Phosphoinositides Are Required for Store-mediated Calcium Entry in Human Platelets*

J. Rosado and S. O. Sage

We have recently observed that small GTP-binding proteins are important for mediation of store-mediated Ca\(^{2+}\) entry in human platelets through the reorganization of the actin cytoskeleton. Because it has been shown in platelets and other cells that small GTP-binding proteins regulate the activity of phosphatidylinositol 3-kinase and phosphatidylinositol 4-kinase, whose products, phosphoinositides, play a key role in the reorganization of the actin cytoskeleton, we have investigated the role of these lipid kinases in store-mediated Ca\(^{2+}\) entry. Treatment of platelets with LY294002, an inhibitor of phosphatidylinositol 3- and phosphatidylinositol 4-kinases, resulted in a concentration-dependent inhibition of Ca\(^{2+}\) entry stimulated by thapsigargin or the physiological agonist, thrombin. In addition, wortmannin, another inhibitor of these kinases, which is structurally unrelated to LY294002, significantly reduced store-mediated Ca\(^{2+}\) entry. The inhibitory effect of LY294002 was not mediated either by blockage of Ca\(^{2+}\) channels or by modification of membrane potential. LY294002 inhibited actin polymerization stimulated by thrombin or thapsigargin. These results indicate that both phosphatidylinositol 3-kinase and phosphatidylinositol 4-kinase are required for activation of store-mediated Ca\(^{2+}\) entry in human platelets and that the mechanism could involve the reorganization of the actin cytoskeleton.

Store-mediated Ca\(^{2+}\) entry (SMCE)\(^1\) is a mechanism present in many cell types; however, the intracellular processes underlying SMCE remain unclear (1). Several hypotheses have considered both direct and indirect coupling mechanisms (2). Indirect coupling assumes the existence of a diffusible messenger generated by the intracellular Ca\(^{2+}\) stores; in contrast, direct coupling models propose a physical interaction between the endoplasmic reticulum (ER) and the plasma membrane (PM; Ref. 2). Recently a new model for SMCE has been proposed in several different cell types. This involves a physical but reversible interaction between the ER and the PM that may require translocation of portions of the ER toward the PM and mechanical support provided by the actin cytoskeleton (3, 4). In support of this hypothesis, small GTP-binding proteins, which modulate vesicular transport through the reorganization of the actin cytoskeleton and the actin cytoskeleton itself, have been suggested to be important for SMCE in different cell types (5–7) including platelets (8, 9).

Phosphoinositides have been shown to play a key role in the reorganization of the actin cytoskeleton (10, 11). Both phosphatidylinositol 3-kinase (PI3-kinase) and phosphatidylinositol 4-kinase (PI4-kinase) and their products are present in all eukaryotic organisms and tissues that have been investigated, including platelets (12, 13). PI3-kinase and PI4-kinase have been shown to be regulated upstream by small GTP-binding proteins in platelets and other cells (14, 15). Two different signal transduction pathways involving phosphoinositides have been identified. In the classically defined phosphatidylinositol (PtdIns) turnover mechanism, phosphorylation at the C-4 position by PI4-kinase yields PtdIns-4-P, the immediate precursor of PtdIns-4,5-P\(_2\), the substrate of receptor-activated phospholipase C (16). Alternatively, phosphoinositides can be phosphorylated at the C-3 position by PI3-kinase to generate several lipid messengers, such as PtdIns-3-P, PtdIns-3,4-P\(_2\), and PtdIns-3,4,5-P\(_3\), which are not substrates for phospholipase C (17). PtdIns-4-P and PtdIns-4,5-P\(_2\) have been shown to regulate cytoskeletal rearrangement through association with several actin-binding proteins (10). In addition, PtdIns-3-P, PtdIns-3,4-P\(_2\), and PtdIns-3,4,5-P\(_3\) can induce actin filament uncapping, thereby facilitating actin polymerization (11, 18).

Here we report for the first time the involvement of PI3- and PI4-kinases in SMCE. In addition, the possible involvement of the actin cytoskeleton in phosphoinositide-mediated SMCE was investigated in human platelets.

EXPERIMENTAL PROCEDURES

**Materials**—Fura-2 acetoxymethyl ester (fura-2/AM) was from Texas Fluorescence. Apyrase (grade VII), aspirin, bovine serum albumin, paraformaldehyde, Nonidet P-40, fluorescein isothiocyanate-labeled phallolidin, valinomycin, wortmannin (WT), thrombin, PtdIns, lanthana- num chloride, and thapsigargin (TG) were from Sigma. LY294002 was from Calbiochem. Anti-phosphotyrosine monoclonal antibody (4G10), anti-PI4-kinase \(\beta\) polyclonal antibody, anti-PI3-kinase p85 polyclonal antibody, and recombinant-Protein A-agarose were from Upstate Biotechnology, \(\gamma\)-\(\text{P}\)-ATP and \(\gamma\)-\(\text{P}\)-phosphophate were from Amersham Pharmacia Biotech. All other reagents were of analytical grade.

**Platelet Preparation**—Fura-2-loaded platelets were prepared as described previously (19), and platelet-rich plasma was incubated at 37 °C with 2 \(\mu\)M fura-2/AM for 45 min. For co-loading with dimethyl BAFTA, 10 \(\mu\)M dimethyl BAFTA/AM was added for the final 30 min of the fura-2 loading period. Cells were collected by centrifugation and resuspended in HEPES-buffered saline containing (HBS in mM): 145 NaCl, 10 HEPES, 10 glucose, 5 KCl, 1 MgSO\(_4\), pH 7.45 and supplemented with 0.1% (w/v) BSA and 40 \(\mu\)M/\(\mu\)l apyrase.

**Measurement of \([\text{Ca}^{2+}]_{\text{i}}\)**—Fluorescence was recorded from aliquots of stirred platelet suspensions at 37 °C using a Cairns Research Spectrophotometer (Cairn Research Ltd., Kent, UK) with excitation wavelengths of 340 and 380 nm and emission at 500 nm. Changes in \([\text{Ca}^{2+}]_{\text{i}}\) were monitored using the fura-2 340/380 fluorescence ratio and calibrated according to the method of Grynkiewicz et al. (20). Ca\(^{2+}\) influx in TG-induced store-depleted platelets was estimated using the integral of the rise in \([\text{Ca}^{2+}]_{\text{i}}\) for 2.5 min after addition of CaCl\(_2\). Thrombin-evoked Ca\(^{2+}\) influx was measured as the integral of the rise in \([\text{Ca}^{2+}]_{\text{i}}\) above basal for 2.5 min after addition of thrombin in the presence of external
**Results and Discussion**

**LY294002 Inhibits PI3- and PI4-kinase Activity and the Synthesis of PtdIns Phosphate**—The activity of PI3- and PI4-kinase 

**β** was determined by immunocomplex assay as described under “Experimental Procedures.” The effect of LY294002 on PI3- and PI4-kinase activity immunocomplexed from human platelets is shown in Fig. 1A. LY294002 inhibited the activity of PI3- and PI4-kinase 

**β** in a concentration-dependent manner, with an IC**50** of 1.5 and 34.4 μM for PI3-kinase and PI4-kinase activity, respectively. These results are similar to those reported by...

**Statistical Analysis**—Analysis of statistical significance was performed using Student's unpaired t test. For multiple comparison, one...

**Concentration dependence of inhibition by LY294002 and wortmannin of TG-evoked store-mediated Ca**

**2** entry in human platelets. Fura-2-loaded human platelets were incubated for 30 min in the presence of different concentrations of LY294002 (1–100 μM) (A and B) or for 10 min in the presence of WT (10 nM and 1 μM) (C) at 37 °C and then stimulated with TG (200 nM) in a Ca**2**-free medium. Three minutes later CaCl**2** (final concentration 300 μM) was added to the medium to initiate Ca**2** entry. Elevations in Ca**2** entry were determined by integration as described under “Experimental Procedures.” Values are mean ± S.E. Significance values indicate differences compared with TG-treated cells in the absence of inhibitor. *p < 0.05; **p < 0.001.

Gratacap et al. (13) and Downing et al. (23). Treatment of human platelets with different concentrations of LY294002 required a concentration-dependent reduction in the 32P labeling of PtdIns phosphate (PtdIns-P) induced by treatment with TG (200 nM). The concentration of LY294002 required for 50% inhibition of [32P]PtdIns labeling was 22.7 μM (Fig. 1B).

**Effect of LY294002 or Wortmannin on TG-evoked SMCE**—In a Ca**2**-free medium TG evoked a prolonged elevation of [Ca**2**]**2** in platelets due to release of Ca**2** from the intracellular stores. The subsequent addition of Ca**2** (final concentration 300 μM) to the external medium resulted in a sustained increase in [Ca**2**]**2** indicative of SMCE (Fig. 2A). Pretreatment of human platelets for 30 min at 37 °C with different concentrations of LY294002 decreased TG-evoked Ca**2** entry in a concentration-dependent manner (Fig. 2, A and B). Treatment of platelets with low concentrations of LY294002 (10 μM), conditions under which PI3-kinase activity is inhibited in vitro (Fig. 1), slightly reduced TG-evoked SMCE by 31 ± 4% (Fig. 2B; p < 0.05). However, pretreatment of platelets with higher concentrations of LY294002 (100 μM), conditions under which PI4-kinase activity is almost completely inhibited in vitro (Fig. 1), significantly reduced TG-induced SMCE to 17 ± 4% of control (Fig. 2B; p < 0.001).

Consistent with the above, treatment of human platelets for 10 min with 10 μM WT, conditions under which PI3-kinase activity has been shown to be inhibited in several cell types (24, 25) including platelets (12, 13), slightly reduced TG-induced SMCE by 18 ± 4% (Fig. 2C; p < 0.05). In addition, treatment of platelets for 10 min with 1 μM WT, a concentration reported to inhibit PI4-kinase activity (22, 24, 25), significantly reduced TG-evoked SMCE by 81 ± 2% (Fig. 2C; p < 0.001). LY294002-treated cells retained their ability to respond to Ca**2**-mobilizing agents such as TG (Fig. 2A). A similar effect was observed with WT (not shown). These findings indicate that treatment of...
Platelets with these agents did not affect their ability to store Ca²⁺ in intracellular compartments.

**LY294002 Reduces Thrombin-evoked [Ca²⁺], Elevation—** Thrombin is a physiological agonist that stimulates a large number of processes in platelets such as calcium mobilization and activation of PI3- and PI4-kinases (19, 26). Treatment of human platelets for 30 min at 37 °C with either 100 μM LY294002 or Me₂SO₄ (control) and then were treated with 250 nM TG and 50 nM ionomycin. Platelet proteins were analyzed by 10% SDS-polyacrylamide gel electrophoresis and subsequent Western blot with a specific antiphosphotyrosine antibody, and the presence of phosphotyrosine residues was quantified by densitometry in Western blots as described. The data represent the integrated optical density for entire lanes under each condition. Results are expressed as -fold increase (mean ± S.E. of six or seven separate experiments).

### Table I

| Time (s) | Control | 100 μM LY294002 |
|---------|---------|------------------|
| 0       | 1.00 ± 0.00 | 0.95 ± 0.03 |
| 45      | 1.09 ± 0.09 | 1.13 ± 0.15 |
| 170     | 1.41 ± 0.09 | 1.43 ± 0.11 |
| 510     | 1.70 ± 0.14 | 1.70 ± 0.10 |

Fig. 3. Effect of LY294002 on thrombin-evoked [Ca²⁺], elevations in the presence and absence of external Ca²⁺. Fura-2-loaded human platelets were incubated for 30 min at 37 °C in the presence of either Me₂SO₄ (control) or LY294002 (10 or 100 μM). The time of experiment either 1 mM CaCl₂ (A and C) or 100 μM EGTA (B and D) were added. Cells were then stimulated with thrombin (0.1 unit/ml) at the time indicated. [Ca²⁺], was monitored as described in the legend to Fig. 2. Traces shown are representative of six or seven separate experiments.

**Table I**

Effects of LY294002 on the phosphotyrosine content of store-depleted BAPTA-loaded platelets

Dimethyl-BAPTA-loaded platelets were preincubated for 30 min at 37 °C with either 100 μM LY294002 or Me₂SO₄ (control) and then were treated with 250 nM TG and 50 nM ionomycin. Platelet proteins were analyzed by 10% SDS-polyacrylamide gel electrophoresis and subsequent Western blot with a specific antiphosphotyrosine antibody, and the presence of phosphotyrosine residues was quantified by densitometry in Western blots as described. The data represent the integrated optical density for entire lanes under each condition. Results are expressed as -fold increase (mean ± S.E. of six or seven separate experiments).

Although our results suggest that both kinases may be implicated in the activation of SMCE, the small effect observed after treatment of platelets with LY294002 or WT at concentrations that specifically inhibit PI3-kinase suggests that PI4-kinase rather than PI3-kinase is in the main responsible for the activation of SMCE or PI4-kinase activity may impair the inhibition of PI3-kinase by phosphorylating PtdIns-3-P molecules remaining in the cell. To our knowledge this is the first time that PI3- and PI4-kinases have been shown to be involved in SMCE.

**Fig. 4. Comparative effects of LY294002 or lanthanum on TG-evoked store-mediated Ca²⁺ entry in human platelets.** Fura-2-loaded human platelets were stimulated with TG (200 nm) in a Ca²⁺-free medium, and 3 min later CaCl₂ (final concentration 300 μM) was added. [Ca²⁺], was monitored as described in the legend to Fig. 2. Addition of LY294002 (100 μM), lanthanum chloride (100 μM), or the vehicle (control) was made 2 min after Ca²⁺ addition as shown by the arrow. Traces are representative of four independent experiments.

**Effect of LY294002 on Tyrosine Phosphorylation Induced by Depletion of the Intracellular Ca²⁺ Stores—** We have previously reported a general inhibitory effect of WT on tyrosine phosphorylation in human platelets (27), which has been extensively reported to be required for SMCE (e.g. Ref. 19). Hence we have now examined the effect of LY294002 on store depletion-evoked tyrosine phosphorylation in these cells. Platelets heavily loaded with the Ca²⁺ chelator dimethyl-BAPTA were used for this study so as to eliminate Ca²⁺-, but not store depletion-dependent tyrosine phosphorylation (28). Dimethyl BAPTA-loaded platelets were incubated for 30 min at 37 °C in the presence of LY294002 or the vehicle, and Ca²⁺ stores were depleted using TG (250 nm) and ionomycin (50 nm). In each experiment we checked that the rise in [Ca²⁺], evoked by TG and ionomycin was abolished by BAPTA loading (data not shown). Samples for protein phosphorylation analysis were taken from the spectrophotometer cuvette 10 s prior, and 45, 170, and 300 s after the addition of TG and ionomycin. As shown in Table I, LY294002 did not significantly modify phosphotyrosine levels at the highest concentration used in this study.
study. The data presented here demonstrated that, in contrast to the effect reported for WT, preincubation for 30 min with the highest concentration of LY294002 used in this study did not have a general inhibitory effect on tyrosine kinase activity stimulated by depletion of intracellular Ca^{2+} stores.

To assess whether the inhibitory effect of LY294002 on SMCE could be mediated by Ca^{2+} chelation or Ca^{2+}-channel blockage we examined the effect of LY294002 after initiation of SMCE. As shown in Fig. 4, addition of 100 μM LY294002 2 min after the addition of Ca^{2+} to store-depleted cells did not modify TG-evoked Ca^{2+} entry for up to 30 min, the preincubation time used in this study. In contrast, addition of 100 μM lanthanum, a nonspecific cation channel blocker, caused a rapid decrease in [Ca^{2+}], over the same time course, indicating that Ca^{2+} entry had been inhibited (Fig. 4). The lack of effect of LY294002 after SMCE has been stimulated using TG indicate that the inhibition of Ca^{2+} entry by LY294002 is not due to nonspecific effects as a chelator of Ca^{2+} or as a Ca^{2+} entry channel blocker. In addition, our results indicate that PI3- and PI4-kinase activity is required for the activation of SMCE but not for its maintenance in human platelets.

Since depolarization of the membrane potential has been shown to reduce the driving force for Ca^{2+} entry we also investigated whether the inhibitory effects of LY294002 could be attributed to changes in membrane potential by studying the effect of this inhibitor on SMCE in the presence of valinomycin, which stabilizes the platelet membrane potential close to the K+ equilibrium potential (29). Treatment of platelets with 100 μM LY294002 inhibited TG-induced SMCE to the same extent in the presence of valinomycin (3 μM) (not shown). Ca^{2+} influx was 57278 ± 9742 nM/sec under control conditions and 7034 ± 1250 nM/sec in LY294002-treated cells in the absence of valinomycin (87% inhibition) and 7032 ± 8058 nM/sec in control and 11488 ± 2093 nM/sec in LY294002-treated cells in the presence of valinomycin (84% inhibition), respectively (n = 3). These findings indicate that the effects of LY294002 are not due to a reduction in the membrane potential. These results together with others presented in this paper indicate that the effect of LY294002 on SMCE is more likely to be explained by inhibition of PI3- and PI4-kinases. These findings are in agreement with previous studies reporting the high specificity of LY294002 as an inhibitor of PI3- and PI4-kinase activity (22).

**LY294002 Inhibits Actin Polymerization in Platelets—**The actin cytoskeleton has been shown to play a key role in the activation and maintenance of SMCE in platelets (8, 9) and others presented in this paper indicate that the effect of LY294002 on SMCE is more likely to be explained by inhibition of PI3- and PI4-kinases. These findings are in agreement with previous studies reporting the high specificity of LY294002 as an inhibitor of PI3- and PI4-kinase activity (22).

In conclusion, the results of the current study demonstrate that the synthesis of phosphoinositides is required for the activation of SMCE in human platelets. The activity of PI3- and PI4-kinase may represent one of the factors involved in the regulation of the phosphoinositide pool required for the activation of SMCE in these cells, probably through the reorganization of the actin cytoskeleton. These data are thus compatible with a physical coupling model for SMCE, which requires actin polymerization for both translocation of the ER toward the PM for initiation of Ca^{2+} entry and cytoskeletal support for its maintenance (8).

**REFERENCES**

1. Sage, S. O., Roast, R., and Rink, T. J. (1990) Biochem. J. 265, 675–680
2. Parekh, A. B., and Penner, R. (1997) Physiol. Rev. 77, 901–950
3. Patterson, R. L., van Rossum, D. B., and Gill, D. L. (1999) Cell 98, 487–499
4. Yao, Y., Ferrer-Montiel, A. V., Montal, M., and Tisien, R. Y. (1999) Cell 98, 475–485
5. Bird, G. S., and Putney, J. W. Jr. (1993) J. Biol. Chem. 268, 21486–21488
6. Fasolato, C., Hoth, M., and Penner, R. (1993) J. Biol. Chem. 268, 20737–20740
7. Holda, J. R., and Blatter, L. A. (1997) FEBS Lett. 403, 191–196
8. Rosado, J. A., Jener, S., and Sage, S. O. (2000) J. Biol. Chem. 275, 7527–7533
9. Rosado, J. A., and Sage, S. O. (2000) Biochem. J. 346, in press
10. Janmey, P. A., and Stossel, T. P. (1989) J. Biol. Chem. 264, 4825–4831
11. Hartwig, J. H., Krug, S., Kovanosavljevic, T., Janmey, P. A., Cantley, L. C., Stossel, T. P., and Toker, A. (1996) J. Biol. Chem. 271, 32986–32993
12. Haurad, J. M., Racaud-Sultan, C., Gironel, D., Albiges-Rizo, C., Giacomini, T., Roques, S., Martel, V., Breton-Deouillon, M., Perrot, B., and Chap, H. (1998) Biochem. J. 329, 329–340
13. Gratapac, M.-P., Payrastre, B., Viaia, C., Mauco, G., Plantavid, M., and Chap, H. (1998) J. Biol. Chem. 273, 24314–24321
14. Fox, J. E. B. (1996) Haemostasis 28, 102–113
15. Gasman, S., Chasserot-Golzar, S., Hubert, P., Aunis, D., and Bader, M. F. (1998) J. Biol. Chem. 273, 16913–16920
16. Berdidge, M., Heslop, J., Irvine, R. F., and Brew, K. D. (1984) Biochem. J. 222, 195–201
17. Carpenter, C. L., and Cantley, L. C. (1990) Biochemistry 29, 11147–11156
18. Hartwig, J. H., Bokoch, G. G., Carpenter, C. L., Janmey, P. A., Taylor, L. A., Toker, A., and Stossel, T. P. (1995) Cell 82, 643–653
19. Sargeant, P., Farndale, R. W., and Sage, S. O. (1993) FEBS Lett. 315, 242–246
20. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) J. Biol. Chem. 260, 340–3450
21. Way, B. A., and Mooney, R. A. (1999) J. Biol. Chem. 274, 26419–26415
22. Meyers, R., and Cantley, L. C. (1997) Biochemistry 35, 537–540
23. Downing, G. J., Kim, S., Nakanishii, S., Catt, K. J., and Balla, T. (1996) Biochemistry 35, 3587–3594
24. Linseman, D. A., McEwen, E. L., Sorensen, S. D., and Fisher, S. K. (1998) J. Neurochem. 70, 349–357
25. Mahaut-Smith, M. P., Rink, T. J., Collins, S. C., and Sage, S. O. (1990) FEBS Lett. 247, 191–196
26. Linseman, D. A., McEwen, E. L., and Fisher, S. K. (1998) Mol. Pharmacol. 53, 827–836
27. Jenner, S., Farndale, R. W., and Sage, S. O. (1996) Biochem. J. 318, 2228–2232
28. Sargeant, P., Farndale, R. W., and Sage, S. O. (1994) Exp. Physiol. 79, 269–272
29. Mull-Schmide, M. P., Rink, T. J., Collins, S. C., and Sage, S. O. (1996) J. Physiol. (Lond.) 428, 723–735
30. Cullen, P. J. (1998) Biochem. Biophys. Acta 1345, 35–47
31. Irvine, R. F., and Cullen, P. J. (1996) Curr. Biol. 6, 537–540
Phosphoinositides Are Required for Store-mediated Calcium Entry in Human Platelets
Juan A. Rosado and Stewart O. Sage

J. Biol. Chem. 2000, 275:9110-9113.
doi: 10.1074/jbc.275.13.9110

Access the most updated version of this article at http://www.jbc.org/content/275/13/9110

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 14 of which can be accessed free at http://www.jbc.org/content/275/13/9110.full.html#ref-list-1