Identification of ecto-PKC on surface of human platelets: role in maintenance of latent fibrinogen receptors

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Numerous reports demonstrate the activity of ecto-protein kinases on the outer surface of the plasma membrane in a number of cell types, including the human platelet (reviewed by Ehrlich et al. in Refs. 9 and 10). The functional significance of this extracellular phosphorylation system and its relationships to the secretion of ATP by stimulated platelets and neurons is rapidly emerging, and reports on the involvement of ecto-protein kinases and the ecto-phosphoprotein phosphatases in development, cell proliferation, coagulation factor activation, collagen/thrombospondin receptor affinity-shifts, T-cell receptor activation, and neuronal plasticity have been cited extensively (2, 3, 6, 41). The phosphorylation of proteins on the surface of intact cells is carried out by several different enzymes shown to have the catalytic specificity of known intracellular protein kinases, such as casein kinase and protein kinase C (PKC) (4, 16, 18, 19, 41). A neuronal ecto-PKC was identified and characterized by demonstrating that: 1) the exogenous substrates preferred by PKC specifically competed for the phosphorylation of specific endogenous proteins on the surface of intact neurons, 2) the PKC pseudosubstrate inhibitory peptide [19–36] produced a selective inhibition of the phosphorylation of specific proteins identified as exclusive substrates of a neuronal ecto-protein kinase, and 3) antibodies directed to the catalytic domain of specific PKC isoforms inhibited the phosphorylation of these specific surface proteins (16).

Ecto-protein kinase phosphorylates proteins using extracellular ATP as a phosphoryl donor. In the circulation, extracellular ATP is present through release from platelets and other cells. The presence of ecto-protein kinase activity has been shown in a variety of cells in the circulatory system, including platelets (4, 11, 14, 27, 35), endothelial cells (15, 31), epithelial cells (32), T lymphocytes (2, 33), human leukemic cells (30), neutrophils (8, 12, 36–39), and macrophages (1). Functional proteins in the circulation that were shown to be phosphorylated by ecto-protein kinases include coagulation Factors V and VIII (18–20), fibronectin (17), fibrinogen and fibrin (34), vitronectin (38), angiotensin II (23), atrial natriuretic hormone (22), basic fibroblast growth factor (39), osteopontin and bone sialoprotein (44), components of the complement system (13, 30), as

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well as β-amyloid precursor protein (40), the collagen receptor (7), platelet glycoprotein IV (CD36) (3, 14), and the αβ-T-cell receptor (2). Recently, we demonstrated that an ecto-PKC operates on the surface of human platelets (4). In these studies, we used a monoclonal antibody termed MAb 1.9 that specifically interacts with the catalytic domain of PKC and inhibits its activity. This antibody was developed by Drs. Daria Mochly-Rosen and Daniel E. Koshland using highly purified PKC and was found to have no effect on the activity of cAMP-dependent protein kinase or Ca²⁺/CaM-dependent protein kinase (24, 25). The effects of this antibody on platelet function (4) led to the conclusion that the phosphorylation of surface proteins by a platelet ecto-PKC protects platelets from spontaneous aggregation and thus can play an important role in homeostatic mechanisms that maintain circulating platelets in a resting, unactivated state.

In the present report we focus on the identification of a specific ATP-binding protein on the platelet surface with properties of ecto-PKC and begin elucidating the mechanisms by which its activity contributes to platelet homeostasis.

**MATERIALS AND METHODS**

Murine monoclonal antibody (MAb 1.9) (lot numbers EUUB06, EJX806, and FA9803), directed against the catalytic domain of purified PKC (23, 24), and myelin basic protein (MBP), were purchased from Life-Technologies, Gibco BRL (Gaithersburg, MD). Radiolabeled [γ32P]ATP, 8-azido-[α32P]ATP (azido-[α32P]ATP), and unlabeled 8-azido-ATP were purchased from ICN Biomedicals (Irvine, CA). Apyrase (grade V), prostaglandin E₁ (PGE₁), ATP, ADP, bovine serum albumin (BSA), dephosphocasein, and phosphotin were purchased from Sigma Chemical (St. Louis, MO). Heparin sodium (derived from porcine intestines) was purchased from Elkins-Sinn (Cherry Hill, NJ). Luciferin-luciferase reagents were purchased from Chrono-Log (Havrstown, PA). Bio-Lyte ampholytes and two-dimensional SDS-PAGE standards were purchased from Bio-Rad (Hercules, CA). Pseudosubstrate PKC peptide inhibitors [19–31] (RFARKGLRQKNV) and [19–36] (RFARKGLRQKNVHEVK) and myristoylated alanine-rich C kinase substrate (MARCKS) protein phosphorylated site domain (psd) (residues 151–175) were purchased from BIOMOL Research (Plymouth, PA).

Collection of blood and preparation of washed platelets. Whole blood was collected by venipuncture from healthy male and female donors (ranging in age from 25 to 50 yr) as described (21). Donors stated that they were free of any medications for at least 2 wk before blood donation. Whole blood was collected by gravity flow into a tube containing the anticoagulant acid-citrate-dextrose (7:1). Platelet-rich plasma was obtained following the centrifugation (200 g) of whole blood for 10 min at 22°C, contained the aggregation and clotting inhibitors PGE₁ (1 µM), apyrase (1 U/ml), and heparin (2 U/ml) (final concentrations). Platelets were isolated by centrifugation of the platelet-rich plasma at 2,200 rpm for 10 min at 22°C and washed three times using a Tyrode albumin-containing solution (pH 7.35) consisting of the following (in mM): 137 NaCl, 2.7 KCl, 1 MgCl₂, 0.36 Na₂HPO₄, 12 NaHCO₃, 2 CaCl₂, 5.5 glucose, and albumin (0.35%). The first wash solution contained heparin, apyrase, and PGE₁; the second wash contained apyrase and PGE₁; and the third wash contained only PGE₁. The final platelet pellets were resuspended in Tyrode solution (pH 7.35) in the absence of any inhibitors. Platelets were counted microscopically using a hemocytometer (21).

Platelet aggregation and ATP release. The experiments were carried out in a Chronolog Lumi-Aggregometer (Chronolog, Havertown, PA). Washed platelet suspensions were added to the aggregometer cuvette at a volume of 8.15 ml and a cell concentration of 2 × 10⁸ cells/ml under constant stirring conditions of 1,100 rpm at 37°C. Platelet aggregation was initiated by the addition of the MAB 1.9 (10 µl) at various concentrations.

Measurement of intracellular Ca²⁺ concentration. To measure intracellular free calcium concentration levels, washed platelets, suspended at a final cell concentration of 2 × 10⁸ cells/ml, were loaded with the calcium-sensitive indicator fura 2 by incubating them with 4 µmol/l fura 2-acetoxymethoxy ester (AM) for 30 min at 37°C. Excess fura 2-AM was removed from the platelets by centrifuging the platelet suspension at 1,000 g for 10 min. Platelets were incubated for an additional 30 min to allow for conversion of the fura 2-AM to fura 2 by intracellular esterases. The platelets were then washed and diluted to the final concentration of 2 × 10⁵ platelets/ml in Tyrode buffer. Measurements of the intracellular calcium concentration were performed by digital fluorescence microscopy with the Attofluor Ratio Imaging System (Atto Instruments) attached to a Zeiss Axiovert 135 microscope (Carl Zeiss). The system was calibrated as provided in the Attofluor Ratio Vision manual. Platelets were added to a 25-mm coverslip chamber at a volume of 700 µl. Measurements were taken at the excitation wavelengths of 334/380 nm and the emission wavelength of 520 nm using the fura-2 Zeiss filter set. MAB 1.9 was added to the platelet suspensions in a volume of 20 µl at various concentrations. At the end of each experiment the nonfluorescent calcium ionophore bromo-A-23187 was added (20 µl; 10 µM). Data were collected in real time and stored in the Attofluor computer for analysis by the AttoGraph software.

Ecto-protein kinase assays and phosphorylation of platelet surface proteins. All phosphorylation reactions (ecto-protein kinase assays) were carried out at room temperature at ~22°C. Phosphorylation reaction mixtures contained a solution of 0.45 ml and a cell concentration of 10⁸ cells/ml in a Tyrode solution (pH 7.35) consisting of the following: washed platelets (30 or 35 µl) (from 8 × 10⁸ to 1.2 × 10⁹ platelets/ml) resuspended in BSA-free Tyrode buffer, [γ32P]ATP solution (0.1 µM final ATP concentration), and BSA-free Tyrode buffer, to a final volume of 50 µl. Phosphorylation reactions were initiated with the addition of [γ32P]ATP. All inhibitors were added 10 min before the addition of radiolabeled ATP. Radiolabeled platelets were dissolved in 6× Laemmli buffer (containing 2% β-mercaptoethanol and 2% SDS, final concentrations) (20). The samples were immediately boiled for 3 min and processed for SDS-PAGE.

Photoaffinity labeling of platelet surface proteins. The photoaffinity probe, 8-azido-ATP, labeled in the α position with 3P, was dissolved in methanol and dried on the walls of an Eppendorf tube as described (26). The dried material was dissolved and mixed in a BSA-free Tyrode solution, pH 7.35. Labeling reaction mixtures contained the following components: washed platelets (30 µl) (8 × 10⁶ platelets/ml) resuspended in BSA-free Tyrode solution, azido-[α32P]ATP solution (various concentrations), and BSA-free Tyrode solution, prepared in a final volume of 50 µl. Labeling reactions were initiated by the addition of radiolabeled azido-ATP. All antibodies and various inhibitors were added 1–10 min before the addition of radiolabeled azido-ATP. The samples were then irradiated with a short wavelength ultraviolet light (254...
nm) at distance of 8 cm for 2 min at 0°C (on ice). Then 10 μl of a 6× Laemmli buffer (containing 2% β-mercaptoethanol and 2% SDS, final concentrations) was added. The samples were boiled for 3 min and processed for SDS-PAGE and autoradiography.

SDS-PAGE and autoradiography. The proteins contained in a 50-μl aliquot of the reaction mixtures, diluted with 10 μl of 6× Laemmli buffer, were separated by SDS-electrophoresis in polyacrylamide gels using a linear 7–15% gradient of acrylamide with a 3% stacking gel. High- and low-molecular weight standards were used to estimate the molecular weight of each protein band. The gels were stained for proteins with Coomassie brilliant blue G-250, destained in 10% acetic acid and 20% methanol, and dried in vacuo. Incorporation of radiolabeled phosphate was detected by exposure of dried gels to Kodak X-Omat AR film with DuPont-Cronex Lightning Plus intensifying screens for ~1–3 days at ~70°C. X-ray films were developed automatically in a Kodak X-OMAT processor. Quantitation of [32P]phosphate incorporation into separate platelet proteins was performed by automatic scanning and by a PhosphorImager Storm 860 System (Molecular Dynamics) using the ImageQuant software program.

Two-dimensional gel electrophoresis and isoelectric point determination. Two-dimensional isoelectric focusing/SDS-PAGE gel electrophoresis was carried out following procedures described (28, 29) with modifications as detailed in the instruction manual provided for the Protean II xi 2-D Cell System (Bio-Rad). The molecular mass standards (in daltons) used for two-dimensional gel electrophoresis were as follows: hen egg white conalbumin (76,000), bovine serum albumin (66,200), bovine muscle actin (43,000), rabbit muscle GAPDH (36,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and equine myoglobin (15,500).

RESULTS

We previously reported that platelet aggregation can be induced by the inhibition of ecto-PKC activity, which occurs when intact human platelets are incubated with the PKC inhibitory monoclonal antibody termed MAb 1.9 (4). We now confirm the specificity of this platelet activation process by using a different type of specific PKC inhibitor, the PKC pseudosubstrate inhibitory peptides. Figure 1A depicts the formation of platelet aggregates when platelets were incubated with the pseudosubstrate peptide PKC [19–36] in the presence of fibrinogen. Platelet aggregates of similar large size were observed upon platelet incubation with the shorter inhibitory pseudosubstrate peptide PKC [19–31]. Platelets that were incubated under the same conditions without PKC peptides did not exhibit formation of such aggregates (Fig. 1B). Furthermore, when platelets were incubated under the same conditions in the presence of PKC [19–36] peptide at the same concentration shown in Fig. 1A, but without including fibrinogen in the incubation medium, platelet aggregation did not take place, and all the fields observed under these conditions were similar in appearance to that shown in the control (see Fig. 1B).

To determine whether the aggregation of platelets caused by PKC pseudosubstrate inhibitory peptides involved inhibition of the phosphorylation of platelet surface proteins, we examined the effects of these peptides on platelet ecto-protein kinase activity. As reported previously (4), incubation of intact platelets with 0.1 μM extracellular [γ-32P]ATP results in the phosphorylation of about seven surfase phosphoproteins of 89, 68, 56, 48, 40, 36, and 22 kDa (see Fig. 1C, lane C). We previously satisfied all the criteria needed as evidence that the incorporation of 32P into proteins that takes place under these reaction conditions occurred in the ectodomains of these platelet surface proteins and is carried out by an ectoenzyme (4, 27). When the same ecto-protein kinase reactions were performed in the presence of PKC pseudosubstrate peptides, a significant decrease was observed in the phosphorylation of six surface proteins (Fig. 1C, lane PS). Interestingly, an enhancement in the phosphorylation of a 56-kDa surface protein was observed in reactions conducted in the presence of PKC inhibitory peptides (see Fig. 1C, lane PS), showing the selectivity of the peptides’ effects. This 56-kDa protein may be a substrate of a platelet ecto-protein kinase other than ecto-PKC, which benefits from the additional availability of extracellular [γ-32P]ATP molecules, which are not used by ecto-PKC when it is specifically inhibited by the PKC pseudosubstrate peptides. The observation of platelet aggregation induced by PKC inhibitory peptides is consistent with our previous report, which demonstrated that aggregation of platelets could be induced by PKC inhibitory antibodies and confirms the conclusion that this aggregation is caused by the inhibition of ecto-PKC activity (4).

When physiological platelet agonists induce platelet aggregation, an increase in the level of intracellular free calcium can be observed. We obtained similar results in the process of platelet activation that is induced by inhibition of ecto-PKC activity. As shown in Fig. 2, the addition of the PKC inhibitory antibody MAb 1.9 to platelet suspensions resulted in a significant increase in the level of intracellular free Ca2+, as detected by fluorescence imaging with fura 2. In the presence of MAb 1.9, the level of free intracellular calcium doubled from a resting value of 90 nM to a plateau level of ~200 nM. This increase in the level of intracellular free calcium induced by MAb 1.9 appeared blunted compared with the large effects of physiological platelet agonists, such as thrombin or collagen that release Ca2+ from intracellular stores. However, this effect of MAb 1.9, as shown in Fig. 2, could be blocked by the prior addition of an antibody directed against the fibrinogen receptor, an anti-GPIIIa antibody (21) termed MAb G10 (Fig. 3). Because antibody G10, directed against the GPIIIa component of the fibrinogen receptor, completely blocks the binding of fibrinogen to the platelet surface (21), it appears that MAb 1.9 induces a fibrinogen-binding-dependent Ca2+ flux through the GPIIb/IIIa integrin complex. Taken together, our present results demonstrate that platelet aggregation and the calcium elevation induced by inhibition of the platelet ecto-PKC is dependent on the binding of fibrinogen to the fibrinogen receptor. Therefore, identification and characterization of the platelet ecto-PKC will contribute to the elucidation of a novel mechanism that regulates the homeostatic maintenance of fibrinogen receptors on the platelet surface.
In the next phase of this investigation, we focused on the identification of the protein that serves as the ecto-PKC on the platelet surface. In designing these experiments, we followed a working hypothesis based on three assumptions: 1) ecto-PKC is an enzyme that has an exposed ATP binding site localized on the outer surface of platelets, 2) the binding of extracellular ATP to this site would be clearly detectable at low ATP concentrations of 100 nM, a concentration that we have found to be optimal for measuring ecto-PKC activity, and 3) the binding of extracellular ATP to this site would be blocked by the PKC monoclonal antibody MAb 1.9 at the concentration range in which this antibody inhibits ecto-PKC activity on the platelet surface and causes platelet aggregation. We carried out the experiments designed to test this hypothesis using the membrane-impermeable photoaffinity probe, azido-[α-32P]ATP, to identify specific ATP binding proteins on the external surface of washed, intact human platelets. The covalent binding of azido-[α-32P]ATP to specific proteins on the surface of intact platelets is shown in Fig. 4. At a concentration of 0.1 µM azido-[α-32P]ATP, we
observed the labeling of a single protein migrating in SDS gels with an apparent molecular mass of 43 kDa. At higher concentrations of azido-\([\alpha^{32}P]\)ATP, 1 and 4 µM, major labeling of proteins with molecular masses of 10, 55, and 180 kDa was observed in addition to the 43-kDa protein, with at least 10 proteins noticed as minor bands in the autoradiogram. When the same labeling procedure was employed, except that the step of illuminating the samples with ultraviolet light, which produces the covalent bond, was omitted, no labeled bands were detected, and the autoradiogram was blank (not shown).

Because we routinely measure ecto-PKC activity using an extracellular ATP concentration of 100 nM, all the binding experiments of the present study were carried out using a concentration of 100 nM azido-\([\alpha^{32}P]\)ATP, a condition in which only one surface protein, the 43-kDa, is labeled (see lane 0.1 µM in Fig. 4, and lane 1 in Fig. 5). Binding of azido-\([\alpha^{32}P]\)ATP to the 43-kDa protein was found to be inhibited in a dose-dependent manner by prior binding of unlabeled 8-azido-ATP. When 100 nM unlabeled azido-ATP was covalently bound to the platelet surface by ultraviolet irradiation before the addition of 100 nM azido-\([\alpha^{32}P]\)ATP, ~35% of the radiolabeled analog binding to the 43-kDa protein was inhibited (see lane 2 in Fig. 5). Higher concentrations of 1, 10, and 100 µM of unlabeled azido-ATP produced a 60, 90, and 100% blockade of subsequent azido-\([\alpha^{32}P]\)ATP binding to the 43-kDa protein, respectively, with 50% inhibition occurring at 500 nM.

The competition of ATP with the binding of azido-\([\alpha^{32}P]\)ATP to the 43-kDa protein is shown in Fig. 6. The addition of a 10-fold excess of unlabeled ATP together with the labeled probe (before the ultraviolet irradiation) resulted in ~40% inhibition of covalent azido-\([\alpha^{32}P]\)ATP binding, whereas over 90% inhibition of the binding of azido-\([\alpha^{32}P]\)ATP to the 43-kDa protein was...
observed in the presence of a 100-fold excess of ATP. The concentration at which 50% inhibition of the binding occurred was calculated as \( \sim 500 \text{nM} \). In contrast, additions of 100-fold excess concentrations of GTP, GDP, or ADP had no inhibitory effect on the binding of azido-\([\alpha^{32}\text{P}]\)ATP to the 43-kDa protein (not shown).

The role of the divalent cations \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) in ATP binding to the 43-kDa protein was examined by utilizing...
ing the chelators EGTA and EDTA. Figure 7 shows that a complete inhibition of the binding of azido-[α³²P]ATP to intact platelets occurred in the presence of EDTA (lane 2), whereas no inhibition was caused by the presence of EGTA (lane 3). These results indicate that removal of calcium ions does not interfere with the binding of azido-[α³²P]ATP to the 43-kDa protein, whereas magnesium ions are critical for the binding of azido-[α³²P]ATP to the 43-kDa protein on the platelet surface.

Previous studies have established the use of low concentrations of proteolytic enzymes incubated with intact cells as one of the test criteria for proving surface localization (10, 11). Using very low concentrations of trypsin, we examined the effect of limited proteolysis of the surface of intact platelets on the 43-kDa protein that binds extracellular ATP. Figure 8B (autoradiogram) demonstrates that a concentration of trypsin as low as 0.0005% already caused partial digestion of the 43-kDa azido-[α³²P]ATP binding protein (shown as 42.6 kDa) resulting in the generation of a major proteolytic fragment of 34 kDa. At the same time, the Coomassie blue protein staining pattern (Fig. 8A, lane 2) did not reveal the appearance of a digestion product of 34 kDa, indicating that the major protein band stained by Coomassie blue in Fig. 8A (also of 43 kDa) was not the source of the 34-kDa azido-[α³²P]ATP-labeled protein fragment. In addition, lanes 3 and 4 of the autoradiogram (Fig. 8B) demonstrate that with an increase in the concentration of added trypsin (from 0.001 to 0.005%), greater inhibition of the azido-[α³²P]ATP-labeled 43-kDa protein occurred and ~80% of this labeled protein band disappeared with a larger amount of the azido-[α³²P]ATP-labeled 34-kDa protein being formed. When 0.01% trypsin was used (Fig. 8B, lane 5), complete digestion of the 43-kDa labeled protein occurred concurrent with the appearance of three major radiolabeled azido-ATP-containing digestion products at 34, 15.9, and 3.2 kDa. On the other hand, the Coomassie brilliant blue-stained proteins shown in Fig. 8A revealed a completely different pattern of digestion. The major proteolytic digestion products of 28, 12, and 8.5 kDa seen in the protein-staining pattern (Fig. 8A) were generated only at higher concentrations of trypsin ranging from 0.005 to 0.15%. A 43-kDa stained protein that is a major band in the staining pattern did not show the beginning of digestion until 0.005 to 0.01% trypsin was used. This differential sensitivity to extracellular trypsin assigns a surface location to the labeled 43-kDa protein and an intracellular location to the comigrating major stained protein.

We reported that platelet aggregation induced by MAb 1.9 is associated with a concurrent inhibition of the phosphorylation of surface proteins measured by adding 100 nM [γ³²P]ATP to the medium (4). In the present study we examined whether the binding of azido-[α³²P]ATP was similarly affected by the presence of MAb 1.9. Indeed, Fig. 9 (lanes 2 and 3) demonstrates that the binding of azido-[α³²P]ATP to the 43-kDa protein on the surface of intact platelets was blocked in the presence of the PKC inhibitory antibody MAb 1.9. Maximal inhibition of the binding of azido-[α³²P]ATP to the 43-kDa protein occurred at concentration range of MAb 1.9, which is optimal for inducing platelet aggregation. The specificity of this inhibition for the MAb 1.9 antibody was shown by the use of another PKC monoclonal antibody termed M6, a PKC type III antibody. M6, which does not induce platelet aggregation even at 100 µg/ml, also did not inhibit the binding of azido-[α³²P]ATP to the 43-kDa protein when used at this concentration (not shown).

MAb 1.9 was shown to have three separate effects: 1) it binds to the catalytic domain of purified PKC and inhibits its activity (24), 2) it inhibits platelet and neuronal ecto-protein kinase activities (4, 6), and 3) MAb 1.9 blocks azido-[α³²P]ATP binding to the 43-kDa surface protein (Fig. 9). Put together, these three effects suggest that the covalent binding of azido-ATP to the 43-kDa protein on the platelet surface should inhibit the activity of the platelet ecto-PKC. Because the binding of azido-[α³²P]ATP to the 43-kDa protein could be blocked by both unlabeled ATP and unlabeled azido-ATP with identical IC₅₀ values, we examined whether the covalent binding of unlabeled 8-azido-ATP would inhibit the platelet ecto-protein kinase activity. As predicted, covalent binding of azido-ATP to intact platelets produced an inhibitory effect on the phosphorylation.
tion of platelet surface proteins by ecto-protein kinase activity (see Fig. 10A). In the absence of such covalent binding, we observed the phosphorylation by \([\gamma^{32}P]ATP\) of five endogenous protein substrates (89, 67, 56, 40, and 36 kDa) of ecto-protein kinase on the surface of intact platelets. The greatest inhibition by covalent binding of azido-ATP was observed in the phosphorylation of the 67- and 89-kDa proteins with 50% inhibition produced by <1 µM of azido-ATP. Inhibition by 50% of the surface phosphorylation of the 39.8, 56.2, and 35.5 proteins was observed at concentrations of azido-ATP of 1, 10, and 50 µM, respectively (Fig. 10B).

The ecto-protein kinase that binds 100 nM azido-ATP was further characterized by examining its ability to phosphorylate several exogenously added proteins. The results revealed that exogenous substrates known to be preferentially phosphorylated by PKC, such as MARCKS and MBP, are excellent substrates for the platelet ecto-protein kinase operating when 100 nM of \([\gamma^{32}P]ATP\) is added to the extracellular medium. The platelet ecto-protein kinase was observed to phosphorylate the exogenously added substrates detailed in Table 1 by detecting radioactive bands (indicative of \(^{32}P\) incorporation) in autoradiograms of SDS-PAGE gels corresponding to the molecular mass of each exogenous substrate added. Thus a phosphorylated protein band of ~3 kDa was observed when MARCKS was added as the exogenous substrate. When MBP was added as the substrate, a major phosphorylated band of ~18–29 kDa was observed. Likewise, a phosphorylated band of ~34 kDa was observed when phosvitin was added as the substrate, whereas a wide band of phosphorylated proteins of 19–25 kDa was observed when dephosphocasein was added as the exogenous substrate. These were the expected molecular masses of the added proteins. The rank order for the phosphorylation of these substrates by ecto-protein kinase operating on the surface of intact platelets in the presence of 100 nM \([\gamma^{32}P]ATP\) was found to be MARCKS > MBP > phosvitin > dephosphocasein. Covalent binding of azido-ATP before the ecto-protein kinase reactions resulted in the inhibition of the phosphorylation of MARCKS by ~75%. The phosphorylation of the other exogenous substrates by extracellular ATP was also inhibited by the covalently bound azido-ATP, and the rank order for the inhibition of the phosphorylation of these substrates by ecto-protein kinase was determined as follows: MARCKS > phosvitin > MBP > dephosphocasein. Interestingly,
MARCKS, the most specific PKC substrate used here, was not only the best substrate for platelet ecto-protein kinase reactions at 100 nM ATP, but this PKC substrate also exhibited the highest degree of sensitivity to inhibition by azido-ATP at the same concentration (see Table 1). Because covalent binding of azido-ATP performed at 100 nM is limited to the 43-kDa protein, this result completes the evidence pointing at this protein as the platelet ecto-PKC.

The 43-kDa azido-[γ-32P]ATP binding protein was analyzed further by IEF/SDS-two-dimensional PAGE to determine its isoelectric point. Figure 11 demonstrates that a radiolabeled spot of ~43 kDa was resolved that migrated in IEF with an isoelectric point of 4.9–5.0.

DISCUSSION

Circulating plasma contains ~100 nM ATP that serves as an extracellular source for purinergic receptors, ecto-ATPases, and ecto-protein kinases. Circulating, nonstimulated platelets in their discoid state express on their surface an ecto-protein kinase activity, which operates optimally at an ATP concentration of 100–500 nM (4). Ecto-protein kinases that operate at the ATP concentration of circulating plasma have the potential of contributing to the homeostatic regulation of circulating cells. Indeed, we found that an antibody which inhibits the activity of the platelet ecto-PKC causes platelet aggregation. The aggregation induced by this ecto-protein kinase inhibitory antibody could be blocked by a membrane-impermeable inhibitor of phos-
Table 1. Covalent binding of 8-azido-ATP inhibits exogenous substrate phosphorylation by platelet surface ecto-protein kinase

<table>
<thead>
<tr>
<th>Exogenous Substrate</th>
<th>Percentage (%) of Exogenous Substrate Phosphorylation During Reincubation With γ-32P]ATP</th>
<th>% Inhibition Due to Preincubation With 8-Azido-ATP</th>
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<tr>
<td>Phosvitin Before</td>
<td>100.0 ± 12.19</td>
<td>51</td>
</tr>
<tr>
<td>After 8-azido-ATP</td>
<td>49.3 ± 5.45*</td>
<td></td>
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<tr>
<td>Dephosphocasein</td>
<td>100.0 ± 14.17</td>
<td>21</td>
</tr>
<tr>
<td>Before</td>
<td>79.0 ± 7.23*</td>
<td></td>
</tr>
<tr>
<td>After 8-azido-ATP</td>
<td>100.0 ± 14.02</td>
<td>38</td>
</tr>
<tr>
<td>Myelin basic protein</td>
<td>100.0 ± 6.50*</td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>62.0 ± 6.50*</td>
<td></td>
</tr>
<tr>
<td>After 8-azido-ATP</td>
<td>100.0 ± 41.63</td>
<td></td>
</tr>
<tr>
<td>MARCKS</td>
<td>25.0 ± 6.55*</td>
<td>75</td>
</tr>
<tr>
<td>Before</td>
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<td>After 8-azido-ATP</td>
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Values are means ± SE of 3 separate experiments. Ecto-protein kinase phosphorylation reactions were performed using intact platelet suspensions to which phosvitin (100 µg/ml), diphosphocasein (100 µg/ml), myelin basic protein (50 µg/ml), or myristoylated Ala-rich C-kinase substrate (MARCKS, 50 µg/ml) was added. Phosphorylation reactions were carried out for 10 min at 37°C with washed platelets at a final cell concentration of 1.2 × 10^9/ml using 100 nM γ-32P]ATP. Intact platelets were preincubated with 8-azido-ATP (100 nM) for 1 min and exposed to ultraviolet light for 2 min before the addition of γ-32P]ATP. Samples were prepared for SDS-PAGE, and data analysis was performed using a PhosphorImager. Control values were set at 100% of substrate phosphorylation with platelets preincubated without 8-azido-ATP. Statistical analysis comparing control and azido-treated groups was performed using a two-tailed unpaired Student’s t-test. *Statistically significant differences were observed between each group at the level of P < 0.05.

phosphoprotein phosphatase activity, the heptapeptide microcystin (4). Therefore, it was concluded that the conservation of a physiological state of phosphorylation of surface proteins by the combined activities of ecto-protein kinase and ecto-phosphoprotein phosphatase contributes to the maintenance of circulating platelets in a nonactivated, discoid shape. In the present study, we have begun elucidating the biochemical pathways that are triggered by the inhibition of surface protein phosphorylation and lead to platelet aggregation. We found that PKC pseudosubstrate inhibitory peptides, similar to PKC inhibitory antibodies, cause the formation of platelet microaggregates before any indication that platelet secretion has occurred. This microaggregate formation was dependent on the addition of exogenous fibrinogen to the platelet suspension. This observation demonstrates that the platelet aggregation induced by the inhibition of ecto-protein kinase is caused directly by the binding of fibrinogen to the platelet surface and the formation of fibrinogen bridges that produce the microaggregates. The formation of fibrinogen bridges between platelets requires activation of latent fibrinogen receptors before fibrinogen binding (5). It must be concluded, therefore, that inhibition of ecto-protein kinase causes activation of latent fibrinogen receptors. This conclusion has been verified by using an anti-GPIIb antibody called G10, which inhibits fibrinogen binding to activated fibrinogen receptors (21). Indeed, we found that MAb G10 inhibited the microaggregation of platelets induced by the PKC antibody and PKC inhibitory peptides.

Another line of evidence pointing to the activation of fibrinogen receptors as the initial mechanism evoked by inhibition of the platelet ecto-PKC emerged from the measurements of changes in the level of platelet intracellular Ca^{2+}. Both the PKC inhibitory antibody (see Figs. 2 and 3) and PKC inhibitory peptides caused an increase in the platelet intracellular ionized Ca^{2+} level at concentrations that cause platelet aggregation and inhibition of platelet ecto-protein kinase activity. Moreover, this increase in intracellular Ca^{2+} levels, induced by inhibition of the platelet ecto-protein kinase, could be prevented by MAb G10, namely, the increase in intracellular Ca^{2+} by MAb 1.9 is dependent on the binding of fibrinogen to its activated receptors. This conclusion was also confirmed from another direction, by showing that the rise in platelet intracellular Ca^{2+} caused by MAb 1.9 does not take place unless fibrinogen is added to the extracellular medium. Taken together, these findings point at the following sequence of events as the mechanism by which inhibitory PKC antibodies and peptides cause platelet aggregation. First, the platelet ecto-PKC activity, which operates on the surface of nonstimulated platelets at 100 nM of extracellular ATP, is inhibited. Second, while the ecto-kinase is inhibited, the continued platelet ecto-phosphatase activity (27) causes a decrease in the phosphorylation state of surface proteins, which can be prevented by microcystin. The decreased state of phosphorylation results in conformational changes in the structure of

![Two-dimensional O'Farrell SDS-PAGE/isoelectric focusing (IEF)](http://ajpheart.physiology.org/)

Fig. 11. Two-dimensional O’Farrell [SDS-PAGE/isoelectric focusing (IEF)]. Intact platelets were photoaffinity labeled with 100 nM 8-azido-[γ-32P]ATP as described in MATERIALS AND METHODS. Equal volumes of 1× IEF buffer were added, and 50 µl of sample was applied to tube gel for IEF. After two-dimensional gel electrophoresis in SDS, gel was stained by silver and dried in vacuo (A). Gel was analyzed by phosphoimage using a PhosphorImager (Molecular Dynamics), and results are shown in B. Molecular weight and isoelectric point (pI) were determined as described in MATERIALS AND METHODS using two-dimensional molecular weight standards. Arrow points to a radiolabeled protein of 43,000 Da with a pI value of 4.9–5.0.
The molecular mass of PKC isozymes in platelets ranges between 70 and 95 kDa (42, 43). A related protein kinase with a molecular mass of 43 kDa could be the kinase termed PKM. PKM represents the catalytic domain of PKC holoenzymes separated from the regulatory mechanism significant to the homeostatic regulation of the cardiovascular system. The importance of the maintenance of the phosphorylation state of surface proteins for platelet homeostasis indicates that the understanding of the biochemical mechanisms underlying platelet physiology would be greatly enhanced by the identification, isolation, and cloning of the platelet surface protein that serves as this ecto-protein kinase. The first step in achieving this goal has been accomplished in the present study by using the membrane-impermeable, photoaffinity probe 8-azido-[α-32P]ATP to identify on the platelet surface an ATP-binding protein with properties of ecto-PKC. Several ATP binding proteins were detected on the platelet surface using this reagent (see Fig. 4), and these may be ATP receptors, ADP receptors, ecto-ATPases, or ecto-protein kinases. However, only one single protein, a 43-kDa component, was identified when using 8-azido-[α-32P]ATP at 100 nM, a concentration optimal for ecto-PKC activity. The interference by an anti-PKC antibody (that cannot penetrate cells) with the binding of 8-azido-[α-32P]ATP to this protein points out the surface location of the ATP binding site of this protein and its identity as the protein named ecto-PKC. The surface location of this protein was confirmed further by selective trypsinization of surface proteins. The major Coomassie-stained band in the pattern of electrophoretically separated platelet proteins is known to be actin, which by molecular weight would comigrate with the 8-azido-[α-32P]ATP binding protein identified here. Indeed, we found (see Fig. 8) that 0.0005% trypsin added to the extracellular medium had no effect on the major stained band representing intracellular actin, whereas at the same time proteolysis of the comigrating 8-azido-[α-32P]ATP binding protein confirmed its surface location.

In addition to the inhibition by MAb 1.9, other properties of the binding of 8-azido-[α-32P]ATP to the 43-kDa protein were also found to be as expected from ecto-PKC. The azido-ATP binding to the 43-kDa protein was inhibited by extracellular ATP (see Fig. 6) but not by ADP. Consistent with the known properties of all protein kinases, magnesium ions were found to be required for the ATP binding activity of the 43-kDa protein (see Fig. 7). The binding of azido-ATP could be readily inhibited by ATP, but not by GTP, at the same concentration, which is consistent with the known properties of PKC but not of casein kinase. Finally, a direct relationship between the ATP binding activity of the 43-kDa protein on the platelet surface and the activity of ecto-PKC was determined. The investigation of this relationship was carried out based on the finding that nonlabeled azido-ATP and ATP itself bound to the 43-kDa protein on the platelet surface at 100 nM with identical properties to that of 8-azido-[α-32P]ATP. Occupancy of the ATP binding site of the surface 43-kDa protein by covalent binding of azido-ATP would prevent subsequent binding of free ATP and thus inhibit the enzymatic activity of this protein, if it was a kinase. Indeed, covalent binding of 100 nM azido-ATP, which bound exclusively to the 43-kDa protein (see Fig. 4), resulted in preferential inhibition of the phosphorylation of MARCKS peptide that is a selective PKC substrate, demonstrating that the 43-kDa surface protein exhibits protein kinase activity with catalytic specificity of ecto-PKC.

The molecular mass of PKC isozymes in platelets ranges between 70 and 95 kDa (42, 43). A related protein kinase with a molecular mass of 43 kDa could be the kinase termed PKM. PKM represents the catalytic domain of PKC holoenzymes separated from the regulatory domain by proteolysis. MAb 1.9, which binds to the platelet 43-kDa surface protein, inhibits its ATP binding activity as well as ecto-PKC activity, is an antibody that blocks the activity of authentic PKM prepared from brain PKC (24). Furthermore, using azido-[α-32P]ATP, we found on the surface of brain neurons a 43-kDa protein with ATP binding properties and ecto-PKC activity as reported here (MV Hogan, A Babinska, Z Pawlowska, E Kornecki, and YH Ehrlich, unpublished observations). Accordingly, ecto-PKC may be a PKM-like protein generated from PKC by proteolysis, or PKM that is synthesized directly by alternate splicing of PKC mRNA, or a new protein that shares (with PKC) properties associated with the catalytic site that binds ATP. Purification of the 43-kDa protein and the cloning of its gene will resolve this question and contribute to the continued elucidation of a novel regulatory mechanism significant to the homeostatic regulation of the cardiovascular system.

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