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Inhibitors of Protein Phosphatase Type 1 and 2A Attenuate Phosphatidylinositol Metabolism and Ca^{2+}-Transients in Human Platelets. Role of a cdc2-Related Protein Kinase†

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ABSTRACT: The addition of either okadaic acid or calyculin A desensitizes human platelets to thrombin. One objective of this study was to determine which step(s) leading to secretion reactions may be affected by these protein phosphatase inhibitors. In a dose-dependent manner, okadaic acid or calyculin A inhibits phosphatidylinositol metabolism and Ca^{2+}-transients. In all cases, calyculin A was approximately 10-fold more potent than okadaic acid, and it had maximal effects at a concentration of 1 μM. Although thrombin-induced rises in $[Ca^{2+}]_i$ were diminished, an increase in the phosphorylation state of myosin light chains (MLC) was still observed. Changes in this phosphorylation were diminished, however, following the addition of thrombin to calyculin A-treated platelets that were loaded with dimethyl-BAPTA. These data demonstrate that calyculin A and okadaic acid lower agonist-induced Ca^{2+}-transients, which in turn prevents responses such as secretion reactions. Calyculin A/okadaic acid-induced phosphorylation events were not diminished in BAPTA-loaded platelets, suggesting that these phosphorylations are Ca^{2+}-insensitive. Thus, a second objective of this study was to identify the protein kinase(s) that was(were) responsible for the calyculin A-induced phosphorylations. In a platelet lysate system, calyculin A caused an increase in the incorporation of [^{32}P]phosphate into p50. This phosphorylation event was identical to that observed in the intact platelet and was not mimicked by cAMP, cGMP, Ca^{2+}, or a Ca^{2+}/phospholipid/diacylglycerol mixture. Kinase activity was removed after the lysate was incubated with p13^{suc1}–Sepharose. This suggests that a p13^{suc1}-sensitive protein kinase, e.g., a cell cycle-dependent protein kinase, is responsible for the calyculin A-sensitive phosphorylation events. To support this notion, cdk2 was detected in p13^{suc1}–Sepharose precipitates using antibodies that were generated to conserve regions of the kinase.

Agonists and antagonists alter the phosphorylation state of platelet proteins (Lyons et al., 1975; Haslam et al., 1979). Consequently, the correlation between protein phosphorylations and regulation of platelet responses has been investigated vigorously by many laboratories [reviewed in Siess (1989)]. However, the extent to which protein phosphorylation regulates platelet responses still needs to be clarified. Studies have shown that the phosphorylation status of a limited number of proteins reflects the activation state of platelets (Kawamoto et al., 1989). Some of the enzymes that modulate the phosphorylation reactions have also been identified (Haslam et al., 1979; Rink et al., 1983; Kaibuchi et al., 1984). Two proteins having molecular weights of 20000 (p20) and 47000 (p47) have been shown to undergo agonist-induced phosphorylation and have been studied extensively in order to identify their functions as well as the role of specific protein kinases in controlling the activation process. From such studies, it is evident that protein kinase C is responsible for phosphorylating p47 (Nishizuka, 1984), whereas the Ca^{2+}/calmodulin-dependent enzyme MLCK[1] is responsible for phosphorylation of p20 (Dabrowska & Hartshorne, 1978). In contrast, activation of other protein

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[1] Abbreviations: BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; MLCK, myosin light chain kinase; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; PGE_1, prostaglandin E_1; PGI_2 prostacyclin.

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kinases, e.g., cyclic nucleotide-dependent protein kinases, leads to the inhibition of platelet responses (Packham et al., 1980).

Protein phosphorylation events reflect a balance between protein kinase and phosphatase activities. Attention has recently been focused on the role of protein phosphatases in cellular events including contraction, mitogenesis, and regulation of Ca^{2+} currents [reviewed in Shenolikar and Nairn (1991)]. Four types of protein phosphatases have been identified in human platelets: protein–serine/threonine phosphatases type 1 and type 2A (Lerea, 1991), type 2B (Tallant & Wallace, 1985; Tallant et al., 1988), and protein–tyrosine phosphatases (Lerea et al., 1989; Smilowtiz et al., 1991). The role that each enzyme plays in regulating platelet responses is not clear. It has been proposed recently that type 1 (and possibly type 2A) protein phosphatase activity is needed for an agonist to initiate platelet secretion reactions (Lerea, 1991).

The above studies demonstrated that perturbation of type 1 and 2A protein phosphatases results in a desensitization of human platelets to activating agents such as thrombin. The desensitization occurred concomitantly with a burst of specific phosphorylation events. These studies raised several questions, including what biochemical events are affected following treatment of platelets with specific phosphatase inhibitors such as okadaic acid and calyculin A? Moreover, which protein kinase(s) is(are) responsible for the burst of phosphorylation that occurs following the addition of okadaic acid or calyculin A? Identifying the protein kinase(s) may define additional key regulators of the activation process. The current work has addressed these questions and revealed that early events that occur during the activation process are inhibited by inhibitors of type 1 and type 2A protein phosphatases. Moreover, a cdc2-like protein kinase, e.g., cdk2, appears to play a major role in desensitizing platelets to agonists such as thrombin.

Experimental Procedures

Materials. Highly purified human thrombin (2694 units/mL) was a gift from Dr. Walter Kisiel, University of New Mexico. Prostaglandin E_1, apyrases, crystallized human albumin, histones (types IIIS and VIIS), cAMP, cGMP, TPCK-treated trypsin, phosphoserine, phosphotyrosine, phosphothreonine, and phosphatidic acid were from Sigma, and ampholines, urea, acrylamide, *N',N*-methylenebis(acrylamide), and HRP-conjugated goat anti-mouse and anti-rabbit antibodies were from Bio-Rad. New England Nuclear supplied the [^{32}P]phosphoric acid (in water) and [γ-^{32}P]ATP (3000 Ci/mmol). 5-Hydroxy[2-^{14}C]tryptamine creatine sulfate (54 mCi/mmol) and the enhanced chemiluminescence reagents were purchased from Amersham. Okadaic acid and calyculin A were purchased from LC Services, Woburn, MA. Fura2/AM and dimethyl-BAPTA/AM were obtained from Molecular Probes, Eugene, OR. Phosphatidylserine and 1,2-dioleoylglycerol were purchased through Avanti. TLC cellulose plates were from Merck. UBI supplied the monoclonal anti-human cdc2Hs kinase (PSTAIR) antibody and a polyclonal anti-mouse cdc2 kinase (C-terminal) antibody. A second rabbit anti-cdc2 antibody was kindly provided by Dr. David W. Litchfield (Manitoba Institute of Cell Biology). Affinity-purified antisera raised against the C-terminal sequence of cdk2 was provided by Dr. David O. Morgan (UCSF). Protein kinase inhibitor (PKi) (residues 11–24) was synthesized by the chemical synthesis facility of the Howard Hughes Medical Institute at the University of Washington, Seattle, WA.

Isolation of Unlabeled and Serotonin/Fura2-Labeled Platelets. Human blood was drawn into vacutainer collection tubes containing acid/citrate/dextrose. Platelet-rich plasma (PRP) was obtained following centrifugation at 1100*g* for 3 min at 21 °C. For release reactions and fluorescence measurements, platelets in the PRP were incubated with 2 μM [^{14}C]serotonin (54 mCi/mL) for 60 min at 37 °C *and* 2.5 μM Fura2/AM for 30 min. Prostaglandin E_1 (0.4 μM) and apyrases (12.5 milliunits/mL) were added, and the platelets were removed by centrifugation for 13 min and washed once in a modified Tyrode's buffer containing 5 mM Hepes, pH 6.4, 140 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5.5 mM glucose, 12 mM $NaHCO_3$, 0.125% (w/v) human serum albumin, 6 milliunits/mL apyrases, and 0.4 μM prostaglandin E_1. Platelets were subsequently suspended in either a modified Tyrode's buffer containing 5 mM Hepes, pH 7.4, 140 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5.5 mM glucose, and 12 mM $NaHCO_3$ (resuspension buffer) at a concentration of 2×10^8/mL or at a concentration of 4×10^8/mL in lysis buffer containing 20 mM Hepes, pH 7.4, 1 mM benzamidine, 20 μg/mL leupeptin, and 1 mM EGTA.

Measurement of Serotonin Release. Platelets (3.3×10^7) were preincubated at 37 °C in the presence of buffer alone or varying concentrations of okadaic acid or calyculin A, after which thrombin was added to start the reactions. At various times, the reactions were stopped by the addition of 37% formaldehyde. Following centrifugation, the radioactivity in the supernatant was measured. The amount of [^{14}C]serotonin released is expressed as a percentage of the amount of [^{14}C]serotonin in 3.3×10^7 platelets.

Measurement of [^{32}P]Phosphatidic Acid and Phosphoproteins from Intact Platelets. Platelets suspended in resuspension buffer were incubated with [^{32}P]orthophosphate (0.2 mCi/mL) for 2 h at 37 °C. Following the incubation period, platelets (3.3×10^7) were pretreated at 37 °C for 2 min with buffer alone or varying concentrations of okadaic acid or calyculin A and then treated with either buffer or thrombin (0.1 unit/mL). For analysis of [^{32}P]phosphatidic acid formation, reactions were stopped by the addition of $CHCl_3$/MeOH/HCl (100:200:2) and the phospholipids were separated by thin-layer chromatography using cellulose thin-layer chromatography plates and detected as previously described (Marche et al. 1982). To examine the changes in the phosphorylation state of platelet proteins, reactions were quenched by adding an equal volume of IEF sample buffer containing 9 M urea, 2% (w/v) NP-40, 5% (w/v) 2-mercaptoethanol, and 2% (v/v) ampholines (pH 3–10; pH 4–6; pH 5–7 ratio of 1:1:1) and immediately placing them in a dry ice/methanol bath. Phosphoproteins were separated by two-dimensional polyacrylamide gel electrophoresis (O'Farrell, 1975) and visualized by autoradiography.

Measurement of Ca^{2+} Fluxes in Platelet Suspensions. Fura2 fluorescence was measured in suspensions using a Perkin-Elmer 204-A fluorescence spectrophotometer with excitation at 340 nm and emission at 510 nm. Platelets were preincubated in a siliconized cuvette in Ca^{2+}-free buffers in the presence of buffer alone, okadaic acid, or calyculin A for 2 min at 37 °C. Thrombin, 0.1 unit/mL, was added, and the platelets were further incubated for 2 min. The fluorescence was continuously monitored during the entire assay period. In studies that make use of dimethyl-BAPTA/AM, platelets in the PRP were loaded simultaneously with Fura2/AM (2.5 μM) and dimethyl-BAPTA/AM (25 μM).

In Vitro Protein Phosphorylation Using Platelet Extracts. Platelet extracts were prepared by sonication procedures. Intact platelets were suspended in "lysis" buffer and sonicated with a Braun sonicator in an ice bath with two 15-s bursts at

a setting of −50. For the most part, phosphorylation studies were conducted in whole extracts. The reaction mixtures (62.5 μL), containing platelet extracts (80% of the total volume), 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM [^{32}P]ATP (5 cpm/fmol), and, when indicated, 1 μM calyculin A, 50 μM cAMP, 50 μM cGMP, 6 μg/mL diolein, 60 μg/mL phosphatidylserine, 50 μM PKi, or calcium (10 μM, final concentration), were incubated for 10 min at 30 °C. Maximal effects of calyculin A were reached using these assay conditions. The reactions were stopped by the addition of 62.5 μL of IEF sample buffer, and the mixtures were processed as described above.

Precipitation of Protein Kinase Activities Using p13^{suc1}–Sepharose. Platelet extracts were subjected to centrifugation at 10000*g* for 10 min at 4 °C. The supernatant fraction was incubated with either Sepharose 4B or with p13^{suc1}–Sepharose for 2 h at 4 °C. Following centrifugation at 10000*g* for 5 min, the supernatants were assayed for calyculin A-induced phosphorylations as described above. To assess whether "nonspecific" trapping occurred, the following experiments were conducted: First, protein kinase C assays were routinely performed to demonstrate that a general loss of enzymatic activities did not occur; second, Sepharose washes were added back to the extract to show that the phosphorylating activity was not restored.

Kinase Assays in p13^{suc1}–Sepharose Pellets. The Sepharose pellets, obtained above, were washed twice with 200 μL of platelet "lysis" buffer and then assayed for histone kinase activity. Phosphorylation of histone was carried out by incubating the Sepharose pellets at 30 °C for 10 min with histones (0.5 μg), 10 mM $MgCl_2$, 2 mM dithiothreitol, and either 100 μM [γ-^{32}P]ATP (2.5 cpm/fmol) or 200 μM [γ-^{32}P]GTP (2.5 cpm/fmol). The reactions were terminated by the addition of SDS gel buffer and proteins were separated by SDS–polyacrylamide gel electrophoresis using 5–15% gradient gels. Phosphorylated histones were visualized by autoradiography, excised, and extracted with 30% H_2O_2. The associated radioactivity was determined using scintillation counting. Incubations in the presence of hexokinase and glucose were carried out as described by Graziani et al. (1983).

Western Analysis of p13^{suc1}–Sepharose Precipitates. Platelet and HL60 extracts were centrifuged at 10000*g* for 10 min, and the supernatants (500 μg) were incubated with p13^{suc1}–Sepharose as described above. The washed Sepharose pellets were solubilized by addition of Laemmli sample buffer (Laemmli, 1970), followed by boiling for 2 min. Proteins were separated on a 5–15% SDS–polyacrylamide gel followed by transfer onto nitrocellulose for 1 h at 50 V. The nitrocellulose was blocked with TTBS (Tween-20-containing Tris-buffered saline) containing 5% nonfat dried milk. The membranes were subsequently incubated with 3% milk containing primary antibody (anti-PSTAIR (5 μg/mL), anti-cdk2 (approximately 2 μg/mL), or anti-cdc2 (1:250) for 2 h at 21 °C, washed twice with TTBS, and incubated with HRP-coupled secondary antibody (goat anti-mouse IgG or goat anti-rabbit IgG) for 1 h at 21 °C. The membranes were washed four times with TTBS, incubated with enhanced chemiluminescence (ECL) detection reagent for 1 min, and exposed to X-AR film for 30–60 s.

Tryptic Phosphopeptide Mapping and Phosphoamino Acid Analysis. The phosphoproteins were excised from dried gels, and the gel pieces were resuspended in 50 mM NH_4HCO_3, homogenized, and removed by centrifugation. For peptide mapping studies, the extracted phosphoproteins were treated with performic acid, washed, and subsequently incubated with

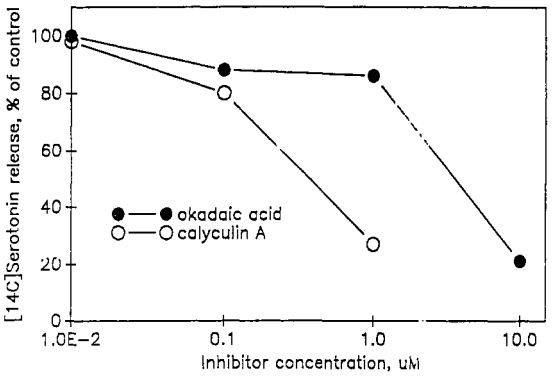

FIGURE 1: Dose response for the effect of calyculin A or okadaic acid on thrombin-induced serotonin release. Platelets were pretreated for 2 min at 37 °C with various concentrations of calyculin A (open symbols) or okadaic acid (solid symbols) and then further incubated with thrombin for 2 additional min. The reactions were stopped, and the amount of [^{14}C]serotonin released was determined. The results are from one experiment that is typical for what was seen in three experiments.

10 μg of TPCK-treated trypsin at 37 °C for 24 h. The peptide fragments were washed several times with water and separated in two dimensions using cellulose plates: by electrophoresis in the first dimension (15% glacial acetic acid, 5% formic acid, pH 1.9) and chromatography in the second dimension (isobutyric acid/pyridine/acetic acid/water 62.5:4.8:2.9:28) (Scheidtmann et al., 1982). The mobility of phosphopeptides was assessed relative to the positions of marker dyes, e.g., DNP-lysine and basic fucshin. For phosphoamino acid analysis, extracted phosphoproteins were hydrolyzed in the presence of 6 N HCl for 1 h at 110 °C. The phosphoamino acids were separated by two-dimensional electrophoresis on cellulose plates using 2.5% formic acid and 7.8% acetic acid (pH 1.9) in the first dimension and 0.5% pyridine and 5% acetic acid (pH 3.5) in the second dimension (Cooper et al., 1983).

RESULTS

Calyculin A/Okadaic Acid Effects on Platelet Functions. The effects of calyculin A and okadaic acid on platelet activation are compared in Figure 1, using serotonin release as the measure of activation. Following a 2-min preincubation, both compounds are potent inhibitors of thrombin-induced serotonin release, with calyculin A being approximately 10-fold more potent. Whether the differential sensitivity is a reflection of their specificity toward type 1 phosphatase or their permeability into platelets is not known.

Although the molecular mechanisms underlying platelet activation are only partly understood, it was important to evaluate the effects of calyculin A and okadaic acid on steps that precede release responses. Thrombin stimulation of platelets has been shown to induce a rapid increase in cytosolic Ca^{2+} levels from intracellular stores (Rink et al., 1982; Davies et al., 1989). In the present study, the effect of phosphatase inhibitors on Ca^{2+} levels was compared to the release of serotonin from platelets in a Ca^{2+}-free medium. In these studies, Ca^{2+} measurements and serotonin release were monitored routinely from platelets that have been loaded with both Fura2 and [^{14}C]serotonin. Preincubation of platelets with calyculin A or okadaic acid inhibited, in a dose-dependent manner, thrombin-induced Ca^{2+}-transients (Figure 2B,D). The concentration of each inhibitor that was needed to affect Ca^{2+} levels corresponded to the concentration that affected secretion (Figure 2A,C).

Pretreatment of platelets with 1 μM calyculin A also inhibits the rapid incorporation of [^{32}P]P_i into phosphatidic acid (a

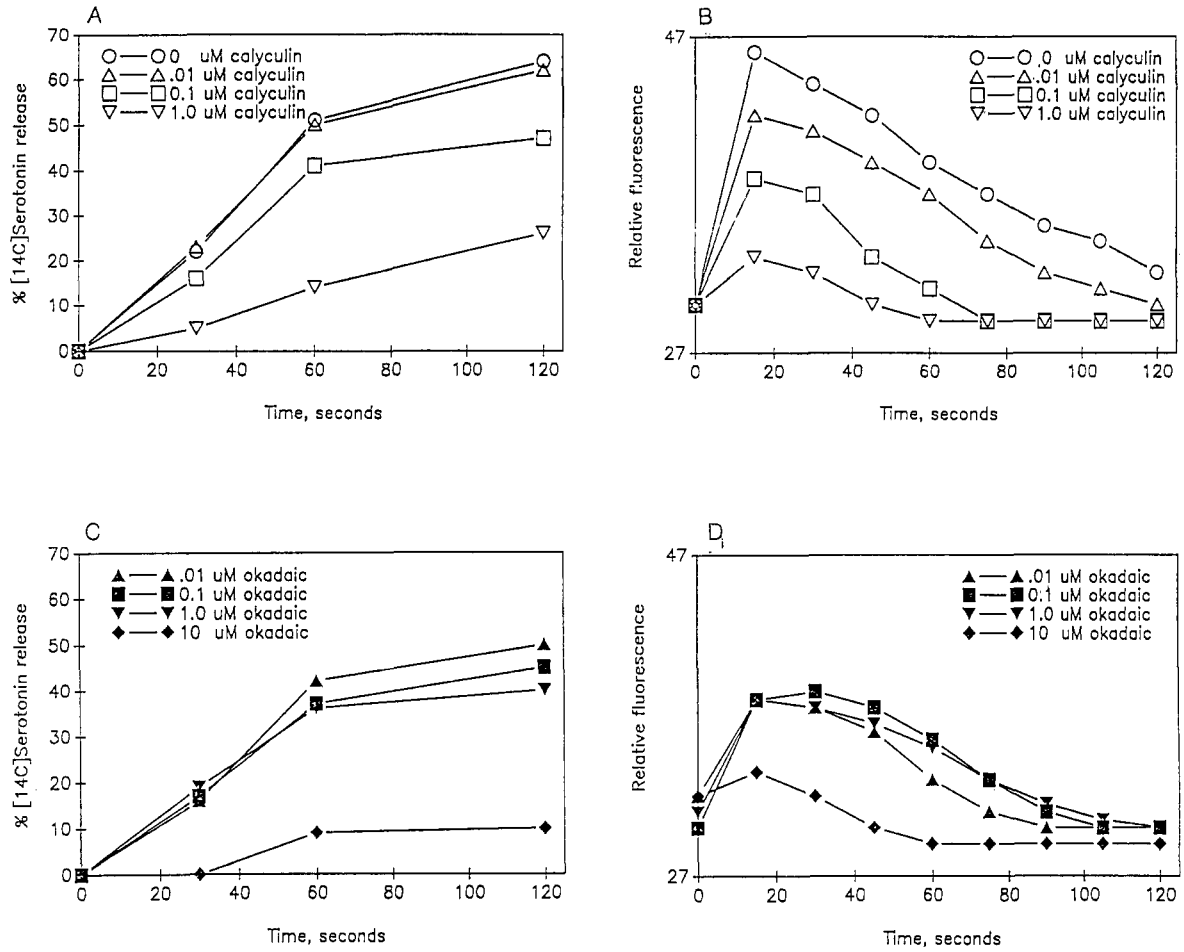

FIGURE 2: Comparative effects of calyculin A or okadaic acid on thrombin-induced serotonin release and Ca^{2+}-transients. Platelets were pretreated for 2 min at 37 °C with the indicated concentrations of calyculin A (open symbols) or okadaic acid (solid symbols) and then further incubated with 0.1 unit/mL thrombin for the indicated time. Panels A and C: The reactions were stopped and the amount of [^{14}C]serotonin released was determined. Panels B and D: To assess Ca^{2+}-transients, the change of emission at 510 nm was continually monitored as described under Experimental Procedures. The results shown in each panel are from one experiment that is typical for what was seen in three to four experiments.

measure of phosphatidylinositol turnover) that occurred after treatment with 0.1 unit/mL thrombin (Figure 3). In contrast, a higher dose of thrombin, 0.5–1 unit/mL, was able to overcome the effect of calyculin A by stimulating the incorporation of [^{32}P]P_i into phosphatidic acid (data not presented). These higher doses of thrombin also overcame the effect of the inhibitors on Ca^{2+} mobilizations (data not presented) and serotonin secretion (Lerea, 1991), suggesting that the compounds "desensitize" platelets to thrombin.

Although calyculin A and okadaic acid attenuate thrombin-induced phosphatidylinositol breakdown and Ca^{2+} mobilization, no differences in thrombin-induced phosphorylation of p47 and MLC (p20) were observed in okadaic acid- or calyculin A-treated platelets as compared with control platelets (Lerea, 1991). Since these compounds affect phosphatase activities, agonist-induced phosphorylation of proteins may proceed normally if phosphatidylinositol metabolism and Ca^{2+} mobilization are diminished and not totally abolished. Experiments were designed to ascertain whether the phosphorylation of p20 in calyculin-treated platelets is in fact a Ca^{2+}-mediated event. The incorporation of [^{32}P]P_i into p20 was measured in both control and BAPTA-loaded platelets that were treated first with calyculin A and subsequently with thrombin. As shown in Figure 4, BAPTA-loaded platelets (panel C) exhibit a 50% decrease of [^{32}P]P_i incorporation (as assessed by scintillation counting) into p20 in comparison with the controls (panel B). This strongly suggests that p20 phosphorylation in calyculin-treated platelets is being mediated

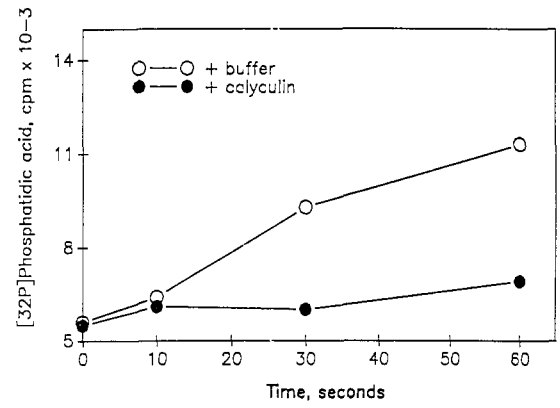

FIGURE 3: Effect of calyculin A on thrombin-induced phosphatidic acid formation. Human platelets were labeled with [^{32}P]orthophosphate for the measurement of phosphatidic acid formation. The assays were conducted as described under Experimental Procedures using 0.1 unit/mL thrombin. The results are from one experiment that is typical for what was seen in three experiments.

by Ca^{2+}. As observed with okadaic acid (Lerea, 1991), treatment with calyculin A alone does not induce the phosphorylation of p20 or p47 (see Figure 6B), but it does increase the phosphorylation of proteins of 50 kDa (Figure 4, panels D–F). Interestingly, these calyculin-induced phosphorylations are not decreased in the BAPTA-loaded platelets, indicating that the calyculin-induced phosphorylation events are not Ca^{2+}-dependent.

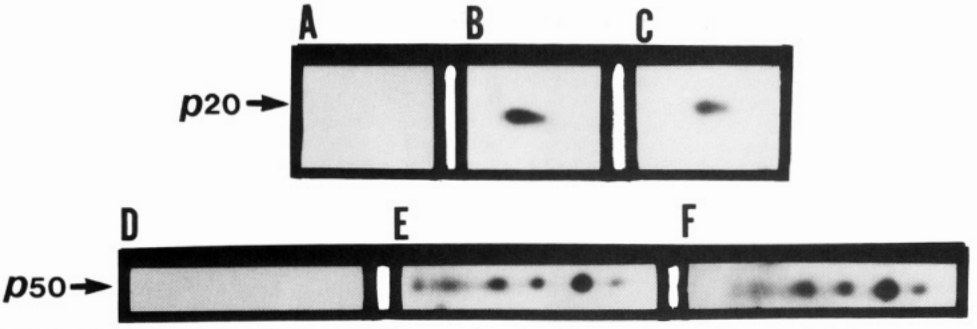

FIGURE 4: Thrombin-induced phosphorylation of p20 and p50 in control-, calyculin-treated, or BAPTA-loaded platelets. Platelets loaded without (panels A, B, D, and E) or with BAPTA (panels C and F) were labeled with [^{32}P]orthophosphate, as described under Experimental Procedures. The platelets were treated with buffer alone (panels A and D), 0.1 unit/mL thrombin (panels B and C), or 1 μM calyculin A (panels B, C, E, and F). The reactions were stopped by the addition of IEF sample buffer, and the phosphoproteins were separated by two-dimensional gel electrophoresis. The spots corresponding to p20 (panels A–C) and to p50 (panels D–F) were excised, and the amount of radioactivity was assessed by Cerenkov measurements.

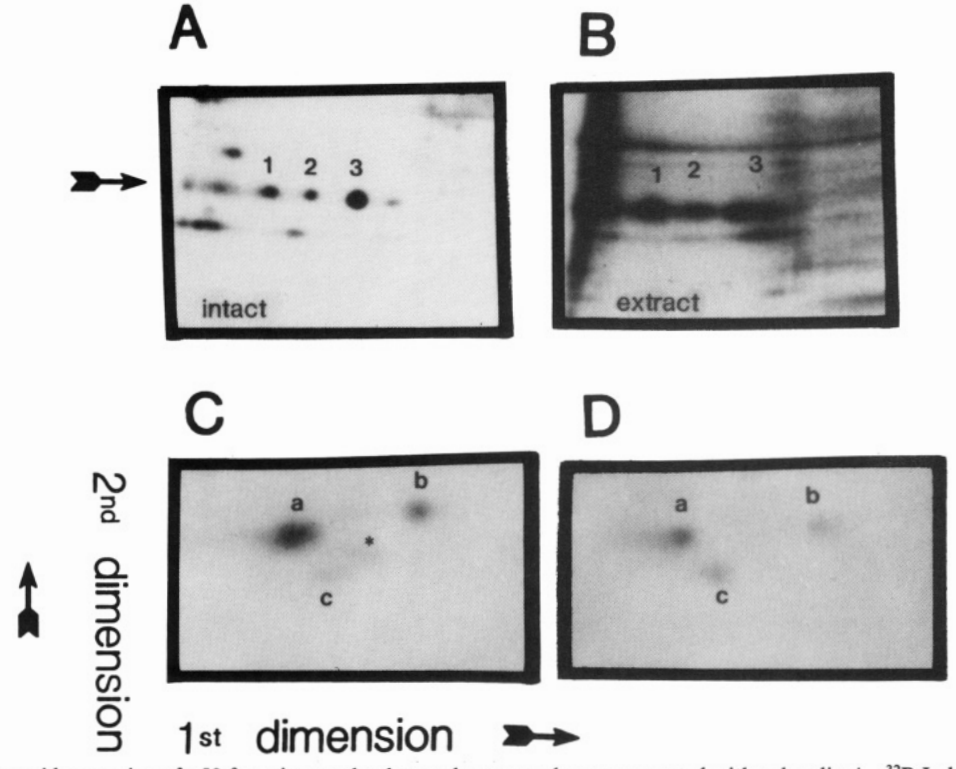

FIGURE 5: Phosphopeptide mapping of p50 from intact platelets and extracts that were treated with calyculin A. ^{32}P-Labeled platelets (panel A) were treated with 1 μM calyculin A, and the phosphoproteins were separated by two-dimensional PAGE. Spot 3 was excised and subjected to phosphopeptide mapping studies (panel C), as described under Experimental Procedures. Similarly, platelet extracts were treated with 1 μM calyculin A in the presence of [γ-^{32}P]ATP, and the phosphoproteins were separated by two-dimensional PAGE (panel B). Spot 1 was subsequently excised and subjected to mapping studies (panel D). The three major phosphopeptide that are common to p50 from intact platelets and extracts are designated as a, b, and c. The additional phosphopeptide found in spot 3 is designated with an asterick. This minor spot is also found in maps of spot 3 isolated form [^{32}P]P_i-labeled extracts (data not shown).

Characterization of the Calyculin A-Stimulated Phosphorylation of p50 in Platelet Lysates. To characterize the phosphorylation reaction that is induced by calyculin A in intact platelets, a cell-free system was designed in order to mimic the response. Platelet extracts were prepared and tested for calyculin A-induced phosphorylation of p50. The addition of calyculin A to extracts results in an increase in phosphorylation of a 50-kDa protein (Figure 5). This phosphorylated protein appears to be the same protein that is phosphorylated in calyculin A-treated platelets for the following reasons: The phosphorylated spots that are identified in extracts comigrate with the 50-kDa phosphoproteins from intact platelets. In addition, phosphoamino acid analyses of each of the spots indicate that calyculin A treatment of intact platelets or extracts specifically increases threonine phosphorylation of p50 (data not shown). Moreover, phosphopeptide analyses were performed to determine the relationship between phosphorylation of p50 in intact platelets and phosphorylation of p50 in vitro. Phosphoprotein spots 1 and 3 from both intact platelets and extracts (see Figure 5, panels A and B) were subjected to tryptic digestion followed by separation on cellulose plates. Regardless of whether the phosphorylation of p50 occurred in the intact system or in extracts, two-dimensional separation of the proteolytic digests resolved three major tryptic phosphopeptides, which are designated as a, b, and c (Figure 5, panels C and D). In all cases, the phosphopeptides migrated similarly with respect to internal tracking dyes. Peptide mapping of spot 3 gave an additional phosphopeptide designated with an asterick (Figure 5, panel C), suggesting that spot 3 is a more highly phosphorylated form of spot 1. From these results shown in Figure 5, it can be deduced that the phosphopeptide digests of p50 that was phosphorylated in vitro

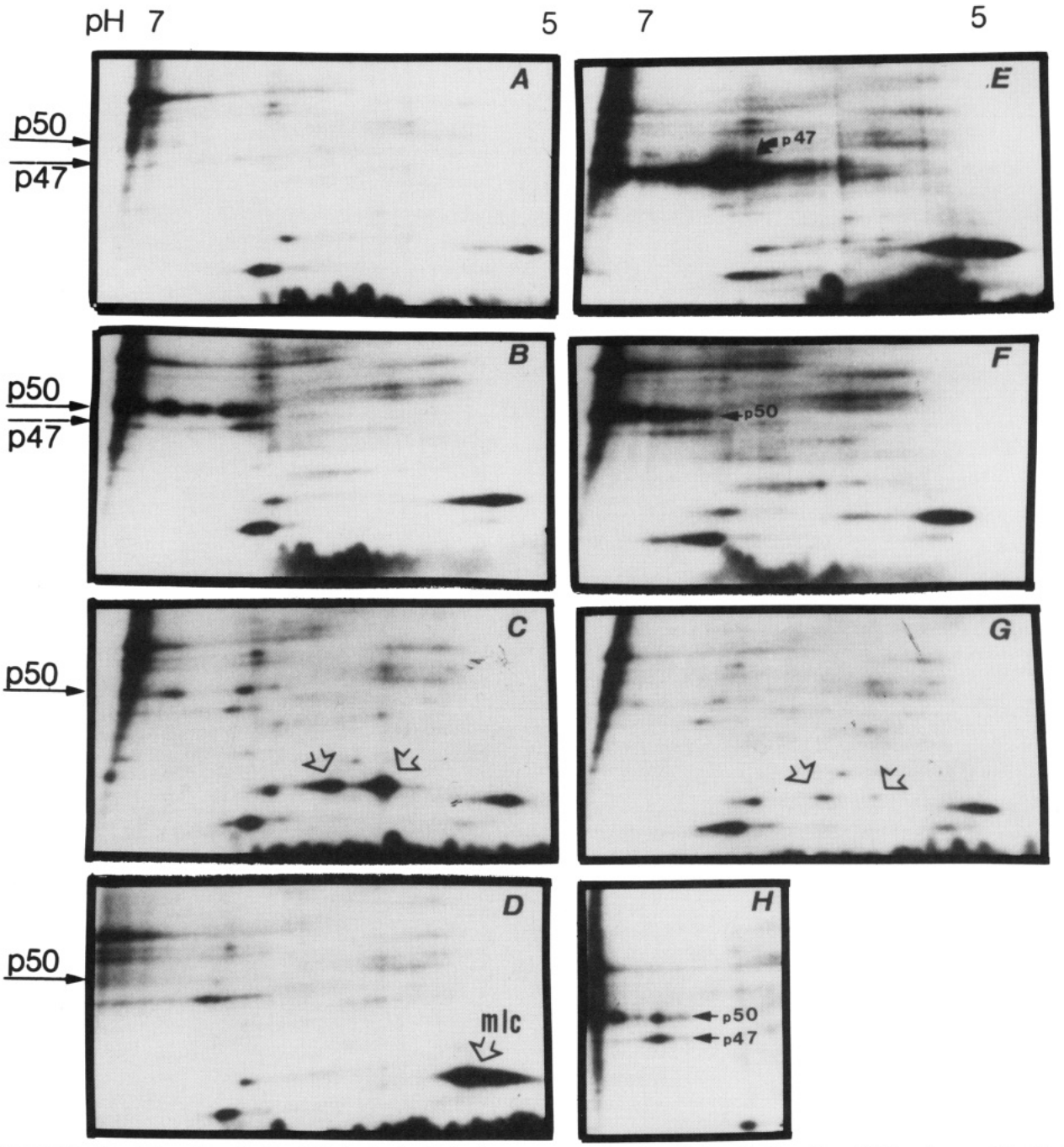

FIGURE 6: Phosphorylation of protein in platelet extracts. Extracts were prepared as described under Experimental Procedures and incubated in the presence of 50 μM [γ-^{32}P]ATP and buffer (panel A), 1 μM calyculin A (panel B), 50 μM cAMP (panel C), 10 μM Ca^{2+} (free concentration, panel D), 10 μM Ca^{2+} and 6 μM/mL diolein and 60 μg/mL phosphatidylserine (panel E), calyculin A and 50 μM PKi (panel F), cAMP and PKi (panel G), or 5 mM *p*-nitrophenyl phosphate and 5 mM β-glycerophosphate and 10 mM sodium pyrophosphate (panel H). Reactions were quenched by the addition of IEF gel buffer, and the proteins were separated by two-dimensional PAGE. Each panel represents at least two different experiments. cAMP-induced phosphorylations that have been typically described within the literature are denoted by the open arrowheads, whereas the phosphorylation of p47, a protein kinase C substrate, and p20, myosin light chain (mlc), also are denoted.

have the same electrophoretic and chromatographic mobilities as those generated from p50 that was phosphorylated in the intact platelet. Together, these data suggest that the same site is being phosphorylated in extracts as in the intact system and that the kinase responsible for this phosphorylation in both situations is likely to be the same.

To test whether known protein kinases are responsible for the increase in phosphorylation of p50, activators or inhibitors of specific protein kinases were added to the extracts. Whereas treatment with calyculin A caused an increase in the incorporation of [^{32}P]P_i into 50-kDa proteins (Figure 6B) in comparison with control assays (Figure 6A), treatment with cAMP (Figure 6C), Ca^{2+} (Figure 6D), a dioleoylglycerol/phosphatidylserine/Ca^{2+} mixture (Figure 6E), and cGMP (data not shown) did not increase [^{32}P]phosphate incorporation into p50. These compounds did, however, increase the incorporation of [^{32}P]phosphate into known substrates. For example, cAMP increases the phosphorylation status of two low molecular weight proteins (Figure 6C, arrowheads), whereas Ca^{2+} alone and in conjunction with diacylglycerol and phosphatidylserine increases the phosphorylation state of MLC and p47, respectively. Cyclic AMP slightly increased the phosphorylation of proteins of 50 kDa, but these phosphorylations appear to differ from those induced by okadaic acid and calyculin A (Lerea, 1991). Moreover, inhibitors of cAMP-dependent protein kinases (e.g., PKi) and casein kinase II (e.g., heparin)

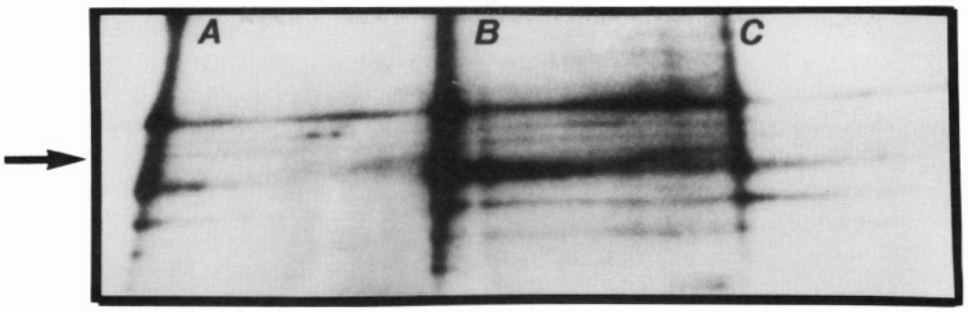

FIGURE 7: Clearance of protein kinase activity by p13^{suc1}–Sepharose. Platelet extracts were incubated with Sepharose 4B (panels A and B) or with p13^{suc1}–Sepharose (panel C) for 2 h at 4 °C. The Sepharose was removed by centrifugation, and phosphorylation studies were conducted using the supernatants as previously described. The phosphoproteins were separated by two-dimensional PAGE as previously described with one modification: only the top 3.5 cm of each IEF gel (total length = 10 cm) was used. Panel A: Supernatants incubated with buffer. Panels B and C: Supernatants incubated with 1 μM calyculin A. The arrow points to p50. Each panel are results from a single experiment that is typical for what was seen in four experiments.

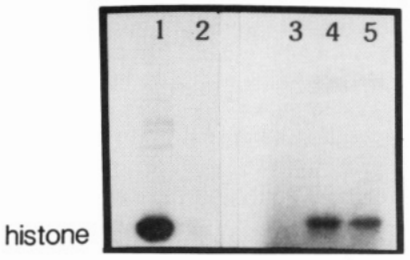

FIGURE 8: Histone phosphorylation using p13^{suc1}–Sepharose pellets. The Sepharose pellets, obtained as described in Figure 7, were washed twice, and histone kinase activity was measured using histones (Sigma type IIIs or VII) and either [γ-^{32}P]ATP (lanes 1 and 2) or [γ-^{32}P]GTP (lanes 3–5), as described under Experimental Procedures. Phosphorylation of histones was conducted in the absence (lanes 1, 3, and 4) or presence (lanes 2 and 5) of hexokinase and glucose. Lane 3 represents the phosphorylation pattern in the absence of histone. The results are from one experiment that is typical for what was seen in four experiments.

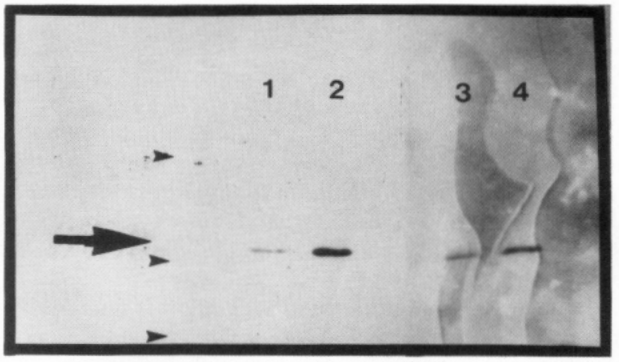

FIGURE 9: Immunodetection of p34^{cdc}-like protein kinase from p13^{suc1}–Sepharose precipitates. Platelet and HL60 extracts were incubated with p13^{suc1}–Sepharose as described under Experimental Procedures. Absorbed proteins were solubilized by SDS and separated on a 5–15% SDS–polyacrylamide gel. Western analysis was performed using a mouse anti-PSTAIR antibody (lanes 1 and 2) and a rabbit anti-cdk2 antibody (lanes 3 and 4). Lanes 1 and 3 represent proteins absorbed from platelet lysates, and lanes 2 and 4 represent proteins absorbed form HL60 extracts. The arrow points to proteins at 32 kDa. The arrowheads point to prestained molecular weight standards of ca. 29 000, 48 000, and 75 000. This result is from one experiment that is typical for what was seen in two to four experiments.

did not decrease calyculin A-induced phosphorylations (Figure 6F and data not shown), further suggesting that these protein kinases are not involved in such phosphorylations. The concentration of PKi that is used in the assay did block cAMP-induced phosphorylation events (Figure 6G). Interestingly, treatment of extracts with a mixture of phosphatase inhibitors, i.e., 5 mM *p*-nitrophenyl phosphate, 5 mM β-glycerophosphate, and 10 mM sodium pyrophosphate, led to the phosphorylation of several proteins including proteins of 50 and 47 kDa (Figure 6H). These data suggest that calyculin A is causing selective phosphorylations by inhibiting specific phosphatases, whereas more general phosphatase inhibitors promote the increase of [^{32}P]phosphate into several proteins.

Precipitation with p13^{suc1}–Sepharose. To assess whether a homolog of *Schizosaccharomyces pombe* p34^{cdc2} is the protein kinase responsible for the phosphorylation of p50, we utilized p13^{suc1} that has been bound to Sepharose to absorb cdc2-related protein kinases from extracts. Cytosolic extracts were incubated with p13^{suc1}-Sepharose, and the unbound material was assayed for p50 phosphorylation activity. Using preabsorbed extracts, no p50 phosphorylation was detected following the addition of calyculin A (Figure 7). In contrast, protein kinase C activity is still detected in the preabsorbed extracts, which suggests that nonspecific depletion of kinase activity was not occurring. To establish whether a cdc2-like kinase is absorbed to the p13–Sepharose, histone kinase activity was measured using either [γ-^{32}P]ATP or [γ-^{32}P]GTP (Figure 8). The precipitated histone kinase was able to utilize GTP as a phosphate donor, although to a lesser extent. This property is unique to a limited number of enzymes, including cdc2 protein kinases (Erikson & Maller, 1989). Since incubation with hexokinase and glucose does not abolish histone kinase activity in the presence of GTP (Figure 8, lane 5) but does in the presence of ATP (Figure 8, lane 2), the utilization of GTP is not caused by the contamination of a GTP–ADP phosphotransferase activity. This has been further substantiated by separating ATP from GTP via thin-layer chromatography and finding that [γ-^{32}P]ATP is not being formed by incubating p13^{suc1}–Sepharose pellets with [γ-^{32}P]GTP (data not shown).

To further assess the presence of p34^{cdc2} or related protein kinases in p13^{suc1}–Sepharose precipitates, Western analyses were performed with a mouse monoclonal antibody that was generated against amino acid residues 30–57 (PSTAIR) of human cdc2. The antibody only recognized a single 32-kDa platelet protein in the p13^{suc1}–Sepharose precipitate (Figure 9, lane 1). This immunoreactive protein migrated with the same molecular weight as HL60 proteins that are also detected by anti-PSTAIR antibodies (Figure 9, lane 2). To determine the identity of this immunoreactive platelet protein, immunoblotting procedures were conducted using antipeptide antibodies prepared to the C–terminal sequences of cdk2 and p34^{cdc2} (Rosenblatt et al., 1992; Litchfield et al., 1991). The anti-cdk2 peptide antiserum recognized the same 32-kDa protein in platelet and HL60 cells as the PSTAIR monoclonal antibody (Figure 9, lanes 3 and 4). In contrast, anti-cdc2 antibodies reacted very weakly to p13^{suc1} precipitates of platelet

lysates but strongly to HL60 lysates (data not shown). These data indicate that the protein kinase absorbing to $p13^{suc1}$–Sepharose is mostly cdk2.

The effect of calyculin A on inducing phosphorylation of p50 may be explained by the inhibition of protein phosphatases 1 or 2A, or from the potential "activation" of cdk2. To distinguish between these two potential mechanisms, quantitative changes in histone H1 kinase activity of the $p13^{suc1}$–Sepharose precipitates were measured. Using H1 as a substrate, a substantial amount of H1 kinase activity was observed in the precipitates of untreated extracts (1200 ± 330 cpm/10 min, mean ±SD, $n = 3$). Only a modest increase in histone kinase activity was found (1800 ± 300 cpm/10 min, mean ± SD, $n = 3$) when extracts were treated with 1 μM calyculin A prior to absorption to $p13^{suc1}$–Sepharose, suggesting that the incorporation of $[^{32}P]P_i$ into p50 is most likely due to the inhibition of protein phosphatases.

Discussion

To define the role of protein serine/threonine phosphatases in platelet functions, inhibitors of type 1 and 2A protein phosphatases, calyculin A and okadaic acid, were used to determine which key steps in the signal pathway leading to secretion are affected. With this approach, it appears that certain responses, such as phosphorylation of p47 and myosin light chains, are not inhibited (Lerea, 1991): This suggests that okadaic acid/calyculin A-sensitive steps occur subsequent to thrombin-induced early events such as phosphatidylinositol hydrolysis and Ca^{2+} mobilization. These early events, however, are affected by treatment with phosphatase inhibitors, which is consistent with the observations of Karaki et al. (1989). This may reflect the concept that calyculin A alters the balance of kinases and phosphatases within cells by inhibiting the latter enzymes. Increases in Ca^{2+} fluxes that are below detectable levels may occur following the addition of thrombin to calyculin-treated platelets. Consistent with this possibility is that the incorporation of $[^{32}P]$phosphate into myosin light chains (p20) is diminished in BAPTA-loaded platelets, strongly suggesting that p20 phosphorylation in calyculin-treated platelets is being mediated by Ca^{2+}. However, the precise molecular mechanism by which okadaic acid and calyculin A affect platelet responses remains to be determined. The agents may desensitize the thrombin receptor by increasing receptor phosphorylation. It has been previously reported that PDGF and EGF receptors are down-regulated following exposure of fibroblasts to okadaic acid (Dean et al., 1991). Moreover, results from recent studies suggest that phosphorylation of the thrombin receptor participates in the desensitization of cells to thrombin (Brass, 1992). Alternatively, calyculin A and okadaic acid may inhibit steps involved in phophoinositide turnover distal to the receptor (Garcia-Sainz et al., 1991).

In our system, addition of calyculin A or okadaic acid consistently stimulates the phosphorylation of the 50-kDa protein. Data from tryptic phosphopeptide map analyses suggest that the multiple phosphorylated spots that are routinely observed result from multiple phosphorylations on one protein. Moreover, calyculin A treatment of intact platelets and extracts induces phosphorylation of the 50-kDa proteins that have similar tryptic phosphopeptide maps, suggesting that the same protein serine/threonine kinase is responsible for the phosphorylation of p50 in both systems. Thus, the platelet extract system, together with a systematic analysis, allows us to identify the protein kinase responsible for the phosphorylation of p50. Cyclic nucleotide-dependent protein kinases have been seen to antagonize platelet functions (Packham et al., 1980), yet several lines of evidence presented in this paper indicate that another specific protein kinase may actually mediate calyculin or okadaic acid effects. First, the addition of cyclic nucleotides fails to affect phosphorylation of p50. Second, an inhibitor of the cAMP-dependent protein kinase does not impede calyculin-induced phosphorylation of p50. The third and most compelling evidence is that $p13^{suc1}$–Sepharose removed the kinase that phosphorylated p50 from platelet extracts without affecting other kinase activities, e.g., protein kinase C. On the basis of the high affinity of $p34^{cdc2}$ for $p13^{suc1}$ (Maller, 1990) and on our findings using $p13^{suc1}$–Sepharose, it appears that a cdc2 or cdc2-related protein kinase is reponsible for the phosphorylation of p50. Recently, Samiei et al. (1991) have demonstrated the presence of a $p34^{cdc2}$ homologue in sheep platelets using antibodies raised against regions corresponding to the PSTAIR and the carboxyl terminus of murine cdc2. However, the present study supports the presence of cdk2 in human platelets. The precise role of cdc2 or cdk2 in platelets remains to be determined; however, the detection of a $p34^{cdc2}$-related protein kinase in platelets indicates a role for such protein kinases in events not necessarily linked to the cell cycle.

How cdk2 is regulated in platelets is not known. Inasmuch cdk2 isolated from HeLa cells contains phosphotyrosine (Elledge et al., 1992), its activity may be controlled by phosphorylation as shown for $p34^{cdc2}$. Using anti-phosphotyrosine antibodies, phosphotyrosine was not detected in $p13^{suc1}$–Sepharose platelet precipitates (Samiei et al., 1991), suggesting that "active" cdc2-related protein kinases may be found in unactivated platelets.

Although most reports have focused on understanding the role of a limited number of protein kinases in platelet functions, it appears likely that other protein kinases may also maintain a balance in the activation state of platelets. Recent data support a role for protein–tyrosine kinases during the activation process (Ferrell & Martin, 1988; Golden & Brugge, 1989; Nakurama & Yamamura, 1989). Moreover, Ferrell and Martin (1989) have shown an abundance of protein kinases, which have yet to be identified, within platelets. Data from the present studies, in addition to others (Samiei et al., 1991), further suggest a role for a cdc2-related protein kinase in regulating platelet responses.

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