Stimulatory antibody-induced activation and selective translocation of protein kinase C isoenzymes in human platelets

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A novel stimulatory monoclonal antibody (Mab) termed Mab.F11 induces granular secretion and subsequent aggregation of human platelets. Mab.F11 recognizes a unique 32 and 35 kDa protein duplex on the platelet membrane surface, called the F11 receptor; binding of Mab.F11 to its receptor results in increased intracellular phosphorylation of P47, the known protein kinase C (PKC) substrate pleckstrin. In order to determine whether the mechanism of action of Mab.F11 involves direct activation of PKC, two types of functional assays for measuring PKC activity were performed. Measurement of PKC activity in digitonin-permeabilized platelets revealed that Mab.F11 produced a rapid, 2–3-fold increase in the control value in the phosphorylation of the PKC peptide substrate, PKC(19–31) Ser²⁵. The increase in PKC activity induced by Mab.F11 was found to be associated with the platelet membrane; a 1.6-fold control value increase in membrane PKC activity occurred rapidly, within 10 s of the

addition of Mab.F11. The translocation from the cytoplasm to the membrane induced by Mab.F11 in PKC isoenzymes α and ζ was reversible, whereas translocation of the PKC isoenzymes δ , β , η' and θ was irreversible, with PKC levels remaining elevated in the membrane for at least 15 min. Taken together, our results demonstrate that in the initial stages of platelet activation by this stimulatory antibody, the enhanced membrane PKC activity reflects the presence of all six isoenzymes. At later stages, PKC activity is reflective of four isoenzymes. These results demonstrate that separate groups of PKC isoenzymes must be involved in different aspects of platelet activation. The long lag period and prolonged activation time of platelets by Mab.F11 renders this agonist most suitable for identifying the isoenzymes and their specific endogenous protein substrates involved in platelet secretion and aggregation induced by platelet membrane protein antibodies.

INTRODUCTION

An important participant in the activation of human platelets is protein kinase C (PKC), which was discovered in 1977 by Nishizuka and his colleagues [1]. To date, 12 isoenzymes of PKC have been identified and classified into three groups: the conventional calcium-dependent cPKC (α , β I, β II and γ), the calcium-independent isoenzymes nPKC (δ , ϵ , η and θ), and the atypical aPKC (ζ , λ , ι and μ) [2–6]. To-date, six isoenzymes of PKC have been identified in human platelets [5,7,8]. One of the functions of PKC in platelets is to phosphorylate pleckstrin, a protein with a molecular mass of 40–47 kDa [9,10] which has been implicated in the process of vesicular secretion [11]. The stimulation of PKC activity in platelets has been shown to occur following the addition of thrombin [12,13], adrenaline (epinephrine) [14], platelet-activating factor (PAF) [15], phorbol 12-myristate 13-acetate (PMA) [16,17] and collagen [18,19]. Recently, Crabos et al. [8] have demonstrated the translocation of PKC isoenzymes α , β and ζ in human platelets stimulated by PMA. Baldassare et al. [7] have demonstrated the translocation of the PKC isoenzymes α , β , δ and ζ in platelets stimulated by thrombin. Our laboratory has confirmed the presence of cPKC α , cPKC β , nPKC δ and aPKC ζ in human platelets and was the first to describe the presence of the PKC isoenzyme nPKC θ in human platelets [20]. We have also identified a novel, high-molecular-

mass PKC isoenzyme, named nPKC η' , of 95 kDa, using an antibody directed against the N-terminal of nPKC η [20]. We have reported that stimulation of human platelets by PAF resulted in a time-dependent translocation of nPKC η' and nPKC θ from the cytosol to the plasma membrane [20], suggesting that PKC isoenzymes η' and θ play a role in the mechanism by which PAF activates platelets.

The presence of autoantibodies directed against platelet surface proteins in the circulating plasma appears to play an important role in the pathophysiology of idiopathic thrombocytopenia purpura (ITP) [21–24] and in thrombocytopenia associated with human immunodeficiency virus infections [25,26]. Many of these circulating antibodies are stimulatory, as they induce platelet activation [21–26]. The role of PKC isoenzymes in platelet activation by stimulatory antibodies has not yet been studied. Mab.F11 is a stimulatory monoclonal antibody developed in our laboratory which recognizes a unique 32 kDa and 35 kDa protein duplex on the platelet surface, termed the F11 receptor [27]. The partial sequence of the platelet F11 receptor has been reported recently and PKC consensus sites were found in peptide fragments prepared both from endoproteinase Glu-C and trypsin digestion [28]. Furthermore, the phosphorylation of the isolated F11 receptor was found to be stimulated by PMA and phosphatidylserine (PS), indicating the potential involvement of PKC in the down-regulation of this receptor [28].

Abbreviations used: ACD, acid–citrate–dextrose; Mab, monoclonal antibody; PAF, platelet-activating factor; PGE₁, prostaglandin E₁; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PRP, platelet-rich plasma; PS, phosphatidylserine.

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In the present study we have examined the effects of Mab.F11 on PKC activation and on the translocation of six specific PKC isoenzymes, and examined the role of the FcγRII receptor and the fibrinogen receptor protein, GPIIIa, in platelet activation by this stimulatory antibody.

MATERIALS AND METHODS

Materials

Radioactive [γ - 32 P]ATP (3000 Ci/mmol) was purchased from Du Pont–New England Nuclear (Boston, MA, U.S.A.). PMSF, PS, 1,2-diolein, ATP, histone type III-s, BSA (fraction V), soybean trypsin inhibitor, leupeptin, dithiothreitol, digitonin, β -glycerophosphate, EDTA, EGTA, benzamide, apyrase grade V, heparin, prostaglandin E₁ (PGE₁) and piperazine-*N,N'*-bis (2-ethanesulphonic acid) (Pipes) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cytoscint was purchased from Fisher Scientific (Springfield, NJ, U.S.A.). Millipore filters (HA type, 0.45 μ m pore diam.) was purchased from Millipore Corporation (Bedford, MA, U.S.A.) and phosphocellulose paper was obtained from Fisher Scientific. The PKC peptide substrate PKC(19–31) Ser²⁵ was purchased from Gibco Company (Grand Island, NY, U.S.A.).

Antibodies

The antibodies used were as follows: anti-cPKC α , polyclonal against peptide sequence ³¹³AGNKVISPEDRRQ³²⁶ (5 μ g/ml; Gibco); anti-cPKC β , monoclonal antibody against the N-terminal regulatory domain (5 μ g/ml; Seikagaku America Co.); and anti-cPKC β , polyclonal against peptide sequence ⁶⁶¹SYTNPEFVINV⁶⁷¹ (5 μ g/ml); anti-cPKC γ , polyclonal against peptide sequence ³⁰⁸NYPLELYERVRTG³¹⁸ (5 μ g/ml; Gibco); anti-nPKC δ , polyclonal against peptide sequence ⁶⁶⁴VNPKYEQFLE-COOH⁶⁷³ (1:100 dilution); kindly donated by Dr. Peter J. Parker, Imperial Cancer Research Fund, London); anti-nPKC ϵ , polyclonal against peptide sequence ³¹³KGFSYF-GEDLMP³²⁴ (5 μ g/ml; Gibco); anti-aPKC ζ , polyclonal directed against peptide sequence ⁵⁷⁷GFEYINLLLSAEESV⁵⁹² (5 μ g/ml; Gibco); anti-nPKC η , polyclonal directed against the peptide sequence ⁵⁶QTSTKQKTNKPTYNEEFC⁷³ (3 μ g/ml) [29]; and anti-nPKC θ , polyclonal against the peptide sequence ³³²CVPT-PGKREPQGISWDSPLDGSNK³⁵⁵ (3 μ g/ml) [30]. The characterization of Mab.F11 and Mab.G10 have been described previously [27]. Mab.IV.3 was purchased from Medarex (W. Lebanon, NH 03784). The supplier of each antibody indicates its specificity to the stated isoenzyme.

Collection of blood and washing of platelets

Blood was collected from healthy individuals free of medication for at least 2 weeks prior to blood collection. All volunteers signed an informed consent form approved by the New York Committee on Human Research, Health Science Center at Brooklyn, State University of New York. Blood was collected in the anticoagulant acid–citrate–dextrose (ACD) at a ratio of 6:1 (blood:ACD, v/v) for preparation of washed platelets or in 3.8% sodium citrate (9:1, v/v) as described previously [27]. Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 120 *g* for 10 min at 24 °C and platelets were isolated by differential centrifugation. Platelet pellets were resuspended in a Tyrode–Hepes solution, pH 7.4, containing: 0.35% BSA, 2.68 mM KCl, 136 mM NaCl, 1 mM MgCl₂, 0.36 mM NaH₂PO₄, 5.5 mM D-glucose, 20 mM Hepes, 2 mM CaCl₂ and the inhibitors heparin (2 units/ml), potato apyrase

(320 μ g/ml) and PGE₁ (1 μ M). Washed platelet pellets were resuspended in a Tyrode–Hepes solution without BSA or inhibitors and counted as described previously [20].

Platelet aggregation

These experiments were performed in a Chronolog Lumi-Aggregometer (Chronolog Corp., Havertown, PA, U.S.A.). Platelet aggregation was initiated by adding Mab.F11 (7 μ g/ml) to 0.45 ml aliquots of PRP or washed platelet suspensions containing (2–4) $\times 10^8$ platelets/ml incubated at 37 °C under stirring conditions.

Assay of PKC activity in permeabilized cells

Washed platelets [(1.2–1.8) $\times 10^9$ platelets/ml] were incubated at 37 °C in Tyrode–Hepes buffer under non-stirring (or stirring) conditions in the presence or absence of Mab.F11. At various time points, aliquots were removed and centrifuged at 10000 *g* for 20 s. The platelet pellets were resuspended in 100 μ l of PKC assay buffer following the procedure of Johnson and Heasley [31] with minor modifications. The buffer consisted of: 137 mM NaCl, 5.4 mM KCl, 0.3 mM NaH₂PO₄·H₂O, 0.4 mM KH₂PO₄, 1 mg/ml glucose, 20 mM Hepes (pH 7.2), 50 μ g/ml digitonin, 10 mM MgCl₂, 25 mM β -glycerophosphate, 100 μ M [32 P]ATP, 5 mM EGTA, 2.5 mM CaCl₂, and 50 μ M of the peptide PKC(19–31) Ser²⁵. Kinase reactions proceeded at 30 °C for 6 min, after which time the complete sample was spotted on to 2 cm $\times$ 2 cm phosphocellulose strips. The strips were washed in two changes of 1% phosphoric acid and distilled water for five min each, placed in minivials containing 5 ml of scintillation fluid and counted in a beta scintillation counter. Endogenous phosphorylation measured in the absence of the peptide substrate was subtracted from all measurements.

Preparation of platelet membrane and cytosol fractions for immunoblotting

Washed platelets (2 $\times 10^9$ /ml) were incubated with Mab.F11 (7 μ g/ml) under non-stirring (or stirring) conditions at 37 °C for various periods of time followed by the addition of an equal volume of cold double-concentrated sonication buffer containing: 20 mM Tris/HCl (pH 7.5), 2 mM EDTA, 50 mM β -mercaptoethanol, 10 mM EGTA, 1 mM PMSF, 5 μ g/ml leupeptin, 5 μ g/ml trypsin inhibitor and 25 mM benzamide as described previously [19]. The platelet suspensions were sonicated with three 10 s bursts and centrifuged at 100000 *g* for 30 min at 4 °C using a Beckman TLA 100 ultracentrifuge (Beckman Instruments, Palo Alto, CA 94304, U.S.A.). The cytosol fractions were collected and the pelleted membrane fractions were resuspended in cold sonication buffer containing 1% Triton X-100 (extraction buffer). The pellets were dispersed, sonicated and centrifuged at 100000 *g* for 30 min at 4 °C. The proteins extracted into the supernatants were removed as the membrane fraction. Protein concentration was determined by the method of Bradford [32].

PKC assays of membrane fractions

Aliquots of washed platelets (700 μ l of 2 $\times 10^8$ platelets) were incubated in an aggregometer under stirring (or non-stirring) conditions at 37 °C with Mab.F11 (7 μ g/ml) for various lengths of time. Control aliquots were incubated for the same amount of time except without the prior addition of Mab.F11. The platelet membranes were obtained as described above. PKC activity was

assayed by measuring the incorporation of ^{32}P from [$\gamma\text{-}^{32}\text{P}$]ATP into histone in membrane fractions as previously described [33–36] with the following modifications. The reaction mixture (final volume, 100 μl) contained the following reagents: 25 mM Pipes (pH 7.0), 10 mM MgCl_2 , 0.2 mM DTT, 1 mM PMSF, 50 mM benzamidine, 25 μM CaCl_2 , leupeptin (10 $\mu\text{g}/\text{ml}$), trypsin inhibitor (50 $\mu\text{g}/\text{ml}$), PS (27 $\mu\text{g}/\text{ml}$), diolein (2 $\mu\text{g}/\text{ml}$) and histone (33 $\mu\text{g}/\text{ml}$). Reactions were initiated by the addition of [$\gamma\text{-}^{32}\text{P}$]ATP (final ATP concentration, 15 μM). Assays were carried out at 30 $^\circ\text{C}$ using the above media in the absence or presence of PS and diolein for 5 min. The reactions were terminated by adding 1 ml of ice-cold 10% trichloroacetic acid containing 1.5% sodium pyrophosphate and 1% NaH_2PO_4 (TCA solution). The samples were poured over filters (HA type) on a manifold (Millipore Corporation) and the filters were washed four times using the TCA solution. The filters were placed into scintillation vials to which 3 ml of scintillation fluid was added. The amount of radioactivity was measured using a Beckman LS6000TA liquid scintillation counter. PKC activity was determined by calculating the difference in the incorporation of ^{32}P in the presence and absence of PS and diolein.

SDS/PAGE

SDS/PAGE was performed using a 3% stacking gel and an 8% running gel as described by Laemmli [37]. Equal aliquots of 0.45 ml of cytosolic and membrane fractions from each time point were mixed with 100 μl of 6 $\times$ Laemmli sample buffer consisting of 62.5 mM Tris, 2% SDS, 10% glycerol, 5% β -mercaptoethanol and 0.001% Bromphenol Blue. Samples were vortexed, boiled for 5 min and applied to 3-cm-wide sample wells. The PKC isoenzymes η and θ , extracted from COS cells transfected with the nPKC θ - and PKC η -cDNA expression plasmids, were used as positive controls. Proteins were transferred for 5 h at 4 $^\circ\text{C}$ at 50 V on to nitrocellulose membranes (Schleicher & Schuell Co., 0.45 μm).

Immunoblotting

The nitrocellulose membranes were rinsed three times and incubated with 1% BSA in TBST [50 mM Tris/HCl (pH 7.5), 0.15 M NaCl and 0.05% Tween-20] for at least 2 h to block the non-specific binding of proteins to membranes. The membranes were cut into 3 mm strips and incubated with specific PKC isoenzyme antibodies. After overnight incubation with PKC antibodies at 4 $^\circ\text{C}$, the nitrocellulose membranes were washed three times for 10 min with 1% BSA in TBST. The membranes were incubated with alkaline phosphatase-conjugated anti-IgG antibody (Sigma Chemical Co.) for 2 h at 22 $^\circ\text{C}$. The membranes were then washed five times in 1% BSA in TBST, and immunoreactive bands were visualized by colour development using 0.41 M Nitro Blue Tetrazolium and 0.38 M 5-bromo 4-chloro-3-indolyl phosphate dissolved in 0.1 M Tris (pH 9.5)/0.1 M NaCl/5 mM MgCl_2 .

Quantification of Immunoblots

The densities of the PKC bands shown in the immunoblots were quantified using the XRS Omnimedia and Sharp scanners (XRS Omnimedia, and Sharp Co.) [23]. The images were numerically quantified according to band density by Image 1.43, a computer program run on a Macintosh IIfx computer. The raw numerical data were divided by the protein concentration of each sample and expressed as density units per μg of protein. The membrane and cytosolic isoenzymes are expressed as percentages of the total of each isoenzyme in the platelets. Differences between the

level of each PKC isoenzyme at various time points were compared by means of paired *t*-tests ($\alpha = 0.05$).

RESULTS

Platelet aggregation and stimulation of PKC activity in human platelets by Mab.F11

A typical aggregation tracing of washed human platelets stimulated by Mab.F11 is depicted in Figure 1. We observed that the latency period measured between the addition of antibody Mab.F11 and the initiation of aggregation was dependent on the concentration of Mab.F11 used. As shown in Figure 1, Mab.F11 produced a latency period of approximately 3 min prior to the initiation of platelet aggregation. This latency period varied from donor to donor, indicating individual differences in sensitivity to this agonist. On the other hand, we consistently observed that the extent of platelet aggregation, measured in relative light transmission units, in response to Mab.F11 was greater than 60% for each donor when platelets were isolated and washed following the centrifugation procedures described in the Materials and methods section.

To determine whether Mab.F11-induced platelet aggregation involves mechanisms associated with the activation of the fibrinogen receptor, we used an antibody shown by immunoblotting to recognize the GPIIb component of the fibrinogen receptor. This antibody, Mab.G10, was reported previously to block the binding of fibrinogen to the platelet surface [27]. Preincubation of platelet suspensions with Mab.G10 completely blocked Mab.F11-induced platelet aggregation, as shown in Figure 1; indicative of the involvement of fibrinogen binding in the antibody-induced platelet aggregation process. Figure 1 also shows that another monoclonal antibody, Mab.IV.3, directed against the platelet Fc γ RII receptor, likewise completely inhibited the stimulatory actions of Mab.F11, indicating the involvement of the Fc γ RII receptor in this process. These same antibodies were used in subsequent experiments, detailed below, to examine the role of the fibrinogen receptor and Fc γ R11 receptor in Mab.F11-induced platelet activation of PKC.

The effects of Mab.F11 on PKC activity were examined directly by utilizing two functional PKC activity assays. The first assay involves the measurement of platelet PKC activity in platelets stimulated by Mab.F11 and subsequently permeabilized with digitonin for assay of PKC activity in whole cells. This procedure probably measures mainly the PKC activity associated

Figure 1 Effect of Mab.G10 and Mab.IV.3 on Mab.F11-induced platelet aggregation

Washed platelets (450 μl , 2×10^8) were incubated at 37 $^\circ\text{C}$ with Mab.F11 (7 $\mu\text{g}/\text{ml}$) alone or with Mab.G10 (7 $\mu\text{g}/\text{ml}$) or Mab.IV.3 (2 $\mu\text{g}/\text{ml}$) added 5 min prior to the addition of Mab.F11. The unlabelled arrow indicates the addition of Mab.F11. The relative increase in light transmission units is a measurement of the extent of platelet aggregation.

Figure 2 Measurement of PKC activity in human platelets stimulated by Mab.F11: inhibition by Mab.G10 and Mab.IV.3

Aliquots of washed platelets [$500 \mu\text{l}$, $(1.2\text{--}1.9) \times 10^9/\text{ml}$] were incubated with Mab.F11 ($7 \mu\text{g}/\text{ml}$) alone (Δ) or preincubated with either Mab.G10 ($7 \mu\text{g}/\text{ml}$) ($\square$) or Mab.IV.3 ($2 \mu\text{g}/\text{ml}$) ($\bullet$) for 5 min at 37°C under non-stirring conditions. Mab.F11 was added ($7 \mu\text{g}/\text{ml}$) to initiate platelet activation. PKC activity was measured in digitonin-permeabilized platelets as described in the Materials and methods section. Each point indicates the average value (mean $\pm$ S.E.M.) of three or more separate experiments.

with the membrane. The second PKC assay involved the direct measurement of PKC activity in membrane fractions isolated from Mab.F11-stimulated platelets. Figure 2 shows the time course of PKC activity in platelets stimulated with Mab.F11 obtained from assays of digitonin-permeabilized platelets. The increase in PKC activity induced by Mab.F11 was 1.6 times the control value. This increase occurred rapidly, within 30 s of the addition of antibody, and continued to increase up to a level of 2.2 times the control value at 60 s. This PKC activity level remained stable for up to 10 min. A similar pattern of increase in PKC activity in intact platelets was observed also under stirring conditions, when the activity of PKC was measured in aggregating platelets.

Preincubation of platelet suspensions with the anti-GPIIIa antibody, Mab.G10, for 5 min at 37°C , under stirring or non-stirring conditions, completely inhibited the Mab.F11-induced stimulation of platelet aggregation and, in parallel, inhibited the Mab.F11-enhanced activation of PKC, as depicted in Figure 2. These results indicate that GPIIIa plays a role in the stimulation of PKC activity by Mab.F11. In addition, preincubation of platelet suspensions with anti-Fc γ R2 antibody, Mab.IV.3, resulted in complete inhibition of the Mab.F11-enhanced PKC activity. These results indicate that, in addition to the fibrinogen receptor, the Fc γ R2 receptor plays an important role in regulating PKC activity when platelets are stimulated by antibodies. In addition, we found that the known PKC inhibitor K252a blocked Mab.F11-induced platelet aggregation with an IC_{50} value of 800 nM (A. Babinska, Y. H. Ehrlich and E. Kornecki, unpublished work). These results indicate that PKC activation is necessary for the functional responses of platelets to Mab.F11.

As shown in Figure 3, the PKC activity of membranes isolated from Mab.F11-stimulated platelets exhibited a rapid, 1.6-fold increase within 10 s of antibody addition. These results were similar to those obtained with digitonin-permeabilized platelets, as shown in Figure 2. At 30 s, the PKC activity increased further to 1.8 times the control value and remained enhanced at this level for the duration of the test period.

Figure 3 PKC activity in isolated platelet membranes following stimulation of platelets by Mab.F11

The platelet membrane fractions were isolated as described in the Materials and methods section. PKC activity in the membrane fraction was assayed by measuring the amount of incorporation of ³²P from [³²P]ATP into histone at different time periods. The y-axis indicates the percentage change in the level of Mab.F11-stimulated membrane PKC as compared with the level of unstimulated platelet membrane PKC. Each point indicates the average value (mean $\pm$ S.E.M.) of three separate experiments.

Figure 4 Detection of various PKC isoenzymes in human platelets

Total platelet proteins, prepared from washed human platelets, were subjected to SDS/PAGE followed by immunoblotting. Nitrocellulose strips 'a' to 'i' and strip 'k' contain $50 \mu\text{g}$ of protein/strip. Recombinant PKC isoenzymes ($5 \mu\text{g}/\text{strip}$) obtained from COS cells transfected with nPKC η -cDNA and nPKC θ -cDNA are shown as strips 'j' and 'k' respectively. Strips 'a', 'c', 'e', and 'g' were incubated with anti-PKC α , β , δ , and ζ antibodies respectively. Strips 'i' and 'j' were incubated with anti-PKC θ , whereas strips 'k' and 'l' were incubated with anti-PKC η antibody. Strips 'b', 'd', 'f' and 'h' were incubated with anti-PKC α , β , δ , and ζ antibodies respectively, in the presence of their corresponding peptide ($10 \mu\text{g}/\text{ml}$).

Identification of PKC isoenzymes in human platelets

To determine which PKC isoenzymes are responsible for the enhanced PKC activity associated with the platelet membrane following platelet activation by Mab.F11, we initially sought to identify the presence of specific PKC isoenzymes in human platelets by use of anti-(PKC isoenzyme)-specific antibodies. We observed that six of the known PKC isoenzymes, namely α , β , δ , ζ , η and θ could be detected immunologically in human platelets (Figure 4). The PKC isoenzymes ϵ and γ were not detectable in human platelets. The molecular masses of the PKC isoenzymes

Figure 5 Translocation of PKC isoenzymes in human platelets stimulated by Mab.F11

Washed human platelets were incubated with 7 μ g/ml of Mab.F11 for the time periods showed above. Membrane and cytosol fractions were prepared and immunoblotting was performed as described in the Materials and methods section by using specific anti-PKC isoenzyme antibodies directed against cPKC α , cPKC β , nPKC δ , aPKC ζ , mPKC η , and nPKC θ . The membrane and cytosolic isoenzymes are expressed as percentages of the total of each isoenzyme in the platelets. Each point indicates the average value obtained from three or more separate experiments. The asterisk (*) indicates a significant difference in value ($P < 0.05$) as compared with control values.

identified in human platelets were determined to be: 80 kDa for α and β , 78 kDa for δ , 75 and 80 kDa (doublet bands) for ζ , 78 and 80 kDa (doublet bands) for θ , and 95 kDa for η (see Figure 4, lanes a, c, e, g, i and k, respectively). The incubation of nitrocellulose strips with specific anti-PKC antibodies together with their corresponding peptides resulted in the complete disappearance of the staining of specific PKC protein bands (as

shown in Figure 4, lanes b, d, f and h, for α , β , δ and ζ , respectively). Lanes j and l show the recognition of recombinant PKC isoenzymes θ and η , obtained from COS cells transfected with nPKC θ -cDNA and nPKC η -cDNA, using PKC θ - and η -specific antibodies, respectively.

Translocation of PKC isoenzymes in human platelets stimulated by Mab.F11

The separate association of PKC isoenzymes with the membrane and the cytosol fractions was determined as a function of time following the stimulation of platelets with Mab.F11. The level of each PKC isoenzyme was measured at various time points in both isolated membrane fractions and cytosolic fractions, and then compared with control, unstimulated levels of each PKC isoenzyme, determined in platelets assayed in the absence of antibody.

As shown in Figure 5, within 10 to 30 s of the addition of Mab.F11, the levels of each of the six PKC isoenzymes investigated here increased significantly within the membrane, with a concurrent decrease in the level of PKC within the cytoplasm.

This rapid translocation to the membrane was maintained for either the entire length of time, as seen with PKC isoenzymes β , δ , η and θ , or it was reversible, as shown for PKC isoenzymes α and ζ . We observed another consistent and significant increase in the level of PKC α within the membrane 15 min after the addition of Mab.F11. It is interesting to note that all of the PKC isoenzymes assayed here are present in detectable quantities in the platelet membrane prior to platelet activation. Among them, the levels of PKC isoenzymes α and δ appear to be the highest ($> 40\%$ of total), PKC isoenzymes ζ , θ and η in the membrane were found to be approximately 25% of the total, whereas the level of PKC isoenzyme β (10% of total) was the lowest of all.

DISCUSSION

In the study reported here we demonstrate directly that PKC activity is required for platelet activation by a stimulatory monoclonal antibody called Mab.F11. The PKC activation by Mab.F11 is accompanied by an increase in membrane-bound PKC activity and by a decrease in cytosolic PKC activity, suggesting translocation of PKC molecules. The pathway for PKC activation by the stimulatory antibody involves its initial interaction with the platelet surface protein recognized specifically by Mab.F11, as well as interaction of Mab.F11 with the platelet surface receptor for the Fc portion of immunoglobulins, Fc γ R2. This was determined by the finding that an antibody raised to the platelet Fc γ R2 receptor blocked the Mab.F11 antibody-induced activation of PKC. Furthermore, the activation of PKC by Mab.F11 appears to be mediated by a component of the platelet fibrinogen receptor, the GPIIb/IIIa molecule. Using a monoclonal antibody raised to the platelet surface glycoprotein GPIIb/IIIa, we have found that the platelet fibrinogen receptor is involved not only in fibrinogen binding and the production of extracellular fibrinogen bridges between aggregating platelets, but also plays a role in producing an intracellular signal that leads to the induction of secretion [27]. In the present study, using the same monoclonal antibody (termed G10), we have found that the intracellular signal transduction pathway which is activated by the fibrinogen receptor leads to stimulation of PKC activity. The inhibition of PKC activation by Mab.IV.3 was not surprising, since it was previously shown that activation of human platelets by monoclonal antibodies that interact with the Fc γ R2 receptor operates through a biochemical pathway that involves PKC [38,39]. Nonetheless, the inhibition of PKC activation by Mab.G10 opens a new direction in studies

investigating the biochemical events of platelet activation via a pathway that requires participation of the fibrinogen receptor.

Human platelets express PKC isoenzymes α , β , δ , ζ , θ and η' , but not PKC γ and ϵ [20]. In the present study, we have confirmed this report and determined, in addition, the redistribution of each PKC isoenzyme between the membrane and cytosol locations following platelet activation by Mab.F11. It is accepted that measuring the redistribution of PKC activity evaluates the translocation of PKC molecules from the cytoplasm to the membrane [7,8]. It should be mentioned, however, that the results of this assay do not exclude the possibility that enzyme activity in the cytoplasm decreases while other enzyme molecules, which are membrane-bound, are activated.

Several of the processes involved in platelet activation, in particular the exposure of fibrinogen receptors on the platelet surface, are likely to involve phosphorylation of membrane-bound proteins. However, the identity of the PKC isoenzymes that phosphorylate different membrane-bound platelet proteins is not yet known. Analysis of the time course of events following platelet activation demonstrated two different responses of PKC isoenzymes: a transient response was elicited by Mab.F11 in the isoenzymes α and ζ and a sustained response was elicited by Mab.F11 in the PKC isoenzymes δ , β , η' and θ . In comparison with other agonists, Baldassare et al. [7] found that thrombin could induce increased membrane-associated PKC α , β and ζ , while the subcellular distribution of PKC δ remained unchanged. Crabos et al. [8] demonstrated that incubation of PMA with platelets resulted in greater translocation of PKC β than α and ζ from the cytosol to the membrane. In the present study, we have found a different profile of activation of PKC isoenzymes, which may be related to the unique mechanism of activation of platelets by stimulatory antibodies.

The selective activation of various PKC isoenzymes, and the differing time course of activation and translocation following platelet stimulation by Mab.F11, suggest that stimulatory antibodies may activate different PKC isoenzymes which may then be involved in different aspects of platelet responsiveness, as compared with other agonists. Mab.F11 initially induces granular secretion followed by aggregation. It appears that the translocation of the PKC isoenzymes α and ζ to the membrane occurs primarily in the early stages of platelet activation by Mab.F11 (those events associated with a long latency), whereas the sustained association of PKC isoenzymes δ , β , η' and θ with the membrane is related to the later irreversible events involved in platelet secretion and aggregation. The prolonged lag-period and the extended time-course characteristic of platelet activation by Mab.F11 [27] have made this agonist most suitable for identifying the specific PKC isoenzymes involved in different aspects of platelet activation. Our future studies are designed to identify the endogenous protein substrate(s) for each of the specific PKC isoenzymes involved in the different phases of platelet function.

Circulating antibodies that stimulate platelet secretion and aggregation are known to be involved in the pathophysiology of disorders such as immune thrombocytopenia [21–26]. Therefore, detailed elucidation of the biochemical pathways operating in the process of platelet activation by stimulatory antibodies is of great clinical significance and may serve in the development of novel therapeutic agents for the treatment of immunological diseases.

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REFERENCES

- 1 Takai, Y., Kishimoto, A., Inoue, M. and Nishizuka, Y. (1977) *J. Biol. Chem.* **252**, 7603–7609
- 2 Nishizuka, Y. (1992) *Science* **258**, 607–614
- 3 Bell, R. M. and Burns, D. J. (1991) *J. Biol. Chem.* **266**, 4661–4664
- 4 Blackshear, P. J. (1988) *Am. J. Med. Sci.* **296**, 231–240
- 5 Selbie, L. A., Schmitz-Peiffer, C., Sheng, Y. and Biden, T. J. (1993) *J. Biol. Chem.* **268**, 24296–24302
- 6 Johannes, L.-J., Prestle, J., Eis, S., Oberhagemann, P. and Pfizenmaier, K. (1994) *J. Biol. Chem.* **269**, 6140–6148
- 7 Baldassare, J. J., Henderson, P. A., Burns, D., Loomis, C. and Fisher, G. (1992) *J. Biol. Chem.* **267**, 15585–15590
- 8 Crabos, M., Imber, R., Woodtli, T., Fabbro, D. and Erme, P. (1991) *Biochem. Biophys. Res. Commun.* **178**, 878–883
- 9 Tyer, M., Rachubinski, R. A., Stewart, M. I., Varrichio, A. M., Shorr, R. G. L., Haslam, R. J. and Harley, C. B. (1988) *Nature (London)* **333**, 470–473
- 10 Tyers, M., Haslam, R. J., Rachubinski, R. A. and Harley, C. B. (1989) *J. Cell. Biochem.* **40**, 133–145
- 11 Haslam, R. J. and Lynham, J. A. (1977) *Biochem. Biophys. Res. Commun.* **77**, 714–722
- 12 Crouch, M. F. and Lapetina, E. G. (1988) *Biochem. Biophys. Res. Commun.* **156**, 149–156
- 13 Sano, K., Nakamura, H., Matsuo, T., Kawahara, Y., Fukuzaki, H., Kaibuchi, K. and Takai, Y. (1985) *FEBS Lett.* **192**, 4–8
- 14 Siess, W. and Lapetina, E. G. (1989) *Biochem. J.* **263**, 377–385
- 15 Salari, H., Duronio, V., Howard, D. L., Demons, M., Jones, K., Reany, A., Hudson, A. T. and Pelech, S. L. (1990) *FEBS Lett.* **263**, 104–108
- 16 Tapley, P. M. and Murry, A. W. (1985) *Eur. J. Biochem.* **151**, 419–423
- 17 Pelech, S. L., Samiei, M., Charest, D. L., Howard, S. L. and Salari, H. (1991) *J. Biol. Chem.* **266**, 8696–8705
- 18 Block, L. M., Abraham, W. M., Groscurth, P., Qiao, B. Y. and Perruchoud, A. P. (1989) *Am. J. Respir. Cell Mol. Biol.* **1**, 277–278
- 19 Walker, G. and Biurguignon, L. Y. W. (1990) *FASEB J.* **4**, 2924–2933
- 20 Wang, F., Naik, U. P., Ehrlich, Y. H., Freyberg, Z., Osada, S.-I., Ohno, S., Kuroki, T., Suzuki, K. and Kornecki, E. (1993) *Biochem. Biophys. Res. Commun.* **191**, 240–246
- 21 McMillan, R., Tani, P., Millard, F. and Woods, V. L. (1987) *Blood* **70**, 1040–1045
- 22 Kiefel, V., Santos, S., Weisheit, M. and Mueller-Eckhardt, C. (1987) *Blood* **70**, 1722–1726
- 23 Fujisawa, K., Tani, P., O'Toole, T. E., Ginsberg, M. H. and McMillan, R. (1992) *Blood* **79**, 1441–1446
- 24 van Leeuwen, E. F., van der Ven, J. T. M., Engelfriet, C. P. and von dem Borne, A. E. G. K. (1982) *Blood* **59**, 23–26
- 25 Klaassen, R. J. L., van der Lelie, J., Vlekke, A. B. J., Weigel, H. M., Eettinck Schattenkerk, J. K. M., Reiss, P. and von dem Borne, A. E. G. K. (1989) *Blut* **59**, 75–81
- 26 Bettaleb, A., Oksenhendler, E., Fromont, F., Duedari, N. and Bierling, P. (1989) *Br. J. Haematol.* **73**, 241–247
- 27 Kornecki, E., Walkowiak, B., Naik, U. P. and Ehrlich, Y. H. (1990) *J. Biol. Chem.* **265**, 10042–10048
- 28 Naik, U. P., Ehrlich, Y. H. and Kornecki, E. (1995) *Biochem. J.* **310**, 155–162
- 29 Osada, S.-I., Mizuno, K., Saido, T. C., Akita, Y., Suzuki, K., Kuroki, T. and Ohno, S. (1990) *J. Biol. Chem.* **265**, 22434–22440
- 30 Osada, S.-I., Mizuno, K., Saido, T. C., Suzuki, K., Kuroki, T. and Ohno, S. (1992) *Mol. Cell. Biol.* **12**, 3930–3938
- 31 Johnson, G. L. and Heasley, L. E. (1989) *J. Biol. Chem.* **264**, 8646–8652
- 32 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 33 Verme, T. B., Velarde, R. T., Cunningham, R. M. and Hootman, S. R. (1989) *Am. J. Physiol.* **257**, G548–G553
- 34 Hagiwara, M., Sumi, M., Usuda, N., Nagatani, T. and Hidaka, H. (1990) *Arch. Biochem. Biophys.* **280**, 201–205
- 35 Pelech, S. L., Charest, D. L., Howard, S. L., Paddon, H. B. and Salari, H. (1990) *Biochim. Biophys. Acta* **1051**, 100–107
- 36 Wakade, A. R., Wakade, T. D., Malhotra, R. K. and Bhawe, S. (1988) *J. Neurochem.* **51**, 975–983
- 37 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 38 Worthington, R. E., Carroll, R. C. and Boucheix, C. (1990) *Br. J. Haematol.* **74**, 216–222
- 39 Carroll, R. C., Worthington, R. E. and Boucheix, C. (1990) *Biochem. J.* **266**, 527–535