

# Interaction of $\text{Ca}^{2+}$ and Protein Phosphorylation in the Rabbit Platelet Release Reaction

ROGER M. LYONS and JAMES O. SHAW, *Divisions of Hematology and Pulmonary Diseases, Department of Medicine, University of Texas Health Science Center at San Antonio, and Audie L. Murphy Memorial Veterans Administration Hospital, San Antonio, Texas 78284*

**ABSTRACT**  $\text{Ca}^{2+}$  flux and protein phosphorylation have been implicated as playing an important role in the induction of the platelet release reaction. However, the interactions between  $\text{Ca}^{2+}$ , protein phosphorylation, and the release reaction have been difficult to study because secretion in human platelets is independent of extracellular  $\text{Ca}^{2+}$ . Thus, we studied rabbit platelets, which, unlike human platelets, require extracellular  $\text{Ca}^{2+}$  for serotonin release to occur. Thrombin, basophil platelet-activating factor (PAF), or ionophore A23187 treatment of intact  $^{32}\text{PO}_4^{3-}$ -loaded rabbit platelets resulted in a 200–400% increase in phosphorylation of two peptides of  $M_r$  41,000 and 20,000, designated as P7P and P9P, respectively. These peptides were similar in all respects to the peptides phosphorylated in thrombin-treated human platelets. When  $\text{Ca}^{2+}$  was replaced in the medium by EGTA, (a) thrombin- and PAF-induced rabbit platelet  $^{3}\text{H}$ serotonin release was inhibited by 60–75%, whereas ionophore-induced release was blocked completely; (b) thrombin-, PAF-, or ionophore-induced P9P phosphorylation was inhibited by 60%; and (c) ionophore-induced P7P phosphorylation was decreased by 60%, whereas that caused by thrombin or PAF was decreased by only 20%. At 0.25–0.5 U/ml of thrombin, phosphorylation preceded  $^{3}\text{H}$ serotonin release with the time for half-maximal release being  $26.0 \pm 1.3$  s SE ( $n = 3$ ) and the time for half-maximal phosphorylation being  $12.3 \pm 1.3$  s SE ( $n = 3$ ) for P7P and  $3.7 \pm 0.17$  s SE ( $n = 3$ ) for P9P. P9P phosphorylation was significantly inhibited ( $P < 0.015$ ) by removal of  $\text{Ca}^{2+}$  from the medium at a time point before any thrombin- or ionophore-induced serotonin release was detectable. Thus, our data suggest that  $\text{Ca}^{2+}$  flux precedes the onset of serotonin secretion and that the

rabbit platelet is an appropriate model in which to study the effects of  $\text{Ca}^{2+}$  on protein phosphorylation during the platelet release reaction.

## INTRODUCTION

Human platelets are not dependent on extracellular  $\text{Ca}^{2+}$  for secretion (the release reaction) to occur (2, 3). Despite this observation, strong circumstantial evidence suggests that  $\text{Ca}^{2+}$  is mobilized from an intracellular source to induce the extrusion phase of secretion (2–6). However, it has been difficult to document that cellular  $\text{Ca}^{2+}$  flux occurs before the onset of secretion (4). This difficulty is caused, in part, by the intracellular origin of regulatory  $\text{Ca}^{2+}$ , a  $\text{Ca}^{2+}$  pool that cannot be readily manipulated.

Cellular protein phosphorylation may also play a critical role in mediating secretion (7). We previously reported that thrombin treatment of intact  $^{32}\text{PO}_4^{3-}$ -loaded human platelets resulted in phosphorylation of two specific peptides of  $M_r$  41,000 and 20,000 (termed P7P and P9P, respectively) as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)<sup>1</sup> (8). The time-course of phosphorylation was similar to that of serotonin secretion (release I [9]); this suggests that phosphorylation of these two peptides may be important in induction of the release reaction. A subsequent report has described a variety of other agents that both induce the platelet release reaction and stimulate phosphorylation of P7P and P9P (10).

P7P was purified to homogeneity and its characteristics were compared to those of several platelet proteins with some features in common with P7P (11). The data indicated that P7P was not actin, tubulin, or the type II regulatory subunit of a cyclic AMP-dependent protein

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<sup>1</sup>Abbreviations used in this paper: DB-cAMP, dibutyryl adenosine 3'-5' monophosphate; PAF, rabbit basophil platelet-activating factor; PGE<sub>1</sub>, prostaglandin E<sub>1</sub>; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; T<sub>1/2</sub>, half-maximal release.

kinase. Thus, the identity and function of this major soluble platelet phosphoprotein, which comprises 0.65% of the total platelet protein, remain unknown. The protein phosphorylated in intact human platelets, which we designated as P9P, has been tentatively identified as a light chain of platelet myosin (12–14). When the myosin light chain was phosphorylated by its  $\text{Ca}^{2+}$ -dependent protein kinase (15), actin-activated myosin ATPase activity was increased (14). The resultant contraction of the actomyosin is presumed to mediate the release reaction (14, 16).

Although the interactions between  $\text{Ca}^{2+}$  flux and protein phosphorylation have been studied extensively (17–23), the relationship of these processes to secretion has been difficult to study in human platelets because secretion in human platelets does not require extracellular  $\text{Ca}^{2+}$ . Unlike human platelets, rabbit platelets contain little intracellular  $\text{Ca}^{2+}$  (24, 25) and are dependent on extracellular  $\text{Ca}^{2+}$  for the release reaction to proceed (5, 6, 26–29).

We report here that in rabbit platelets, protein phosphorylation during the release reaction is similar to that in human platelets, that release and the state of phosphorylation of P7P and P9P depend on extracellular  $\text{Ca}^{2+}$ , and that protein phosphorylation precedes the release of dense-body constituents.

## METHODS

Carrier-free  $\text{H}_3^{32}\text{PO}_4$  (25 mCi/ml) and 5-[1,2- $^3\text{H}$ ]serotonin creatinine sulfate (25 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. XRP-1 X-Omat medical x-ray film was obtained from Eastman Kodak Co., Rochester, N. Y. Homogeneous Staphylococcal V8 protease was purchased from Miles Laboratories, Inc., Elkhart, Ind. and RNase (Type X11A) was purchased from Sigma Chemical Co., St. Louis, Mo. Prostaglandin  $\text{E}_1$  ( $\text{PGE}_1$ ) was a gift from Dr. John Pike, Upjohn Co., Kalamazoo, Mich. Homogeneous human thrombin with a clotting activity of 2,700 U/mg was a gift from Dr. John Fenton, New York State Department of Health, Albany, N. Y. Ionophore A23187 was a gift from Dr. Robert Hammill, Eli Lilly & Co., Indianapolis, Ind. Highly purified rabbit basophil platelet-activating factor (PAF) was provided by Dr. Neal Pinckard and Dr. Donald Hanahan, University of Texas Health Science Center at San Antonio, San Antonio, Tex. Crude PAF stabilized in 2.5 mg/ml of bovine serum albumin was prepared from immunoglobulin (Ig)E-sensitized rabbit basophils as described by Henson (30). Sodium dodecyl sulfate (SDS) (sequanal grade) was purchased from Pierce Chemical Co., Rockford, Ill. Acrylamide, recrystallized three times, was purchased from Polysciences, Inc., Warrington, Pa.

**Platelet preparation and loading with [ $^3\text{H}$ ]serotonin and  $^{32}\text{PO}_4^{3-}$ .** Platelets were isolated by a modification of the method of Ardlie et al. (31) described by Pinckard et al. (32). Blood was anticoagulated with  $\frac{1}{10}$  vol of acid citrate dextrose and platelet-rich plasma was prepared by centrifugation at 400 g for 20 min at 22°C in the swinging bucket rotor of a Beckman TJ-6 centrifuge (Beckman Instruments, Inc., Fullerton, Calif.). The platelets were loaded with serotonin by incubation with 0.8  $\mu\text{Ci}$  of [ $^3\text{H}$ ]serotonin/ml of platelet-rich plasma for 15 min at 37°C. All subsequent steps were carried out at 22°C as described by Pinckard et al. (32). The cells

were resuspended at  $2 \times 10^9/\text{ml}$  in Tyrode's gelatin buffer (136.9 mM NaCl, 1.7 mM KCl, 2.0 mM  $\text{MgCl}_2$ , 12.1 mM  $\text{NaHCO}_3$ , 5.6 mM D-glucose, with 7.5 mg of potato apyrase, and 0.1 g of Sigma type II gelatin/100 ml), pH 7.4.

A sample of the serotonin-loaded platelets, adjusted to contain 0.1 mCi/ml of  $\text{H}_3^{32}\text{PO}_4$  at pH 7.4, was incubated at 22°C (90 min for rabbit platelets and 60 min for human platelets) to load the cells with  $^{32}\text{PO}_4^{3-}$ . The remaining platelets were incubated similarly without  $^{32}\text{PO}_4^{3-}$ .

**Secretion studies.** Secretion and phosphorylation studies were carried out simultaneously at 37°C in 1.5 ml polypropylene microtest tubes (Beckman Instruments Inc.) at a final platelet concentration of  $0.67 \times 10^9/\text{ml}$ . Secretion studies were performed in a final vol of 105  $\mu\text{l}$  consisting of 37.5  $\mu\text{l}$  platelets ( $2 \times 10^9/\text{ml}$ ), 37.5  $\mu\text{l}$  Tyrode's buffer (pH 7.4, 0.1 g of gelatin/100 ml) with either EGTA or  $\text{Ca}^{2+}$ , and 30  $\mu\text{l}$  of the same buffer without EGTA or  $\text{Ca}^{2+}$  and containing stimulating or inhibiting substances. The ingredients were thoroughly mixed before preincubation and again on addition of the stimulus. The final concentrations of EGTA or  $\text{Ca}^{2+}$  in the reaction mixture were 5 or 0.93 mM, respectively. The platelets were preincubated for 2–3 min with  $\text{Ca}^{2+}$  or EGTA before stimulation and after stimulation the reaction was quenched instantaneously at the desired time by adjusting the mixture to contain 100 mM formaldehyde and 5 mM EDTA as described by Costa and Murphy (33). The extent of secretion was assessed from the radioactivity in the supernate after centrifugation at 12,000 for 1 min in an Eppendorf model 5412 microcentrifuge (Brinkman Instruments, Inc., Westbury, N. Y.). The data are expressed as a percentage of the total platelet [ $^3\text{H}$ ]serotonin measured after lysis of the platelets with 1% Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.). The radioactivity was measured by liquid scintillation spectrometry after the addition of Aquasol-2 (New England Nuclear) diluted with an equal volume of toluene.

**Phosphorylation studies.** Phosphorylation studies were performed in a final vol of 37.5  $\mu\text{l}$  with additions proportional to those described above for the secretion studies. After stimulation, the reaction was quenched instantaneously with "SDS buffer" to give a final concentration of 1.1% SDS, 0.1 M 2-mercaptoethanol, 2.5 mM EDTA, 0.0008% bromophenol blue, and 10% glycerol, and the samples heated at 100°C for 2 min. After quenching, the entire sample was subjected to SDS-polyacrylamide slab gel electrophoresis in the system of Neville (34) or Laemmli (35), as described (11). Radioactivity was assessed from the peak absorbance on the densitometric tracing of the autoradiograph and is expressed as percent change in comparison with the control (11). Alternatively, the band of interest was located on the SDS gel by comparison to its autoradiograph, cut from the gel, and counted for [ $^{32}\text{P}$ ]phosphate in a Beckman liquid scintillation spectrometer. All assays were carried out in duplicate with unstirred samples. Preliminary studies indicated that no aggregation took place under the described incubation conditions as assessed by light microscopy of glutaraldehyde fixed specimens or by platelet aggregometry (Payton Associates Inc., Buffalo, N. Y.).

**Characteristics of platelet phosphoproteins.** Platelets were loaded with  $^{32}\text{PO}_4^{3-}$  as described above and treated with 0.5 U of thrombin/ml for 60 s before the proteins were solubilized by heating at 100°C for 2 min in 1% SDS. Portions (in triplicate) were then incubated at 37°C for 30 min with either Staphylococcal V8 protease (88  $\mu\text{g}/\text{ml}$ ) or RNase (27  $\mu\text{g}/\text{ml}$ ). The reaction was stopped by heating at 100°C for 2 min in the presence of 0.1 M 2-mercaptoethanol. Changes in radioactivity in comparison to controls, incubated in the absence of enzyme, were assessed after SDS-PAGE and autoradiography.

The phospholipid content was assessed in samples of the

SDS-solubilized,  $^{32}\text{PO}_4^{3-}$ -loaded, thrombin-treated platelets by extraction into chloroform-methanol by the method of Cohen et al. (36). The protein, precipitating at the interface, was harvested, washed twice in chloroform-methanol and resolubilized in SDS buffer in preparation for SDS-PAGE.

Stability to 10% trichloroacetic acid for 10 min at 95°C was assessed in one of two ways. A portion of the  $^{32}\text{PO}_4^{3-}$ -loaded thrombin-treated platelets in 1% SDS was adjusted to contain 10% trichloroacetic acid and heated. The sample was then placed at 4°C for 30 min to precipitate the protein that was sedimented by centrifugation at 3,000 *g* for 20 min at 4°C. Excess trichloroacetic acid and SDS were removed by washing the precipitate three times with 10 ml of acetone at 22°C before solubilizing the sample in SDS buffer and subjecting it to SDS-PAGE. Alternatively, stability to hot trichloroacetic acid was determined after SDS-PAGE of  $^{32}\text{PO}_4^{3-}$ -loaded, thrombin-treated platelets. Immediately after electrophoresis, the slab gel was cut into two equal parts, and incubated in 200 ml of 10% trichloroacetic acid for 15 min at 22°C. One part was then placed into 10% trichloroacetic acid for 10 min at 95°C while the control remained at 22°C. Both were then processed in the usual manner.

Stability to 0.5 M NaOH for 10 min at 95°C was assessed only after SDS-PAGE because no clear protein bands could be identified in gel if the protein was exposed to NaOH before SDS-PAGE. Exposure of the gel to NaOH after electrophoresis did not significantly alter the protein pattern as observed after staining with Coomassie Blue.

Stability in  $\text{NH}_2\text{OH}$  was assessed after SDS-PAGE in a manner similar to the experiment with trichloroacetic acid. Each half of the gel was incubated for 15 min at 4°C in 200 ml of 0.8 M NaCl, 50 mM Na acetate, pH 5.4. One-half was then incubated in another 200 ml of the same buffer while the other half was placed in 200 ml of 0.8 M  $\text{NH}_2\text{OH}$ , 50 mM Na acetate, pH 5.4. After 10 min at 4°C, both gel pieces were warmed to 30°C and kept at that temperature for 15 min before transfer into the staining solution.

**Miscellaneous methods.** Platelet number was assessed on a Thrombocounter (Coulter Electronics, Inc., Hialeah, Fla.). Thrombin was assayed as previously described by Seegers and Smith (37) and PAF activity was assayed as described by Pinckard et al. (32). Lactate dehydrogenase was assessed by the method of Bergmeyer (38). Preliminary experiments indicated that the formaldehyde-EDTA did not alter lactate dehydrogenase activity.

## RESULTS

**Effect of thrombin, ionophore A23187, and PAF on protein phosphorylation in intact platelets.** Optimum loading of rabbit platelets with  $^{32}\text{PO}_4^{3-}$  was observed after 90 min of incubation at 22°C as assessed by incorporation of  $^{32}\text{P}$  into  $^{32}\text{P}$ -phosphopeptides analyzed by SDS-PAGE and autoradiography. Over 20 bands of radioactivity were readily visible when unstimulated  $^{32}\text{PO}_4^{3-}$ -loaded platelets were examined (Fig. 1). Exposure of  $^{32}\text{PO}_4^{3-}$ -loaded platelets to thrombin, PAF, or ionophore resulted in increased phosphorylation of mainly two bands of radioactivity designated as P7P and P9P in keeping with the nomenclature for the human system (Fig. 1).

At saturating concentrations (see below), thrombin increased phosphorylation of P7P by  $370.4 \pm 31.0\%$  (mean  $\pm$  SE, *n* = 26) and of P9P by  $208.5 \pm 15.7\%$  (*n*

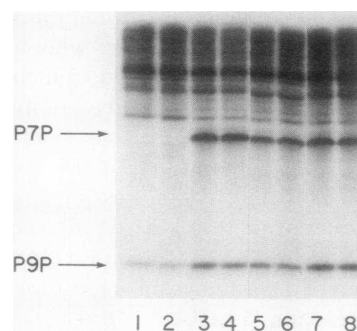


FIGURE 1 Autoradiograph of SDS-polyacrylamide gel of  $^{32}\text{PO}_4^{3-}$ -loaded rabbit platelet. Rabbit platelets were loaded with  $^{32}\text{PO}_4^{3-}$  as described in Methods. Each sample was adjusted to contain 0.93 mM  $\text{CaCl}_2$  and exposed to either buffer alone (1 and 2), 1 U/ml of thrombin (3 and 4), 26 U/ml of PAF (5 and 6), or 0.8  $\mu\text{M}$  ionophore (7 and 8) for 120 s at 37°C. The reactions were quenched with SDS buffer and the samples electrophoresed on a 12% total acrylamide Laemmli polyacrylamide gel.

= 26), PAF increased phosphorylation of P7P by  $297.4 \pm 28.0\%$  (*n* = 26), and of P9P by  $203.9 \pm 17.9\%$  (*n* = 26); whereas ionophore increased phosphorylation of P7P by  $354.1 \pm 46.7\%$  (*n* = 19) and of P9P by  $258.9 \pm 29.3\%$  (*n* = 19). Highly purified PAF gave results similar to the crude preparation. With each stimulus, the increase in P7P phosphorylation was significantly greater than the increase in P9P phosphorylation (*P* < 0.001 for thrombin and PAF and *P* < 0.03 for ionophore when compared by a paired *t* test (39)).

**Characterization of P7P and P9P (Table I).** Exposure of SDS-solubilized,  $^{32}\text{P}$ -labeled proteins from thrombin-treated rabbit platelets to homogeneous Staphylococcal V8 protease resulted in over 90% de-

TABLE I  
Characteristics of P7P and P9P in Rabbit Platelets

| Treatment           | P7P        | P9P  |
|---------------------|------------|------|
|                     | % decrease |      |
| Protease V8         | 92.1       | —    |
| TCA                 |            |      |
| A                   | 20.0       | 23.1 |
| B                   | 17.0       | 26.0 |
| RNase               | 3.5        | +6.0 |
| Chloroform-methanol | 6.9        | 16.1 |
| Hydroxylamine       | +1.9       | +1.0 |
| NaOH                | 94.7       | 96.1 |

The data are expressed as percent decrease in radioactivity from a control value and represent the mean of three observations. The experiments were performed as described in Methods with A indicating exposure to hot TCA before SDS-PAGE and B indicating exposure to hot TCA after SDS-PAGE. A plus (+) sign indicates an increase rather than a decrease in radioactivity.

crease in radioactivity in P7P; this suggests that it is a protein. P9P could not be evaluated in this experiment because it was obscured by radioactive proteolytic fragments from larger  $M_r$  peptides. P7P and P9P are not RNA because both were stable on exposure to hot trichloroacetic acid or RNase. P7P and P9P are not phospholipids because >80% of each remained in the protein precipitate after lipid extraction. The phosphate in P7P and P9P is not present as phosphohistidine or phospholysine, as indicated by the stability of the  $^{32}\text{P}$ -label in hot trichloroacetic acid (40), or as acyl phosphates because the  $^{32}\text{P}$ -label was stable when the SDS-solubilized  $^{32}\text{P}$ -phosphoproteins were exposed to hydroxylamine. However, the  $^{32}\text{P}$ -label in both P7P and P9P was unstable when incubated in hot NaOH. These data, similar to those published for P7P and P9P from human platelets (8), indicate that rabbit platelet P7P and P9P are proteins and suggest that the  $^{32}\text{P}$  is present as phosphomonoesters of serine and/or threonine.

**Comparison of protein phosphorylation in rabbit and human platelets.** Samples of rabbit and human platelets were loaded with  $^{32}\text{PO}_4^{3-}$ , exposed to either thrombin or buffer alone and analyzed by SDS-PAGE and autoradiography. As can be seen in Fig. 2, the Coomassie Blue staining patterns of the rabbit and human platelet proteins were similar. Although several differences between the rabbit and human system were seen when the  $^{32}\text{P}$ -phosphopeptide patterns of the control samples were examined, thrombin stimulated phosphorylation of proteins of 48,000 and 20,000  $M_r$  in both rabbit and human platelets. Other less prominent changes in the phosphorylation pattern were observed after thrombin treatment, but these were not examined further.

Rabbit P7P migrated at a different apparent  $M_r$  in different SDS-PAGE systems. Thus, P7P migrated above actin at a  $M_r$  of 48,000 in the system described by Neville (Fig. 2) and below actin at an apparent  $M_r$  of 41,000 in the Laemmli system (Fig. 3). This is identical to the anomalous migration that we have observed for human P7P (11).

To evaluate other possible similarities between phosphorylation of proteins in rabbit and human platelets, we examined the effects of agents that increase intracellular cyclic AMP on the phosphorylation of rabbit platelet proteins. As can be seen in Fig. 3, dibutyl adenosine 3'-5'-monophosphate (DB-cAMP) and  $\text{PGE}_1$  each completely inhibited PAF-induced P7P and P9P phosphorylation. Similar but less striking effects of  $\text{PGE}_1$  and DB-cAMP were observed when either thrombin or ionophore A23187 were the stimuli (data not shown). These data are similar to those previously observed in stimulated human platelets (8). In addition, DB-cAMP and  $\text{PGE}_1$  induced phosphorylation of four proteins of  $M_r$  56,000 (not seen in Fig. 3), 48,000, 40,000, and 24,000 in agreement with studies in human

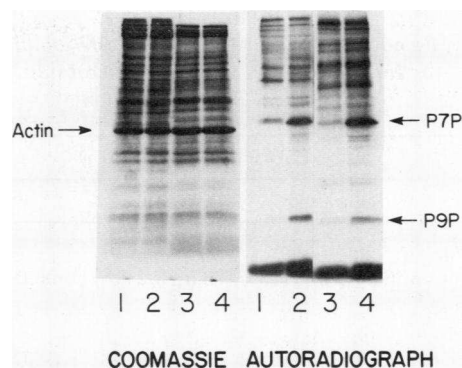


FIGURE 2 SDS-PAGE electrophoresis of  $^{32}\text{PO}_4^{3-}$ -loaded rabbit and human platelets. Rabbit platelets (1 and 2) and human platelets (3 and 4) were loaded with  $^{32}\text{PO}_4^{3-}$  adjusted to contain 0.93 ml  $\text{CaCl}_2$  and exposed to either buffer alone (1 and 3) or 0.5 U/ml of thrombin for 120 s at  $37^\circ\text{C}$  (2 and 4) before SDS-PAGE was performed on a 13% total acrylamide Neville gel.

platelets (41). When examined on Laemmli SDS-PAGE (Fig. 3), neither the 48,000 nor the 40,000  $M_r$  phosphoprotein comigrated with P7P, and the 24,000  $M_r$  phosphoprotein was separated from P9P.

**Effect of extracellular  $\text{Ca}^{2+}$  on serotonin release and platelet protein phosphorylation (Table II).** Ionophore-induced serotonin release depended on the presence of extracellular  $\text{Ca}^{2+}$ . In contrast, the removal of extracellular  $\text{Ca}^{2+}$  failed to inhibit thrombin- or PAF-induced release completely. Thus, release induced by these two stimuli only partially depends on the extracellular  $\text{Ca}^{2+}$  concentration. There was no platelet lysis

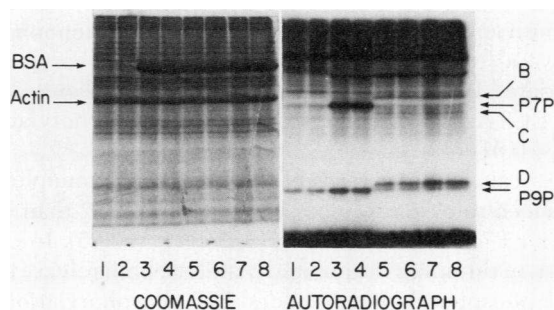


FIGURE 3 SDS-PAGE of  $^{32}\text{PO}_4^{3-}$ -loaded rabbit platelets showing the effect of PAF, DB-cAMP, and  $\text{PGE}_1$ . Rabbit platelets were loaded with  $^{32}\text{PO}_4^{3-}$  and adjusted to contain 0.93 mM  $\text{CaCl}_2$ . Samples were treated with buffer alone (1 and 2); 26 U/ml of PAF for 120 s (3 and 4); 1 mM DB-cAMP, and 5.5 mM theophylline for 60 s before 26 U/ml PAF for 120 s (5 and 6); 2.8  $\mu\text{M}$   $\text{PGE}_1$ , and 5.5 mM theophylline for 60 s before 26 U/ml PAF for 120 s (7 and 8). B, C, and D indicate the phosphoproteins of  $M_r$  48,000, 40,000, and 24,000, respectively, which were phosphorylated in the presence of  $\text{PGE}_1$  or DB-cAMP. Electrophoresis was carried out in the system of Laemmli at 12% total acrylamide. Bovine serum albumin (BSA) was present as a carrier for PAF. BSA by itself did not alter platelet protein phosphorylation (data not shown).

TABLE II

Effect of Removal of Extracellular  $\text{Ca}^{2+}$  on Thrombin-, PAF-, or Ionophore A23187-induced Serotonin Release and Phosphorylation

|                     | % Inhibition in absence of $\text{Ca}^{2+}$ |                     |                     |
|---------------------|---|---------------------|---------------------|
|                     | Thrombin                                    | PAF                 | Ionophore           |
| Serotonin release   | 60.7 $\pm$ 4.9 (12)                         | 73.5 $\pm$ 3.2 (18) | 96.0 $\pm$ 1.0 (12) |
| P7P phosphorylation | 17.7 $\pm$ 6.3 (12)                         | 23.8 $\pm$ 6.5 (18) | 58.5 $\pm$ 8.2 (12) |
| P9P phosphorylation | 54.9 $\pm$ 6.8 (12)                         | 56.6 $\pm$ 5.5 (18) | 64.0 $\pm$ 4.4 (12) |

Platelets were loaded with [ $^3\text{H}$ ]serotonin and  $^{32}\text{PO}_4^{3-}$  as described in Methods and exposed to either thrombin, PAF, or ionophore A23187 in the presence of 0.93 mM  $\text{CaCl}_2$  or 5 mM EGTA. The data are expressed as percent inhibition when  $\text{Ca}^{2+}$  was replaced by EGTA and shown as mean $\pm$ SE with the number of experiments in parenthesis. The assays were carried out after 60–120 s of incubation at concentrations of stimuli where secretion was maximal. These were 0.25–0.5 U/ml for thrombin, 26–65 U/ml for PAF, and 0.4–0.8  $\mu\text{M}$  for ionophore A23187.

under these conditions as assessed by lactate dehydrogenase release.

P7P and P9P phosphorylation in the absence of stimuli was not altered when 0.93 mM  $\text{Ca}^{2+}$  was replaced with 5 mM EGTA in the medium (data not shown). At concentrations of stimuli where secretion was maximal, ~60% of P9P phosphorylation induced by all three stimuli and P7P phosphorylation induced by ionophore, was  $\text{Ca}^{2+}$ -dependent. In contrast, P7P phosphorylation initiated by thrombin or PAF was inhibited only 20% by EGTA, significantly less than P9P phosphorylation ( $P < 0.01$ ) (42).

As can be seen in Table II, PAF- and ionophore-induced release was more dependent on  $\text{Ca}^{2+}$  than was either P7P or P9P phosphorylation ( $P < 0.05$ ). In contrast, in thrombin-stimulated platelets, both release and P9P phosphorylation exceeded P7P phosphorylation in their requirement for extracellular  $\text{Ca}^{2+}$  ( $P < 0.001$ ) (42).

We considered the possibility that chelation of divalent cations other than  $\text{Ca}^{2+}$  might alter either the release reaction or platelet protein phosphorylation. However, the presence of excess  $\text{Mg}^{2+}$  in the absence of  $\text{Ca}^{2+}$  (0.7 mM EGTA, 2 mM  $\text{MgCl}_2$ ) did not support either thrombin-induced secretion or phosphorylation (data not shown). In addition, divalent cations with affinities for EGTA greater than that of  $\text{Ca}^{2+}$  (e.g.,  $\text{Zn}^{2+}$ ,  $\text{Mn}^{2+}$ ) were not important because  $\text{Ca}^{2+}$ -EGTA did not inhibit either secretion or phosphorylation (data not shown).

Effects of different concentrations of stimuli on serotonin release and P7P and P9P phosphorylation in the presence or absence of  $\text{Ca}^{2+}$ . The effect of increasing concentrations of thrombin, PAF, and ionophore on serotonin release and protein phosphorylation (assessed simultaneously) were examined. In the presence of  $\text{Ca}^{2+}$ , the half-maximal concentrations for release and phosphorylation of P7P and P9P proteins were similar for each stimulus with values of 0.09 U/ml for thrombin (Fig. 4), 8–10 U/ml for PAF (Fig. 5), and 0.3–0.4  $\mu\text{M}$  for ionophore (Fig. 6). No ionophore-induced release occurred at concentrations of up to 0.8  $\mu\text{M}$  in the absence of extracellular  $\text{Ca}^{2+}$  (Fig. 6). With that exception,  $\text{Ca}^{2+}$ -independent [ $^3\text{H}$ ]serotonin release and phosphorylation of P7P and P9P, increased to a maximum with increasing concentrations of the stimulus.

*Time-course of release and phosphorylation of P7P and P9P in the presence of extracellular  $\text{Ca}^{2+}$*  (Figs. 7–9). The time-course of thrombin-induced [ $^3\text{H}$ ]serotonin release was similar to that described by Detwiler and Feinman (4). There was a concentration-dependent lag phase, during which no serotonin release occurred, followed by a rapid-release phase and then a plateau. At concentrations of thrombin that caused maximum release (0.25–0.5 U/ml), the lag phase lasted 8–10 s and the time for half-maximal release ( $T_{1/2}$ ) for release was 26.0 $\pm$ 2.5 s SE ( $n = 3$ ). In contrast, P7P and P9P were phosphorylated without a lag phase and with a more rapid  $T_{1/2}$  than release (12.3 $\pm$ 1.3 s SE ( $n = 3$ ) and 3.7 $\pm$ 0.17 s SE ( $n = 3$ ), respectively). For example, in the experiment in Fig. 7, phosphorylation of both proteins was maximal at a time when only 5% of the [ $^3\text{H}$ ]serotonin had been released, after which the degree of phosphorylation decreased. This rapid time-course of phosphorylation did not appear to be an artifact of inadequate quenching because no phosphorylation was observed when thrombin and the SDS buffer were added to the platelets simultaneously.

When PAF was the stimulus (Fig. 8), little or no lag phase for [ $^3\text{H}$ ]serotonin release or P7P and P9P phosphorylation was observed. After rapidly reaching a peak, the  $^{32}\text{P}$  in P7P and P9P decreased. At concentrations of PAF that induced maximum release (26–65 U/ml), the  $T_{1/2}$  was 5–8 s for release and 3.5–5 s for both P7P and P9P phosphorylation.

With ionophore (Fig. 9), the time-course for [ $^3\text{H}$ ]serotonin release was similar to that observed for thrombin with a concentration-dependent lag phase consistently observed. However, there was much more variability between different preparations of platelets than was observed when thrombin was the stimulus. At concentrations of ionophore that caused maximum release (0.4–0.8  $\mu\text{M}$ ), the lag phase was between 7 and 20 s, whereas the  $T_{1/2}$  for release ranged between 23 and 65 s. The  $T_{1/2}$  for P7P phosphorylation had a range of 9–30 s and the  $T_{1/2}$  for P9P phosphorylation a range of 6–18 s.

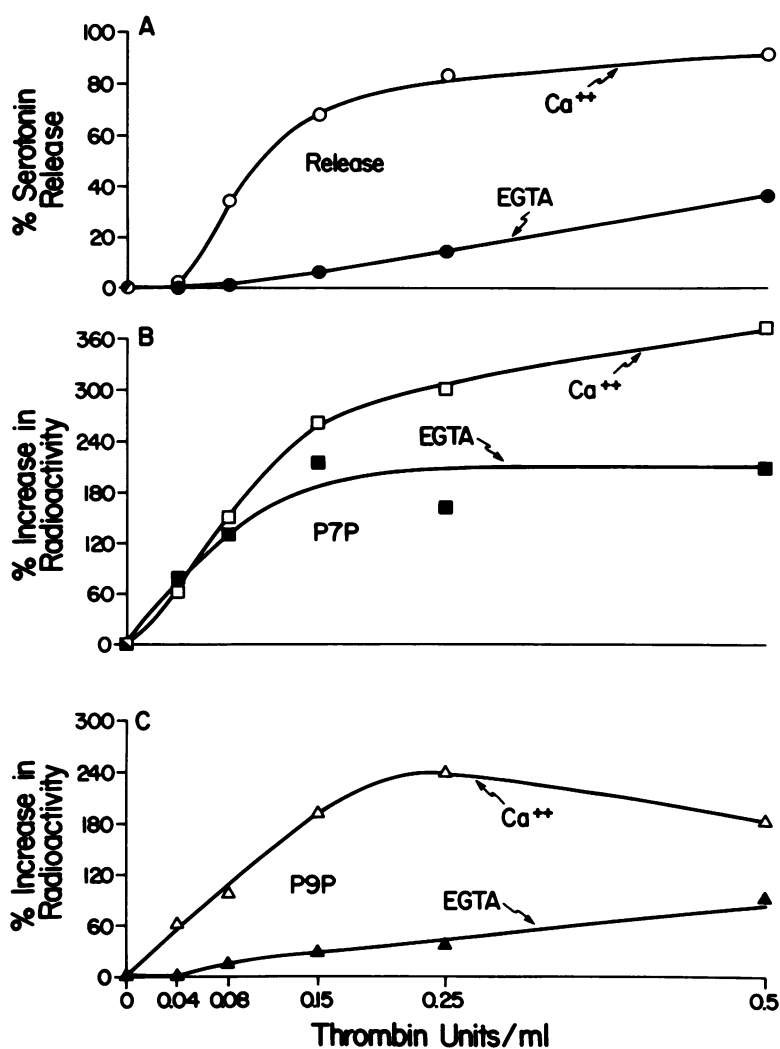


FIGURE 4 Effect of increasing concentrations of thrombin on [<sup>3</sup>H]serotonin secretion and platelet protein phosphorylation. Rabbit platelets, loaded with [<sup>3</sup>H]serotonin and <sup>32</sup>PO<sub>4</sub><sup>3-</sup> as described in Methods, were treated with increasing concentrations of thrombin for 120 s at 37°C in the presence of either 0.93 mM CaCl<sub>2</sub> (open symbols) or 5 mM EGTA (closed symbols). Control experiments showed that the pH of the medium was not altered under these conditions. [<sup>3</sup>H]serotonin secretion and phosphorylation were assessed simultaneously as described in Methods. Each point is the mean of two observations. A depicts [<sup>3</sup>H]serotonin release. B and C depict phosphorylation of P7P and P9P, respectively.

In each of four experiments, the rate of P9P phosphorylation was fastest followed in order by P7P phosphorylation and serotonin release. As can be seen in Fig. 9, at a time point when no release had occurred (10 s), P9P phosphorylation was half maximal and P7P phosphorylation was underway.

*Effect of the removal of extracellular Ca<sup>2+</sup> on the time-course of release and phosphorylation of P7P and P9P.* The removal of Ca<sup>2+</sup> from the extracellular fluid resulted in inhibition of thrombin-, PAF-, and ionophore-induced release at all time points where release had occurred in the presence of Ca<sup>2+</sup> (Figs. 7A, 8A, 9A).

When thrombin and PAF were the stimuli, removal of Ca<sup>2+</sup> had less inhibitory effect on P7P phosphorylation than on P9P phosphorylation while the effect of Ca<sup>2+</sup> removal on ionophore-induced phosphorylation was similar for both P7P and P9P. Ca<sup>2+</sup>-dependent P7P and P9P phosphorylation was most evident at the later time points, generally when phosphorylation was decreasing from its maximum (Figs. 7–9).

In some experiments with thrombin or ionophore as stimulus, we noted that removal of Ca<sup>2+</sup> appeared to have an effect on phosphorylation at a time point before release had occurred. To examine this possibility

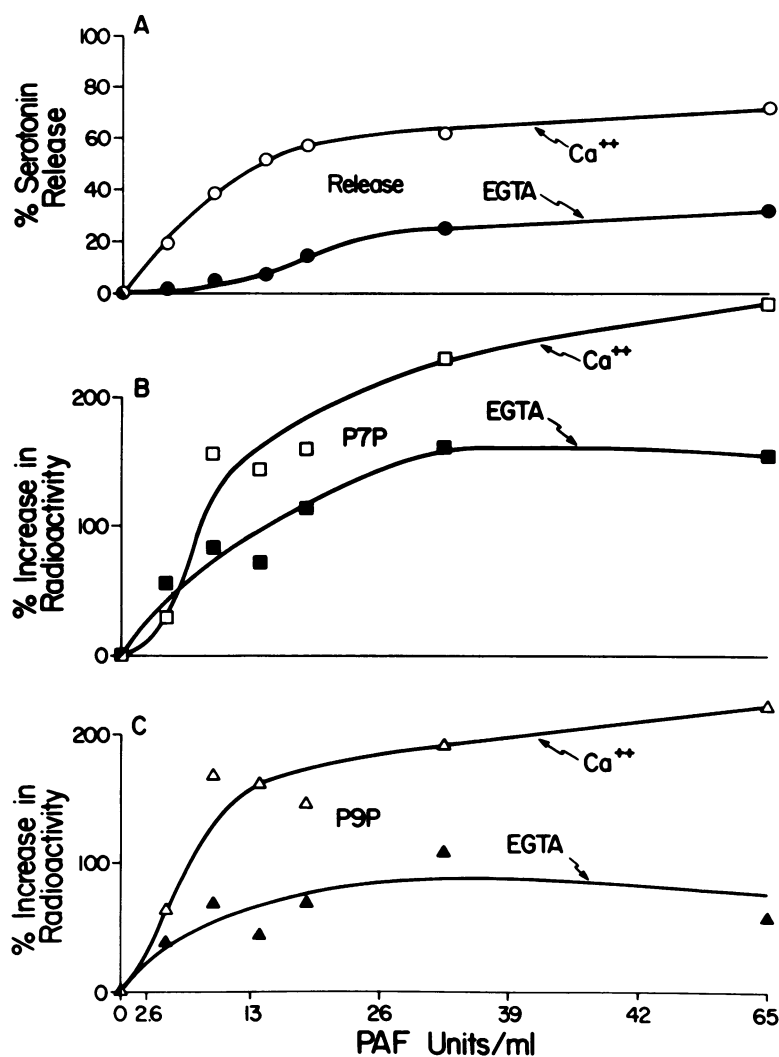


FIGURE 5 Effect of increasing concentrations of PAF on [ $^3\text{H}$ ]serotonin secretion and platelet protein phosphorylation. Conditions for this experiment were similar to those described for Fig. 4 with the exception that PAF was the stimulus.

in greater detail, multiple replicates of thrombin- or ionophore-induced platelet protein phosphorylation were performed at an early time point at which no [ $^3\text{H}$ ]serotonin release had occurred (determined in simultaneous release experiments) (Table III). Under these conditions,  $\text{Ca}^{2+}$  removal significantly inhibited P9P but not P7P phosphorylation. These data indicate that phosphorylation precedes release and that an effect of  $\text{Ca}^{2+}$  removal on phosphorylation of P9P can be detected before release begins.

## DISCUSSION

Our results indicate that the phosphorylation patterns observed during induction of the release reaction are similar in rabbit and human platelets, and that the rab-

bit platelet is an appropriate model in which to study the effects of  $\text{Ca}^{2+}$  on protein phosphorylation.

The pattern of the time-course of release was dependent on the stimulus employed (Figs. 7, 8, 9). As with human platelets (4), the sequence of thrombin- and ionophore-induced serotonin release was characterized by a lag phase, a rapid exponential phase followed by a plateau. As expected, the length of the lag phase was inversely proportional to the dose of the stimulus (4). In contrast, little or no lag phase was detected when PAF was the stimulus (Fig. 8). This observation suggests that the induction phase of release is either bypassed by PAF, or is so short that it was not detected at the earliest time point we measured (5 s). The latter possibility is unlikely because no lag was observed in the time-course of release even at concen-

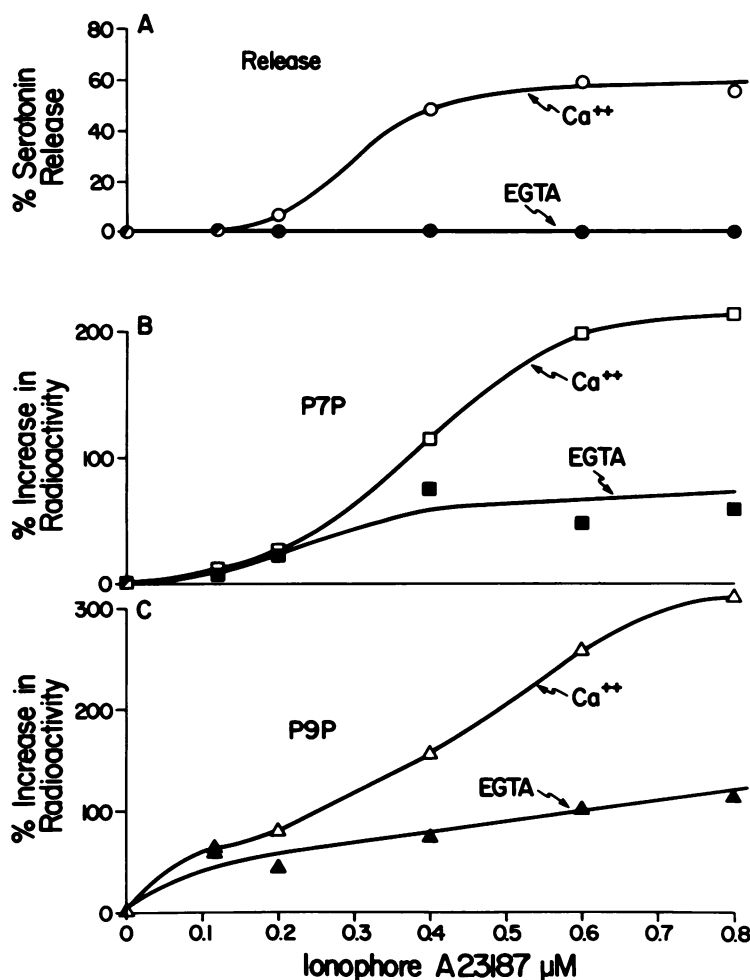


FIGURE 6 Effect of increasing concentrations of ionophore A23187 on [ $^3\text{H}$ ]serotonin secretion and platelet protein phosphorylation. Conditions for this experiment were similar to those described in Fig. 4 with the exception that ionophore A23187 was the stimulus.

trations of PAF where <30% of maximum release took place.

In rabbit platelets, the majority of thrombin- or PAF-induced release (60–75%) was inhibited when extracellular  $\text{Ca}^{2+}$  was replaced by EGTA (Table II). Ionophore-induced release was blocked almost completely under these conditions and did not increase even when high concentrations of stimulus (0.8  $\mu\text{M}$ ) were used (Fig. 6). This is in contrast to human platelets where there is no relationship between extracellular  $\text{Ca}^{2+}$  and thrombin- or ionophore-induced secretion when examined under nonaggregating conditions (2, 3, 5).

Two possibilities (not mutually exclusive) may explain the difference between rabbit and human platelet secretion in their requirement for extracellular  $\text{Ca}^{2+}$ . First, it is possible that the human platelet plasma membrane is less permeable to  $\text{Ca}^{2+}$  than is the rabbit platelet plasma membrane. A second explanation is that rabbit platelets have inadequate intracellular  $\text{Ca}^{2+}$  to induce se-

cretion. Rabbit platelets have substantially less total  $\text{Ca}^{2+}$  content (24) and less dense-body  $\text{Ca}^{2+}$  than human platelets (25). However, we calculate that nondense-body  $\text{Ca}^{2+}$  is 2.1–4.5  $\mu\text{M}/10^{11}$  cells in both human (43) and rabbit platelets (14, 44–46). The location of the intracellular  $\text{Ca}^{2+}$  important in secretion is controversial as studies in human platelets have suggested that the dense tubular system, mitochondria,  $\alpha$ -granules, and surface membrane are all potential sources (5, 6). The possibility of a low  $\text{Ca}^{2+}$  content in one of these compartments of the rabbit platelet has not yet been evaluated.

We noted that the requirement of extracellular  $\text{Ca}^{2+}$  for serotonin release decreased as the concentration of thrombin or PAF increased (Figs. 4 and 5). It is possible that the serotonin secretion that occurs in the absence of extracellular  $\text{Ca}^{2+}$  is completely independent of  $\text{Ca}^{2+}$ , or that at high stimulus concentrations,  $\text{Ca}^{2+}$  from an intracellular source is mobilized. If the



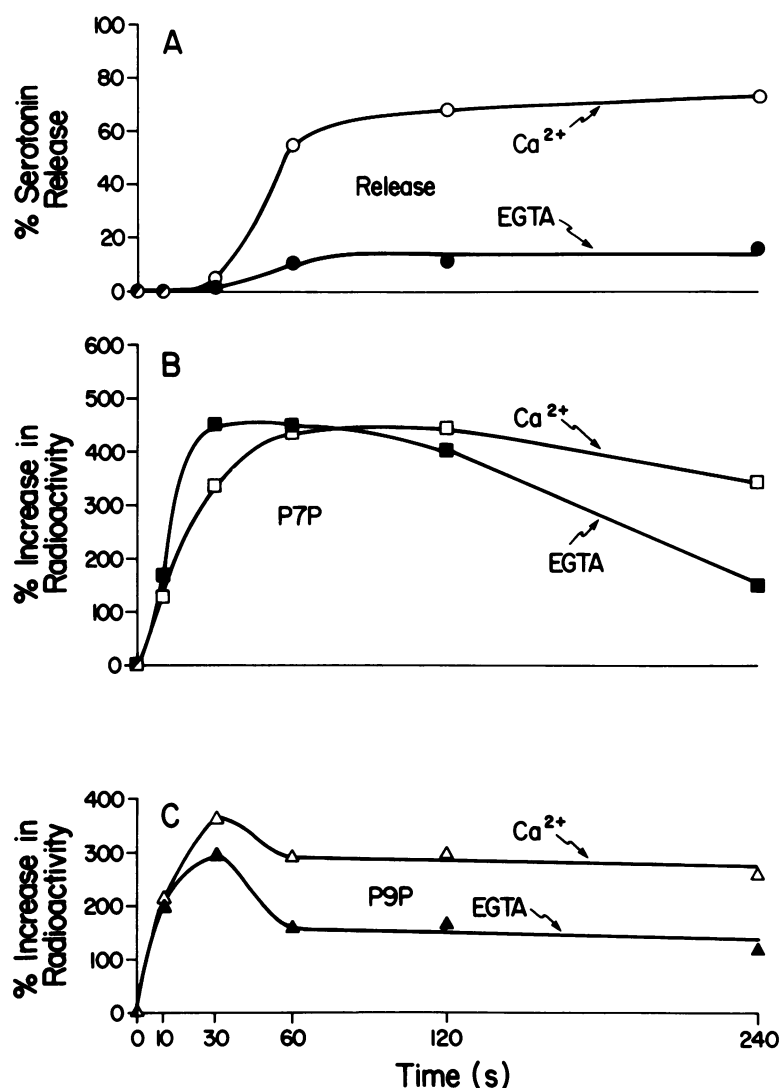


FIGURE 7 Time-course of secretion and protein phosphorylation in rabbit platelets induced by thrombin. [<sup>3</sup>H]serotonin and <sup>32</sup>PO<sub>4</sub><sup>3-</sup>-loaded rabbit platelets were treated with 0.08 U of thrombin/ml in the presence of 0.93 mM CaCl<sub>2</sub> or 5 mM EGTA for various time periods. Release and phosphorylation were assessed as described in Methods. A depicts [<sup>3</sup>H]serotonin secretion. B and C depict phosphorylation of P7P and P9P, respectively.

latter is true, then one must explain why ionophore did not also mobilize this intracellular source of Ca<sup>2+</sup>. The possibility that an intracellular source of Ca<sup>2+</sup> could be mobilized by thrombin and PAF but not by ionophore is supported by three observations. First, at mitogenic concentrations, ionophore induces its initial effects nearly exclusively at the lymphocyte plasma membrane (17). Second, in analogy to thrombin- or PAF-stimulated rabbit platelet secretion, a portion of mast cell histamine secretion, caused by the surface binding secretagogue 48/80, is Ca<sup>2+</sup>-independent, whereas, as with rabbit platelets, ionophore-induced mast cell secretion is entirely Ca<sup>2+</sup>-dependent (46–48).

Third, the presence of a second intracellular source of Ca<sup>2+</sup>, capable of supporting the rabbit platelet release reaction, is indicated by our recent observation (unpublished observation) that EGTA and 8-(*N,N*-diethylamino)octyl 3,4,5-trimethoxy-benzoate, an agent thought to immobilize intracellular Ca<sup>2+</sup> (49), were additive in inhibiting PAF-induced release.

Initial experiments at a time point where release was maximal indicated that P9P phosphorylation (with all three stimuli) and ionophore-initiated P7P phosphorylation were inhibited ~60% by Ca<sup>2+</sup> removal. Thrombin- and PAF-induced P7P phosphorylation were inhibited only 20%. However, detailed examination

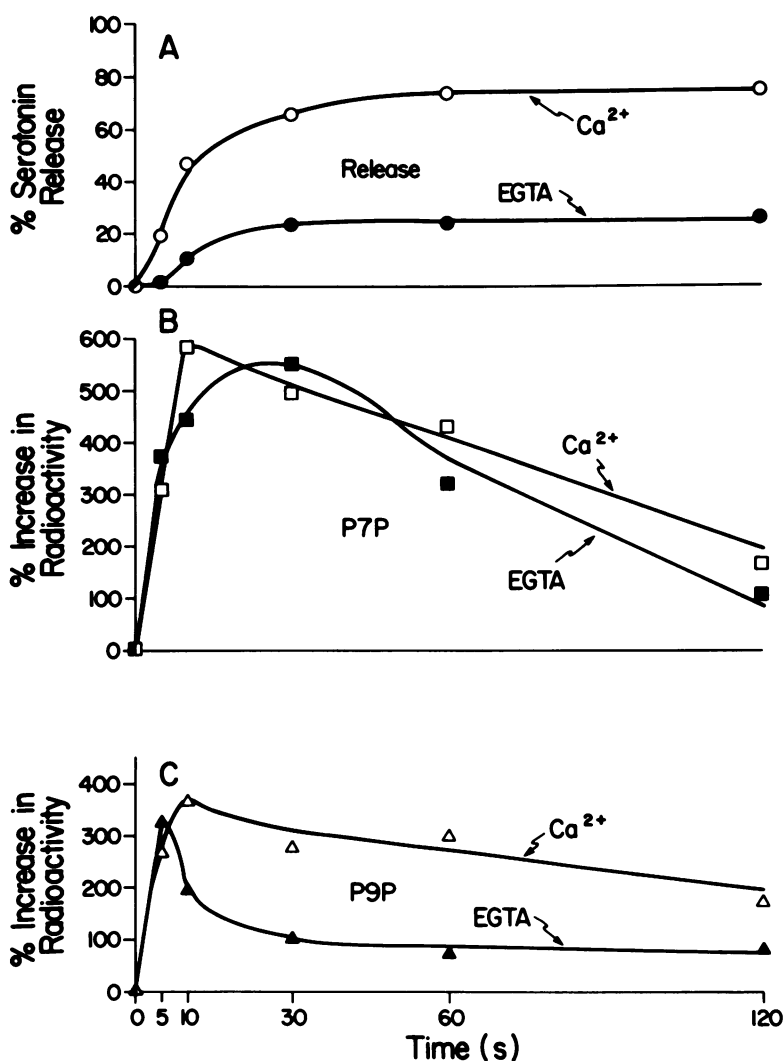


FIGURE 8 Time-course of secretion and protein phosphorylation in rabbit platelets induced by 26 U/ml of PAF in the presence of  $\text{CaCl}_2$  or EGTA. Conditions are similar to those described in Fig. 7.

of the time-course of phosphorylation indicated that in most cases the initial extent of phosphorylation of P7P was independent of extracellular  $\text{Ca}^{2+}$ , whereas initial P9P phosphorylation was only slightly inhibited by  $\text{Ca}^{2+}$  removal.

The major differences between phosphorylation in the presence or absence of  $\text{Ca}^{2+}$  were observed after secretion had reached its maximum. This "late" decrease in phosphorylation has been interpreted in other systems as indicating an increase in phosphatase activity, rather than a decrease in kinase activity (50).  $\text{Ca}^{2+}$ -modulation of phosphatase activity has been described, although other divalent cations have a more potent effect in regulating phosphoprotein phosphatases (51, 52). It remains possible that decreased stimulation of a  $\text{Ca}^{2+}$ -dependent protein kinase (17) may

also play a role in the decreased phosphorylation of P7P and P9P observed in the absence of extracellular  $\text{Ca}^{2+}$ . In this regard, platelet myosin light-chain (i.e., P9P) kinase has been isolated in  $\text{Ca}^{2+}$ -dependent form (15). The  $\text{Ca}^{2+}$  dependency is mediated by a  $\text{Ca}^{2+}$ -binding protein identical to the  $\text{Ca}^{2+}$ -dependent regulatory protein (17, 19, 20, 53, 54). The absence of adequate  $\text{Ca}^{2+}$  could result in a decreased kinase activity, which itself could potentially increase phosphatase activity (52). Thus, the late  $\text{Ca}^{2+}$ -dependent decreases in rabbit platelet protein phosphorylation (Figs. 7–9) could be secondary to decreased P7P and P9P kinase activity or to decreased phosphorylation of a phosphatase inhibitor with subsequent increased phosphatase activity (52).

Our data indicate that  $\text{Ca}^{2+}$  from an extracellular

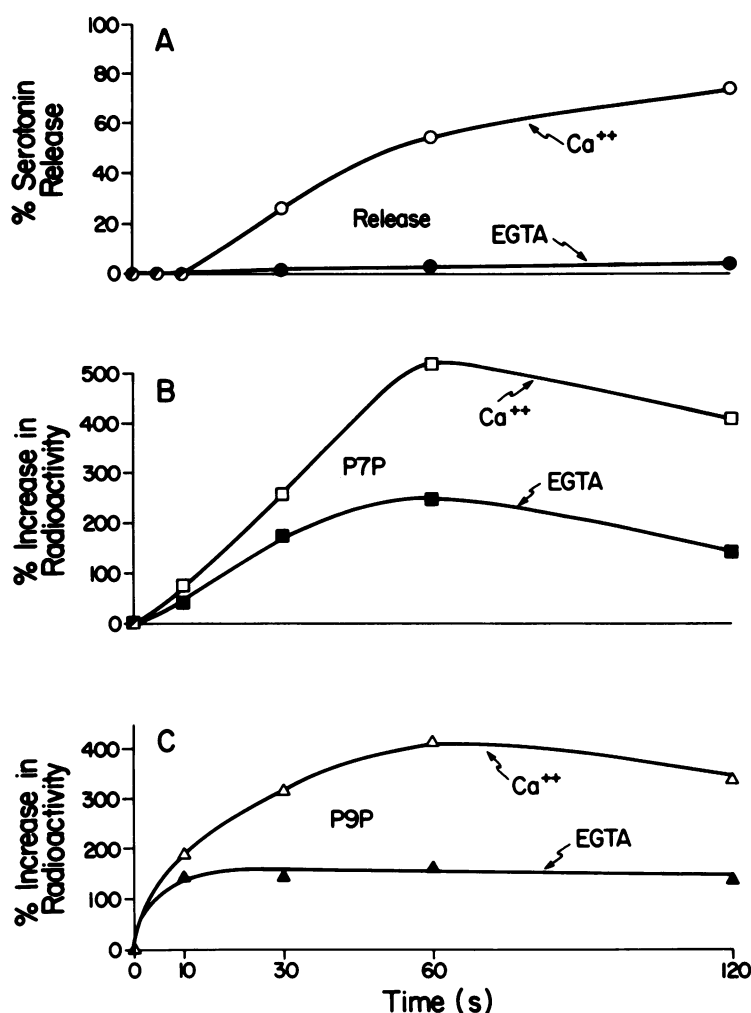


FIGURE 9 Time-course of secretion and protein phosphorylation in rabbit platelets induced by 0.4  $\mu\text{M}$  of ionophore A23187 in the presence of  $\text{CaCl}_2$  or EGTA. Conditions are similar to those described in Fig. 7.

source is not the major stimulus for the increase in the phosphorylation of P7P and P9P observed during secretion. Agents that increase intracellular cyclic AMP in platelets do not stimulate phosphorylation of these phosphoproteins (Fig. 3) and an increase in platelet guanosine 3'-5' monophosphate is not observed in unstirred platelets, where release but not aggregation occurs (55), i.e., conditions similar to our experiments. Thus, alternatives for the observed increased  $^{32}\text{P}$  incorporation into protein, apart from activation of cyclic nucleotide-dependent protein kinases must be considered. These include protease activation of the kinase, inhibition of the phosphatase, increased availability of the kinase or the substrate, or increased accessibility of sites on the protein that can be phosphorylated (56). Lastly, it is possible that protein kinase activation is dependent on a  $\text{Ca}^{2+}$  pool not available (or used) for secretion of dense-body contents.

The different patterns of phosphorylation of P7P and P9P in the time-course and  $\text{Ca}^{2+}$ -dependence experiments suggests that these two proteins do not share the same kinase(s) and phosphatase(s). However, it remains possible that these differences are the result of compartmentalization of the substrates, kinases, phosphatases, or modulators (e.g.,  $\text{Ca}^{2+}$ ) within the platelet, as has been described for cyclic AMP-dependent protein kinases (52, 57, 58), phosphoprotein phosphatases (59), and the  $\text{Ca}^{2+}$ -dependent regulatory protein (60).

When the time-courses of thrombin- or ionophore-induced phosphorylation and serotonin secretion were examined simultaneously, both P7P and P9P phosphorylation began and were often completed during the lag phase, i.e., before any secretion could be detected. This observation was not a result of the different methods of reaction quenching because simultaneous addition of stimulus and quencher gave identi-

TABLE III  
Effect of Removal of Extracellular  $\text{Ca}^{2+}$  on Thrombin or Ionophore-induced Phosphorylation at a Time Point Preceding Release

|                      | Thrombin                 |           | Ionophore      |
|----------------------|--------------------------|-----------|----------------|
|                      | arbitrary densitometry U |           |                |
| Experiment 1         |                          |           |                |
| P7P Ca <sup>2+</sup> | 93.0±6.9 (12)            | NS        | 23.3±5.4 (12)  |
| EGTA                 | 116.0±10.6 (12)          |           | 36.9±11.3 (12) |
| P9P Ca <sup>2+</sup> | 240.8±10.5 (12)          | P < 0.001 | 72.7±11.8 (12) |
| EGTA                 | 170.8±10.1 (12)          |           | 38.8±4.6 (12)  |
| Experiment 2         | cpm                      |           |                |
| P7P Ca <sup>2+</sup> | 138.5±20.7 (12)          | NS        |                |
| EGTA                 | 199.2±34.9 (12)          |           |                |
| P9P Ca <sup>2+</sup> | 219.4±11.2 (12)          | P < 0.001 |                |
| EGTA                 | 131.8±15.7 (12)          |           |                |

[ $^3\text{H}$ ]serotonin secretion and protein phosphorylation were assessed in the presence of 0.93 mM  $\text{CaCl}_2$  or 5 mM EGTA as described in Methods. The data in Experiment 1 are expressed as percent increase in phosphorylation over a control value from unstimulated platelets and the data in Experiment 2 are expressed as counts per minute with the background (from unstimulated platelets) subtracted (mean±SE[n]). In both experiments, the thrombin concentration was 0.08 U/ml and the incubation time was 10 s. The concentration of ionophore A23187 was 0.4  $\mu\text{M}$  and the reaction was quenched after an incubation time of 8 s. No serotonin secretion was detectable at these time points in any of the three experiments.

cal results to controls without added stimulus. Thus, protein phosphorylation in rabbit platelets (P9P preceding P7P phosphorylation) is an event that appears to occur early, before the onset of serotonin secretion. Moreover, removal of  $\text{Ca}^{2+}$  appeared to decrease P9P phosphorylation at a time point preceding detectable release. Thus, a small but significant blockade of P9P phosphorylation appeared when extracellular  $\text{Ca}^{2+}$  was removed; this suggests that P9P phosphorylation during the lag phase is partly dependent on extracellular  $\text{Ca}^{2+}$ . This conclusion is consistent with the observations of others, which suggests a role for  $\text{Ca}^{2+}$  mobilization in the induction phase of secretion in platelets (2–6). However, as indicated by Detwiler et al. (4) no experimental evidence has been presented that conclusively establishes that  $\text{Ca}^{2+}$  flux precedes release. Our experiments, although indirect, provide evidence for an effect of  $\text{Ca}^{2+}$  that occurs before the onset of secretion. Further studies, with the  $\text{Ca}^{2+}$ -dependent rabbit platelet as a model, may help clarify the role of  $\text{Ca}^{2+}$  flux in the various metabolic phenomenon involved in the release reaction.

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