2, thereby enhancing accumulation of this species. Ca^{2+} may, in addition or alternatively, enhance kinase activity (3-kinase or 4-kinase) specific for PtdIns(3,4)P_2 forma-

tion. Added Ca^{2+} may also be serving to enhance $\alpha_{IIb}\beta_3$ association and thereby promote increases in PtdIns(3,4)P_2 [24]. Since the response of PtdIns(3,4,5)P_3 is not depressed by Ca^{2+}, it is unlikely that Ca^{2+} is enhancing 5-phosphatase action on this phosphoinositide.

RGDS, in impairing $\alpha_{IIb}\beta_3$ function, may also affect PtdIns(3,4)P_2 by interfering with Ca^{2+} influx, inasmuch as it inhibits the net increase in intracellular Ca^{2+} in stimulated platelets [14,15]. RGDS is able to impair significantly the sedimentation of $\alpha_{IIb}\beta_3$ and PtdIns 3-kinase in a Triton X-100-insoluble (15000 *g*, 4 min) cytoskeletal fraction of platelets activated with thrombin for 45 s without affecting total PtdIns 3-kinase activity (Triton-soluble and insoluble) [25]. This points to some sort of association of activated PtdIns 3-kinase with a platelet integrin $\alpha_{IIb}\beta_3$–membrane cytoskeletal complex that could potentially affect the later phase of PtdIns(3,4)P_2 accumulation.

Tyrosine phosphorylation has been recognized as an important positive modulator of PtdIns 3-kinase, leading to 3-phosphorylated phosphoinositide accumulation [7], and tyrosine phosphorylation of platelet protein is known to be perturbed by Ca^{2+} ionophore or RGDS [11,13,26]. Therefore we have examined whether the effects of added Ca^{2+} or RGDS on any proteins undergoing stimulated tyrosine phosphorylation are consistent with alterations of the PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 (Fig. 3).

We have assumed that for a tyrosine-phosphorylated protein to be causally involved in the above alterations in PtdIns(3,4)P_2 metabolism, it must demonstrate a middle-to-late-phase tyrosine phosphorylation (or dephosphorylation) that is stimulated by Ca^{2+} and inhibited by RGDS only in the presence of added Ca^{2+}. Similarly, for a tyrosine-phosphorylated protein to regulate PtdIns(3,4,5)P_3 metabolism, it must at least demonstrate an early-phase tyrosine phosphorylation (or dephosphorylation) that is not affected by Ca^{2+} or RGDS. No such candidate protein bands could be identified in our analyses of total platelet lysate. We cannot, however, rule out a role for specific tyrosine-phosphorylated proteins that may exist at very low levels and/or are not resolved by SDS/PAGE.

It has been suggested that only platelets prepared by gel filtration (as opposed to centrifugation) exhibit rapid tyrosine phosphorylations that are inhibited by RGDS [13]. However, detection of tyrosine phosphorylation with the enhanced chemiluminescence technique reveals a pattern of response in our platelets prepared by centrifugation similar to that for gel-filtered platelets, indicating that $\alpha_{IIb}\beta_3$ plays a similar role in both types of preparation.

We have shown that the accumulation of PtdIns(3,4)P_2 is regulated differently from that of PtdIns(3,4,5)P_3. Future studies should aim to elucidate the mechanism by which Ca^{2+} and integrin $\alpha_{IIb}\beta_3$ exert their effects.

While this work was in progress, the inhibition of PtdIns(3,4)P_2 accumulation at 5 min by RGDS or in thrombasthenic platelets was reported [18].

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REFERENCES

1. Kucera, G. L. & Rittenhouse, S. E. (1990) J. Biol. Chem. **265**, 5345–5348
2. King, W. G., Kucera, G. L., Sorisky, A., Zhang, J. & Rittenhouse, S. E. (1991) Biochem. J. **278**, 475–480
3. Huang, R.-S., Sorisky, A., Church, W. R., Simons, E. R. & Rittenhouse, S E. (1991) J. Biol. Chem. **266**, 18435–18438
4. Nolan, R. D. & Lapetina, E. G. (1990) J. Biol. Chem. **265**, 2441–2445
5. Sultan, C., Breton, M., Mauco, G., Grondin, P., Plantavid, M. & Chap, H. (1990) Biochem. J. **269**, 831–834
6. Cunningham, T. W., Lips, D. L., Bansal, V. S., Caldwell, K. K., Mitchell, C. A. & Majerus, P. W. (1990) J. Biol. Chem. **265**, 21676–21683
7. Carpenter, C. L. & Cantley, L. C. (1990) Biochemistry **29**, 11147–11156
8. Eberle, M., Traynor-Kaplan, A. E., Sklar, L. A. & Norgauer, J. (1990) J. Biol. Chem. **265**, 16725–16728
9. Downes, C. P. & Carter, A. N. (1991) Cell. Signalling **3**, 501–513
10. Ferrell, J. E., Jr. & Martin, G. S. (1988) Mol. Cell. Biol. **8**, 3603–3610
11. Ferrell, J. E., Jr. & Martin, G. S. (1988) Proc. Natl. Acad. Sci. U.S.A. **86**, 2234–2238
12. Golden, A. & Brugge, J. S. (1989) Proc. Natl. Sci. U.S.A. **86**, 901–905
13. Golden, A., Brugge, J. S. & Shattil, S. J. (1990) J. Cell Biol. **111**, 3117–3127
14. Yamaguchi, A., Yamamoto, N., Kitagawa, H., Tanoue, K. & Yamazaki, H. (1987) FEBS Lett. **225**, 228–232
15. Sinigaglia, F., Bisio, A., Torti, M., Balduini, C., Bertolino, G. & Balduini, C. (1988) Biochem. Biophys. Res. Commun. **154**, 258–264
16. Huang, R.-S., Kucera, G. L. & Rittenhouse, S. E. (1991) J. Biol. Chem. **266**, 1652–1655
17. Holmsen, H., Walsh, P. N., Koike, K., Murphy, S., Holme, S., Johnson, M. M., Dangelmaier, C. A., Egan, J. J., Benzel, J. E. & Tuszynski, G. P. (1987) Br. J. Haematol. **67**, 335–344
18. Sultan, G., Plantavid, M., Bachelot, C., Grondin, P., Breton, M., Mauco, G., Levy-Toledano, S., Caen, J. P. & Chap, H. (1991) J. Biol. Chem. **266**, 23554–23557
19. Phillips, D. R., Charo, I. F., Parise, L. V. & Fitzgerald, L. A. (1988) Blood **71**, 831–843
20. Fujimoto, T., Fujimura, K. & Kuramoto, A. (1991) Thromb. Haemostasis **66**, 598–603
21. Rybak, M. E., Renzulli, L. A., Bruns, M. J. & Cahaly, D. P. (1988) Blood **72**, 714–720
22. Rybak, M. E. & Renzulli, L. A. (1989) J. Biol. Chem. **264**, 14617–14620
23. Stephens, L. R., Hughes, K. T. & Irvine, R. F. (1991) Nature (London) **351**, 33–39
24. Fitzgerald, L. A. & Phillips, D. R. (1985) J. Biol. Chem. **260**, 11366–11374
25. Zhang, J., Fry, M., Waterfield, M., Jaken, S., Liao, L., Fox, J. E. B. & Rittenhouse, S. E. (1992) J. Biol. Chem. **267**, 4686–4692
26. Takayama, H., Nakamura, T., Yanagi, S., Taniguchi, T., Nakamura, S.-I. & Yamamura, H. (1991) Biochem. Biophys. Res. Commun. **174**, 922–927