Articles

Thrombin-Induced Effects Are Selectively Inhibited following Treatment of Intact Human Platelets with Okadaic Acid†

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ABSTRACT: The involvement of protein phosphatases in regulating platelet activation was studied. The major portion of the phosphorylase phosphatase activity found in platelet lysates appears to be of the type 1 variety. The identification of this enzyme was based on the finding that greater than 80% of protein phosphatase activity was inhibited by the heat-stable inhibitor protein inhibitor 2 and, while only 20% of the phosphorylase phosphatase activity in platelet extracts was inhibited by 2 nM okadaic acid, greater than 95% of the activity was inhibited in the presence of 1 μM okadaic acid. Increases in protein phosphorylations occurred and thrombin-induced release of serotonin was prevented as a result of artificially inhibiting the enzyme with okadaic acid in intact platelets. This implies either that the regulation of okadaic acid sensitive protein phosphatases is necessary for some agonist-induced effects or that okadaic acid sensitive phosphatases are required for maintaining platelets in a responsive state.

Platelets play a critical role in the coagulation process and depend on extracellular signals for the regulation of their responsiveness. For example, positive effectors such as thrombin trigger a series of biochemical events that cause platelets to change shape, secrete intracellular substances such as serotonin, and aggregate [reviewed in Siess (1989)]. In contrast, negative effectors such as prostacyclin (PGI_2)[1] and PGE_1 maintain platelets in a nonreactive state (Siess, 1989). It has been recognized since the mid-1970's that protein phosphorylation events play important roles in the actions of these effectors (Lyons et al., 1975; Haslam et al., 1979), but the biochemical steps that are affected by phosphorylation are still not known.

Increases in protein kinase activity have been presumed to be responsible for the observed changes in phosphorylation of platelet proteins; the activation process can be mimicked by artificially increasing specific activators of protein kinase C or myosin light chain kinase (MLCK) such as diacylglycerol or Ca^{2+}, respectively (Feinman & Detwiler, 1974; Massini & Luscher, 1974; Kinlough-Rathbone et al., 1977; Rink et al., 1983; Kaibuchi et al., 1984). In contrast, the effects of several antagonists can be mimicked by substances that cause an increase in intracellular cAMP levels, with the subsequent activation of the cAMP-dependent protein kinase (Packham et al., 1980). While much attention has been directed toward these protein kinases, the protein substrates that mediate their actions remain to be identified. While activation of protein kinase C and MLCK leads to the phosphorylation of at least two proteins, pleckstrin (p47) and myosin light chain (p20), respectively (Dabrowska & Hartshorne, 1978; Nishizuka, 1984; Tyers et al., 1989), and the activation of the cAMP-dependent protein kinase causes phosphorylation of several proteins, including those with molecular weights of 22K, 24K, and 50K (Haslam et al., 1979), the physiological significance of these serine/threonine phosphorylation events is still not known.

Phosphorylation is a reversible process, but little is known about the identity of platelet protein phosphatases or their involvement in controlling platelet reactivity. It is increasingly clear from other systems that these enzymes play pivotal roles in cellular functions (Dent et al., 1990; Shenolikar & Nairn, 1991). The goal of the present investigation was to determine the role of specific protein phosphatases in platelets. To this end, we used inhibitor 2, an inhibitor of type 1 protein phosphatase (Shenolikar & Nairn, 1991), and okadaic acid, a tumor promoter isolated from *Halichondria okadaii* that has been found to be a potent inhibitor of type 1 and type 2A protein phosphatases (Haystead et al., 1989). Results with platelet lysates indicate that the major phosphatase present is of the type 1 variety. Moreover, okadaic acid blocked activation of intact platelets at doses that also led to increased phosphorylation of three platelet proteins.

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, phenylmethanesulfonyl fluoride, leupeptin, benzamidine, Triton X-100, and digitonin were from Sigma, and ampholines, urea, acrylamide, and N,N'-methylenebis(acrylamide) were from Bio-Rad. New England Nuclear supplied the [γ-^{32}P]ATP (3000 Ci/mmol), [^{32}P]phosphoric acid (in water), and the cAMP-^{125}I-RIA kit. 5-Hydroxy[2-^{14}C]tryptamine creatine sulfate (54 mCi/mmol) was purchased from Amersham. Phosphorylase *b*, phosphorylase kinase, and inhibitor 2 were generously provided by Dr. Timothy Haystead, University of Washington, Seattle. Okadaic acid was purchased from Moana BioProducts, Hawaii, and Bethesda Research Laboratories. When supplied in dimethylformamide, the okadaic acid was dried under nitrogen and suspended at

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; Hepes, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid; MLCK, myosin light chain kinase; PGE_1, prostaglandin E_1; PGI_2, prostacyclin; PRP, platelet-rich plasma.

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a concentration of 1 mM in 10% dimethyl sulfoxide.

Isolation of Platelets. Human blood was drawn into vacutainer collection tubes containing acid/citrate/dextrose. Platelet-rich plasma was obtained following centrifugation at 1100*g* for 3 min at 21 °C. Following the addition of prostaglandin E_1 (0.4 μM) and apyrases (12.5 milliunits/mL), platelets were removed by centrifugation at 1100*g* for 13 min and washed once in a modified Tyrode's buffer A 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 buffers as described below. Platelets were also isolated from platelet-rich plasma in the absence of PGE_1 by filtration through Sepharose 2B, as described by Nachmias (1980).

Preparation of Platelet Lysates. Soluble and particulate fractions were prepared by sonication procedures or homogenization using a "no clearance" Teflon pestle. Intact platelets were isolated and suspended at a concentration of 1.5×10^9 cells/mL in a buffer that contained 25 mM imidazole, pH 7.0, 4 mM EDTA, 1 mM EGTA, 250 mM sucrose, 5 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, and 2 μM benzamidine. The suspension was then sonicated in an ice bath with two 15-s bursts at a setting of −50 with a Braun sonicator, disrupted by rapid decompression following equilibration of platelets with nitrogen at 1200 lb/in.^{2} for 20 min, or homogenized with 15 strokes using a "no clearance" Potter–Elvehjem-type tissue grinder. Soluble and particulate fractions were obtained following centrifugation at 4 °C at 13000*g* for 5 min or at 100000*g* for 60 min. Particulate material was solubilized in $^1/_{20}$th volume of homogenization buffer, which contained 0.5% Triton X-100. Following a 10-min incubation period, the particulate fraction was diluted to yield a final Triton concentration of 0.1%.

Digitonin Lysis of Platelets. To isolate platelet cytosol, a digitonin permeabilization procedure was used, as described by Akkerman and co-workers (Akkerman et al., 1980). Isolated platelets were suspended at a concentration of 4×10^8 cells/mL in a modified Krebs–Ringer buffer containing 136 mM NaCl, 2.6 mM KCl, 0.5 mM $MgCl_2$, 5.5 mM glucose, 0.5 mM phenylmethanesulfonyl fluoride, 10 μg/mL leupeptin, and 2 μM benzamidine. Platelet suspensions (450 μL) were mixed with 50-μL digitonin solutions to obtain the final concentrations indicated in Figure 1, incubated for 60 s at 21 °C, layered onto a cushion of 250 μL of a mixture of dibutyl phthalate and dinonylphthalglycerol (3:2, v/v), and centrifuged for 45 s in a Beckman microfuge. The amount of phosphatase activity, lactate dehydrogenase activity, and [^{14}C]serotonin found in the upper layer (cytosol) was assessed.

Measurement of Phosphorylase Phosphatase Activity in Platelet Extracts. Inasmuch as the strategy of the present investigation was to assess in platelets the role of protein phosphatases type 1 and 2A, the only enzymes to have significant phosphorylase phosphatase activity in cells (Ingebritsen et al., 1983), [^{32}P]phosphorylase *a* (1000 cpm/pmol) was prepared as described by Cohen et al. (1989a), adjusted to a concentration of 30 μM ($\epsilon^{1\%}$ at 280 nM = 1.3), and stored at 4 °C. Phosphatase activity in both the soluble and particulate fractions was assayed by measuring the release of ^{32}P from the radiolabeled substrate. The reaction mixtures (30 μL), containing platelet extracts in 25 mM imidazole, pH 7.0, 4 mM EDTA, 5 mM dithiothreitol, 1 mg/mL bovine serum albumin, 1 μM [^{32}P]phosphorylase *a*, and, when indicated, okadaic acid or inhibitor 2, were incubated for 10 min at 30 °C. The reactions were stopped by the addition of 200 μL of trichloroacetic acid (10%, w/v). The amount of ^{32}P found in the aqueous phase was measured in an LKB scintillation counter. One unit of phosphatase activity is defined as the amount that liberates 1 μmol of phosphate per minute. To ensure that all ^{32}P measured was in fact free inorganic phosphate, P_i–molybdate complexes with isobutyl alcohol/benzene (1:1) were extracted routinely (Foulkes et al., 1981).

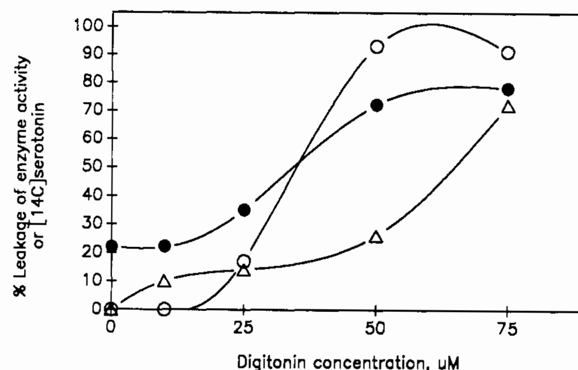

FIGURE 1: Leakage of lactate dehydrogenase, protein phosphatases, and [^{14}C]serotonin from digitonin-permeabilized platelets. Unlabeled or [^{14}C]serotonin-labeled platelets were incubated with varying concentrations of digitonin, and the soluble fraction was removed as described under Experimental Procedures. Lactate dehydrogenase activity (open circles), protein phosphatase activity (solid circles), and [^{14}C]serotonin (open triangles) found in the soluble fraction were then assessed. This graph is representative of six different experiments, and in all experiments, lactate dehydrogenase and phosphatase activities leaked out together and prior to [^{14}C]serotonin leakage.

Measurement of Serotonin Release. Platelets in the PRP were incubated with 2 μM [^{14}C]serotonin (54 mCi/mL) for 60 min at 37 °C. The platelets were collected as described above and suspended at a concentration of 7.5×10^8 cells/mL in modified Tyrode's buffer B containing 5 mM Hepes, pH 7.4, 140 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5.5 mM glucose, and 12 mM $NaHCO_3$. Thrombin-induced serotonin release assays were conducted at 37 °C in a final volume of 62.5 μL as follows: Platelets (3.3×10^7) were preincubated in the presence of buffer alone or varying concentrations of okadaic acid, 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 amount of 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]Phosphoproteins. Platelets were suspended in modified Tyrode's buffer B at a concentration of 7.5×10^8 cells/mL and 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 cells) were pretreated at 37 °C for 2 min with buffer alone, varying concentrations of okadaic acid, or forskolin (50 μM), and then treated with either buffer or thrombin (0.2 unit/mL). Reactions (62.5 μL) were quenched by adding an equal volume of 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 reactions were immediately placed in a dry ice/methanol bath. Phosphoproteins were separated by two-dimensional polyacrylamide gel electrophoresis (O'Farrell, 1975) and visualized by autoradiography.

Measurement of cAMP Levels. Platelets (3.3×10^7 cells) were incubated with buffer, 10 μM okadaic acid, or 10 μM PGE_1 at 37 °C for 2 min. The reactions were quenched by the addition of trichloroacetic acid (10% final concentration). Following extraction of the acid with water-saturated ether, cAMP levels were measured by using a radioimmunoassay kit.

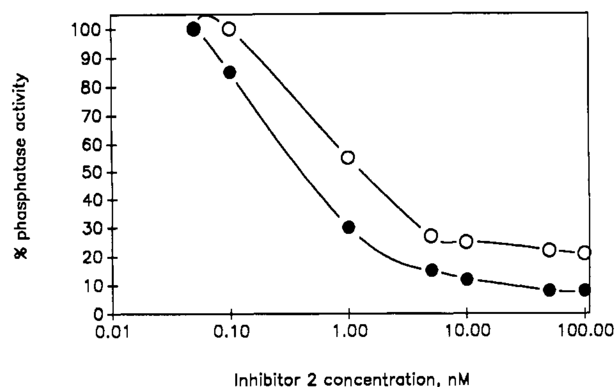

FIGURE 2: Inhibition of platelet phosphorylase phosphatase activity by inhibitor 2. Platelet lysates were prepared by using sonication procedures, as described under Experimental Procedures. Soluble fractions (open circles) and particulate fractions (solid circles) of the extracts were pretreated with increasing concentrations of inhibitor 2 for 5 min, and the amount of phosphatase activity remaining was assessed by using [^{32}P]phosphorylase *a*. This result is representative of three separate experiments.

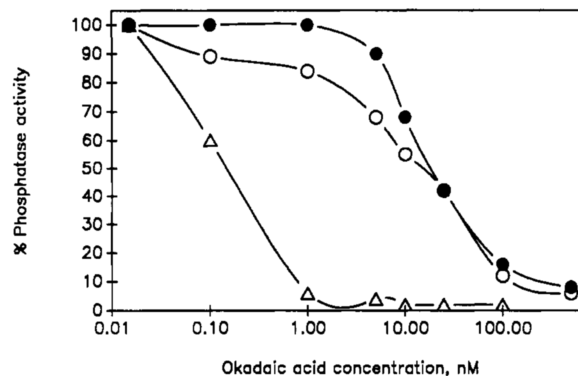

FIGURE 3: Inhibition of platelet phosphorylase phosphatase activity by okadaic acid. Platelet lysates were prepared by using sonication procedures, as described under Experimental Procedures. Soluble fractions (open circles) and particulate fractions (solid circles) of the extracts were pretreated with increasing concentrations of okadaic acid for 5 min, and the amount of phosphatase activity remaining was assessed by using [^{32}P]phosphorylase *a*. Parallel assays were conducted by using an equivalent amount of activity of protein phosphorylase 2A isolated from human erythrocytes (open triangles) to ensure that the concentration of okadaic acid was accurate. This result is representative of three separate experiments.

RESULTS

Serine/Threonine Protein Phosphatase Type 1 and 2A Activity in Human Platelets. The activities of type 1 and type 2A protein phosphatase in human platelet extracts were assessed with phosphorylase *a* as substrate. Phosphorylase phosphatase activity increased 3–4-fold with dilution, an observation described for other tissue extracts (Ingebritsen et al., 1983), and reached a specific activity of 5 ± 2 milliunits/10^9 platelets (mean ± SE, $n = 6$). Moreover, activity was found to be associated with both the particulate and soluble fractions of platelets. When sonicated lysates were used, approximately 80% of phosphorylase phosphatase activity was found in the soluble fraction. This value may be high since sonication can generate small membrane vesicles that fail to sediment at 13000*g*. The amount of soluble protein phosphatase was examined in more detail using post-100000*g* supernatants from lysates that were prepared from platelets homogenized with either a no-clearance Teflon pestle or a nitrogen cavitation: In all cases, similar amounts of soluble phosphatase were measured. In addition, rapid separation of cytosol and particulate fractions of human platelets was achieved by using a digitonin lysis technique described by Akkerman and co-workers (Akkerman et al., 1980). In these experiments, the leakage of protein phosphatase activity from platelets permeabilized with increasing concentrations of digitonin was compared to the leakage of lactate dehydrogenase activity and [^{14}C]serotonin (Figure 1). After treatment with 25–50 μM digitonin, 70–80% of the protein phosphatase activity was recovered in the soluble fraction along with lactate dehydrogenase and prior to the leakage of serotonin ($n = 6$ experiments). Thus, three different experimental approaches were used to obtain a cytosolic fraction, each demonstrating that the major portion of the phosphorylase phosphatase activity was soluble.

To determine whether the activity was that of type 1 or type 2A protein phosphatase, selective inhibitors of the enzymes were employed. Dephosphorylation of phosphorylase *a* by platelet lysates was inhibited by inhibitor 2, a known heat-stable inhibitor of protein phosphatase 1. From the results shown in Figure 2, it is clear that protein phosphatase 1 accounts for greater than 80% of the phosphatase activity found in soluble fractions and 90% of the phosphatase activity found in particulate fractions, of sonicated platelet lysates. This is further demonstrated in experiments that make use of okadaic acid: Levels of okadaic acid required to inhibit platelet phosphatase activity (Figure 3) were similar to the reported concentration needed to block type 1 protein phosphatase in vitro (Cohen et al., 1989b). The IC_{50} for inhibition of phosphorylase phosphatase activity from approximately 1 × 10^6 platelets is 15–30 nM. In contrast, the IC_{50} for the inhibition of an equivalent amount of type 2A phosphatase activity obtained from human erythrocytes is approximately 0.2 nM (Figure 3). Together, these data suggest that most of the phosphorylase phosphatase activity in human platelets is of the type 1 variety and that only approximately 20% of the activity is of type 2A. The same relative amounts of these enzymes were found in platelets that were immediately solubilized with 0.2% Triton X-100, and in fractions obtained from platelets that were homogenized by using nitrogen cavitation or a no-clearance homogenizer (data not shown).

Effect of Okadaic Acid on Serotonin Release. The effect of okadaic acid on platelet responses was determined by using thrombin-induced serotonin release from [^{14}C]serotonin-labeled platelets as the measurement of platelet activation. The concentration of thrombin used in most of the experiments, 0.2 unit/mL, reflects physiological levels (Shuman et al., 1979) and has effects which are tightly regulated by platelet antagonists (Lerea et al., 1987). A short preincubation of platelets with 10–15 μM okadaic acid resulted in a substantial decrease in the ability of thrombin to activate them (Figure 4). In these experiments, platelets isolated by centrifugation procedures were routinely used. Similar results were obtained in experiments that made use of gel-filtered platelets that were isolated in the absence of PGE_1 (data not shown). The inhibition by this concentration of okadaic acid was consistently observed with different preparations of the drug. Preincubation of platelets with 0.1 and 1 μM okadaic acid did not alter the release of serotonin induced by 0.2 unit/mL thrombin (Figure 5). Although 10 μM okadaic acid was also required to inhibit serotonin release induced by 0.1 unit/mL thrombin (Figure 5), it was not sufficient to prevent serotonin secretion induced by 0.5 or 1 unit/mL thrombin.

It was important to exclude the possibility that okadaic acid increased platelet cAMP levels, since high levels of the cyclic nucleotide inhibit secretion reactions (Lerea et al., 1987). When platelets were exposed to buffer alone or to 10 μM okadaic acid, cAMP levels remained low (data not shown). In parallel reactions, platelet cAMP increased from basal levels

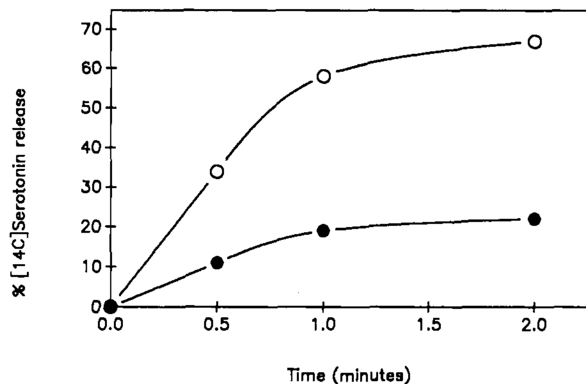

FIGURE 4: Time course of [^{14}C]serotonin release in the presence of okadaic acid. [^{14}C]Serotonin-labeled platelets were preincubated at 37 °C for 2 min with either buffer alone (open circles) or 15 μM okadaic acid (solid circles) and then incubated with 0.2 unit/mL thrombin. At the times indicated, aliquots were removed, and the amount of [^{14}C]serotonin released was assessed, as described under Experimental Procedures.

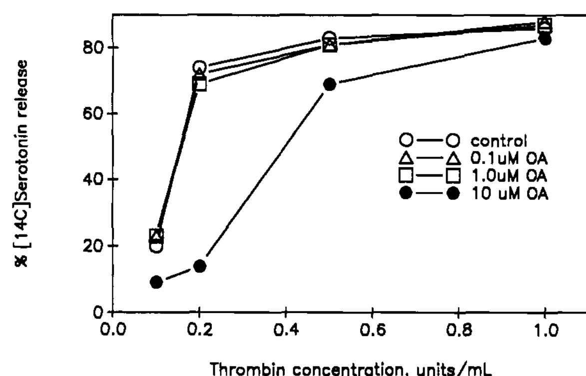

FIGURE 5: Dose response for the effect of okadaic acid on thrombin-induced serotonin release. Platelets were pretreated for 2 min at 37 °C with 0 (open circles), 0.1 (open triangles), 1 (open squares), or 10 μM (solid circles) okadaic acid and then further incubated with the indicated amount of thrombin for 2 additional min. The reactions were stopped, and the amount of [^{14}C]serotonin released was determined. The results represent two to four different experiments.

of 5–10 pmol/10^8 platelets to values of 40 pmol/10^8 platelets following exposure to PGE_1 (10^{-5} M). These data suggest that okadaic acid inhibits serotonin secretion independently of cAMP levels.

Okadaic Acid Induced Phosphorylation of Proteins in Intact Platelets. Previous studies have shown that okadaic acid can readily enter cells and have an effect on the phosphorylation state of intracellular proteins (Haystead et al., 1989). To establish a relationship between the effect of okadaic acid on secretion reactions and protein phosphorylation, ^{32}P-labeled platelets were exposed to the phosphatase inhibitor, and proteins were separated by two-dimensional gel electrophoresis. Upon the addition of 10 μM okadaic acid, several changes in protein phosphorylation occurred; most notable was the prominent increase in phosphorylation of three polypeptides, all with an apparent molecular weight of 50K (Figure 6A,B). This pattern of labeling was observed in 10 separate gels. No changes in the phosphorylation of proteins were observed following the exposure of platelets to 0.1 μM okadaic acid. Exposure of platelets to 1 μM okadaic acid caused a prominent increase in the content of [^{32}P]phosphate in only one of the protein spots, i.e., the most basic of the 50-kDa polypeptides (data not shown).

Interestingly, known inhibitors of platelet functions, such as PGE_1, PGI_2, and forskolin, also induce protein phosphorylation within platelets. The phosphorylation pattern of ^{32}P-labeled proteins from forskolin-treated platelets (Figure 6C), however, was different from that observed on two-dimensional gels of phosphoprotein from okadaic acid treated platelets (Figure 6B). In particular, when ^{32}P-labeled platelets were treated with either forskolin or PGE_1, increases in the phosphorylation of 22- and 24-kDa proteins were observed in the region of the open arrows (Figure 6B,C). Interestingly, these proteins became hyperphosphorylated in ^{32}P-labeled platelets that were treated with forskolin in the presence of okadaic acid (data not shown), suggesting that they are substrates for okadaic acid sensitive phosphatases, but that phosphorylation occurs only if the cAMP-dependent protein kinase is active. While forskolin and PGE_1 also cause an increased incorporation of [^{32}P]phosphate into two polypeptides of 50 kDa, whether the 50-kDa polypeptides that become labeled with [^{32}P]phosphate in the presence of okadaic acid are the same as those proteins that become labeled following treatment with prostaglandins or forskolin remains to be determined. When coelectrophoresed, the most acidic component of the 50-kDa polypeptides phosphorylated following treatment with okadaic acid comigrated in two out of three experiments with the most basic component of the 50-kDa polypeptide phosphorylated in the presence of agents such as forskolin or PGE_1 (data not shown).

To investigate the possibility that inhibition of thrombin-induced events proximal to secretion might account for the effect of okadaic acid on serotonin release, thrombin-induced phosphorylations were measured. The addition of thrombin (0.2 unit/mL) to ^{32}P-labeled platelets resulted in the rapid increase in [^{32}P]phosphate in two proteins: p47, a substrate of protein kinase C that migrates as a 45-kDa protein in Figure 6D, and p20, myosin light chain (Figure 6D). When platelets were treated first with okadaic acid, thrombin still increased the phosphorylation of p47 and p20 (Figure 6E), suggesting that early thrombin-induced events such as phosphatidylinositol hydrolysis and calcium mobilization were okadaic acid insensitve. In parallel assays, the same diluted stock of okadaic acid inhibited serotonin release by 80%. Interestingly, when thrombin was added to okadaic acid treated platelets, a decrease in labeling of the two acidic p50 spots was routinely observed. This may reflect the activation of protein phosphatase 2B, a Ca^{2+}-dependent enzyme whose activity is not affected by the concentrations of okadaic acid used in the present study (Cohen et al., 1990).

Discussion

Three types of protein phosphatases have been identified in human platelets: a phosphorylase phosphatase (Gergely et al., 1980), protein serine/threonine phosphatase type 2B (Tallant & Wallace, 1985; Tallant et al., 1988), and protein tyrosine phosphatases (Lerea et al., 1989). The role that each of these phosphatases may play in regulating platelet responses is not clear. On the basis of the amount of activity found in platelets, Gergely and co-workers (Gergely et al., 1980) proposed that, in addition to its role in glycogen metabolism, phosphorylase phosphatase is likely to affect other platelet processes. The present studies attempted to test this hypothesis by inhibiting its activity in vivo. The addition of 10 μM okadaic acid to intact platelets inhibited thrombin-induced release of serotonin. The concentration of okadaic acid required to affect cellular functions depends on the intracellular concentration of type 1 and type 2A phosphatases (Cohen et al., 1989b). In other cells, the concentration of these enzymes ranges from 0.1 to 1 μM (Cohen et al., 1989b). We considered the possibility that okadaic acid affected platelet activation in a similar manner to known antagonists, such as PGI_2 and

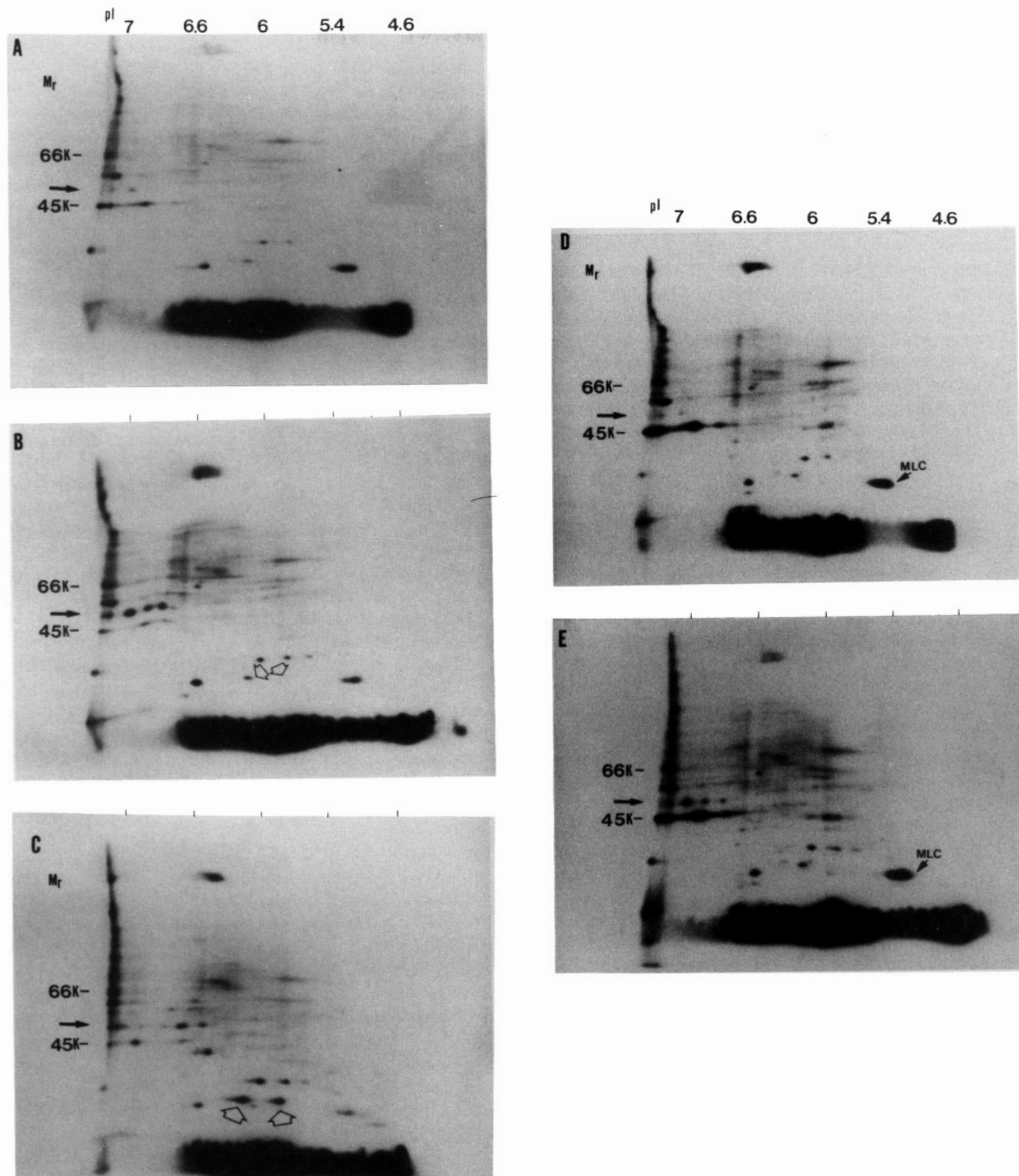

FIGURE 6: Effect of okadaic acid on the phosphorylation of platelet proteins. Platelets were labeled with [^{32}P]orthophosphate as described under Experimental Procedures. The platelets were pretreated for 2 min with buffer alone (panels A and D), 10 μM okadaic acid (panels B and E), or 50 μM forskolin (panel C), and then incubated for an additional 2 min following the addition of either buffer (panels A–C) or 0.2 unit/mL thrombin (panels D and E). The reactions were stopped by the addition of IEF sample buffer and the phosphoproteins separated by two-dimensional gel electrophoresis. The autoradiograms shown in panels A–C each represents five different experiments performed in duplicate. Those shown in panels D and E each represents two different experiments.

PGE_1. Still, it is not likely that the effects of okadaic acid are due to changes in adenylate cyclase activity since okadaic acid did not change cAMP levels. Furthermore, agents that increase cAMP inhibit other thrombin-induced responses such as phosphorylation of p47 and myosin light chains, whereas these thrombin-induced phosphorylation events still occurred in the presence of okadaic acid. This observation is intriguing in that the okadaic acid sensitive step occurs subsequent to early thrombin-induced events such as phosphatidylinositol hydrolysis and Ca^{2+} mobilization but prior to the secretory process. Comparisons between okadaic acid and PGE_1/forskolin-stimulated protein phosphorylations reveal additional qualitative differences. Okadaic acid treatment led to consistent increases in the phosphate content of polypeptides of 50 kDa, whereas treatment with agents that raise cAMP levels stimulated 22-kDa, 24-kDa, and also 50-kDa phosphorylations. Finding unique protein phosphorylations following okadaic acid treatment is consistent with results that demonstrate that the

inhibitory effect of okadaic acid is different from that of cAMP. The possibility remains that the 50-kDa proteins which become phosphorylated in okadaic acid and PGE_1/forskolin-treated platelets, are related despite the likelihood that the molecular mechanism by which okadaic acid acts is distinct from that of PGE_1. One might speculate that okadaic acid enhances the inhibitory effects of agents such as PGE_1, but addition of 1 μM okadaic acid to platelets treated with varying concentrations of PGE_1 did not enhance the effect of the prostaglandin with respect to serotonin release (unpublished observations).

Okadaic acid most likely acts via the inhibition of protein phosphatases. This is supported by the following: (1) a correlation is observed between the phosphorylation of a 50-kDa protein and the inhibition of serotonin secretion (this study); (2) endogenous protein kinases within the platelet, e.g., protein kinase C and myosin light chain kinase, are not inhibited (this study); and (3) okadaic acid does not affect the activity of several other protein kinases including the cAMP-dependent protein kinase (Haystead et al., 1989; Cohen et al., 1990).

The results of the present study show that human platelet responses induced by low levels of thrombin may be blocked by okadaic acid, whereas responses to high levels of thrombin are not affected. Aggregation of rabbit platelets induced by 1 unit/mL thrombin was inhibited by micromolar concentrations of okadaic acid (Karaki et al., 1989). Whether or not this reflects a species difference remains to be determined.

In summary, okadaic acid makes human platelets resistant to activating agents such as thrombin, which is analogous to the *net* effect of PGE_1 and forskolin. This finding raises the intriguing possibility that okadaic acid sensitive protein phosphatases might be instrumental in the regulation of platelet activation. The role that protein phosphatases play in platelet responses is not yet clear, but it should be noted that protein phosphatase activities appear to be regulated by many hormones (Shenolikar & Nairn, 1991). Thus, an important question that remains to be answered is their possible regulation by platelet agonists and antogonists.

Acknowledgments

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Registry No. Protein phosphatase, 9025-75-6; thrombin, 9002-04-4; serotonin, 50-67-9; phosphorylase phosphatase, 9025-74-5.

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