Inositol 1,4,5-Trisphosphate-Mediated Ca^{2+} Release from Platelet Internal Membranes Is Regulated by Differential Phosphorylation†

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ABSTRACT: Platelets are activated by an increase in cytosolic Ca^{2+}, and a portion of this increase is derived from inositol 1,4,5-trisphosphate ($InsP_3$)-mediated Ca^{2+} release from internal stores via the $InsP_3$ receptor. Cytosolic cAMP inhibits platelet activation, and experiments were designed to determine if cAMP-dependent phosphorylation affects the rate of $InsP_3$-mediated Ca^{2+} release. Western blotting of platelet internal membranes with anti-$InsP_3$ receptor and anti-actin binding protein antibodies revealed that the platelet contains type 1 $InsP_3$ receptor and that it is distinct from actin binding protein. The platelet $InsP_3$ receptor was shown to be phosphorylated by endogenous, membrane-bound kinases as well as by exogenous protein kinase A. Prior phosphorylation of the $InsP_3$ receptor by endogenous kinases inhibited additional protein kinase A-dependent phosphorylation by 60%. Furthermore, endogenous phosphorylation resulted in a 2-fold increase in the $InsP_3$-mediated Ca^{2+} release rate relative to dephosphorylated controls. Following endogenous phosphorylation, additional phosphorylation by protein kinase A returned the Ca^{2+} release rate to control values, while protein kinase A-dependent phosphorylation of dephosphorylated membranes did not affect the release rate. These results suggest that the $InsP_3$ receptor within intact platelets is phosphorylated by endogenous kinases which results in a high $InsP_3$-mediated Ca^{2+} release rate, and that increases in cAMP result in additional phosphorylation that inhibits Ca^{2+} release, thus contributing to inhibition of platelet activation.

Inositol 1,4,5-trisphosphate ($InsP_3$)[1] is an intracellular messenger, formed from the action of phospholipase C upon phosphatidylinositol 4,5-bisphosphate in plasma membranes, which serves to elicit the release of Ca^{2+} from intracellular stores (Ferris & Snyder, 1992a; Furuichi & Mikoshiba, 1995). In platelets, Ca^{2+} is an integral component of activation (Heemskerk & Sage, 1994). The intracellular Ca^{2+} concentration is regulated by Ca^{2+}-ATPases and Ca^{2+} channels located both in the plasma membrane and on an endoplasmic reticulum-like structure termed the dense tubular system (Heemskerk & Sage, 1994; Haynes, 1993). Consequently, the dense tubular system is involved in maintaining low resting Ca^{2+} and in providing Ca^{2+} for activation. A portion of the Ca^{2+} needed for activation is derived from $InsP_3$-mediated Ca^{2+} release from internal stores via the $InsP_3$ receptor ($InsP_3R$), a 260 kDa ligand-gated Ca^{2+} channel (Furuichi & Mikoshiba, 1995). Three genes code for different isoforms of the $InsP_3$ receptor in mammals, $InsP_3$-R1, $InsP_3R2$, and $InsP_3R3$, and these proteins exhibit differential tissue distribution and may possess different functional properties (Furuichi & Mikoshiba, 1995). Platelet activation is inhibited by agonists that increase cAMP, and at least part of this effect is due to reduced cytosolic Ca^{2+} (Haynes, 1993). It has been shown that cAMP-dependent phosphorylation of dense tubular system membranes increases the rate at which Ca^{2+} is sequestered both in isolated membranes (Kaser-Glanzmann et al., 1977; Adunyah & Dean, 1987) and in intact platelets (Tao et al., 1992a), and this process may contribute to inhibition of platelet function by cAMP. In addition, this laboratory (Adunyah & Dean, 1987) and others (Tohmatsu et al., 1989) reported that PKA-dependent phosphorylation inhibits Ca^{2+} release from $InsP_3$-sensitive stores, but another laboratory failed to see this effect of cAMP-dependent phosphorylation (O'Rourke et al., 1989). Thus, cAMP may decrease cytosolic Ca^{2+} in platelets both by stimulating Ca^{2+} transport via the Ca^{2+}-ATPase and by inhibiting Ca^{2+} release via the $InsP_3$ receptor in the dense tubular system.

In other tissues, cAMP-dependent phosphorylation of $InsP_3R$ has been shown to have differing effects. For example, rat brain exhibits a 10-fold decrease in sensitivity to $InsP_3$ (Supattapone et al., 1988a), while canine brain shows an increase in the extent of Ca^{2+} release from $InsP_3$-mediated stores upon cAMP-dependent phosphorylation (Volpe & Alderson-Lang, 1990). In liver, microsomal preparations show a 5-fold increase in sensitivity to $InsP_3$ (Burgess et al., 1991), and nuclei show enhanced $InsP_3$-mediated Ca^{2+} release without a change in $InsP_3$ binding characteristics (Matter et al., 1993).

The data in this paper demonstrate that (a) the platelet contains $InsP_3R1$ and it is distinct from the microfilament-

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[1] Abbreviations: $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3R$, $InsP_3$ receptor; $InsP_3R1$, type 1 $InsP_3$ receptor; PKA, cAMP-dependent protein kinase; ABP, actin binding protein; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid.

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associated protein actin binding protein (ABP), a major substrate for cAMP-dependent protein kinase in the platelet exhibiting a molecular weight similar to that of the $InsP_3R$, (b) the platelet $InsP_3R$ can be phosphorylated by cAMP-dependent protein kinase and endogenous membrane-bound kinases, and (c) phosphorylation of the receptor can either stimulate or inhibit $InsP_3$-mediated Ca^{2+} release rates depending upon the phosphorylation state of the receptor.

EXPERIMENTAL PROCEDURES

Materials. Outdated human platelets were obtained from the Louisville chapter of the American Red Cross. Whole brain from male Sprague-Dawley rats was generously provided by Dr. Russell Prough (Department of Biochemistry, University of Louisville). Rabbit polyclonal antibodies against the N-terminal region of the $InsP_3$ receptor were developed as previously described (Dean & Quinton, 1995). Rabbit serum containing polyclonal antibodies against the 19 C-terminal amino acids of the type 1 $InsP_3$ receptor was generously provided by Dr. Thomas Südhof [Department of Molecular Genetics, University of Texas Southwestern Medical Center; see Mignery et al. (1990)]. Mouse anti-ABP monoclonal antibody G-2 was prepared as previously described (Brown & Binder, 1992). Poly(vinylidene difluoride) transfer membrane (Immobilon) was purchased from Millipore (Bedford, MA). Tris, SDS, and β-mercaptoethanol were purchased from BioRad. Bicinchoninic acid protein assay reagents and immobilized protein A were purchased from Pierce. Chemiluminescence reagents, autoradiography film, and [γ-^{32}P]ATP were purchased from Dupont NEN. Thapsigargin, okadaic acid, and $InsP_3$ were purchased from LC Laboratories (Woburn, MA). Rhod-2 was purchased from Molecular Probes (Eugene, OR). A23187 was purchased from Calbiochem. Protein phosphatase 2A was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). The catalytic subunit of PKA, PKA peptide inhibitor, and all other reagents were purchased from Sigma Chemical Co.

Tissue Preparation. Platelet internal membranes were purified according to Dean (1988). Rat brain microsomes were prepared by homogenizing whole rat brain in 10 mM Tes buffer, pH 7.5, containing 1 mM PMSF and 10 μg/mL leupeptin (2 mL per gram of tissue). Homogenization was performed with several strokes in a Kontes glass homogenizer followed by further homogenization with a Tekmar Model SDF tissue homogenizer for 3 min at low speed. The homogenate was centrifuged at 3000*g* for 10 min, and the pellet was discarded. The 3000*g* supernatant was then centrifuged at 10000*g* for 20 min, and the pellet was discarded. The 10000*g* supernatant was finally centrifuged at 100000*g* for 20 min, and the supernatant was discarded. The resulting pellet was resuspended in 10 mM Tes buffer, pH 7.5, containing 100 mM KCl, 20% glycerol, 1 mM EGTA, 1 mM dithiothreitol, 1 mM PMSF, 10 μg/mL leupeptin, and 10 μg/mL antipain. Protein concentrations were determined using the bicinchoninic acid protein assay.

Western Blot Analysis. Proteins were electrophoresed on 5% SDS−polyacrylamide gels (Laemmli, 1970) followed by transfer to Immobilon membrane using 20 mM CAPS buffer at pH 11 containing 10% methanol at 300 mA and 4 °C for 65 min (Dean & Quinton, 1995). After transfer, the blots were washed in TTBS (30 mM Tris, pH 7.5, 150 mM NaCl, and 0.1% Tween-20) and incubated in 2% ovalbumin in TTBS for 1 h. The blots were probed for $InsP_3R$ with either a 1:900 dilution of anti-N-terminal peptide antibody or a 1:10 000 dilution of anti-$InsP_3R1$ antibody, or for ABP with a 1:50 dilution of anti-ABP monoclonal antibody G-2, by incubation with the appropriate primary antibody for 1 h, followed by incubation for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody at a dilution of 1:10 000. All antibodies were diluted with 2% bovine serum albumin in TTBS. The antigens were visualized by incubating the blots with chemiluminescence reagents and exposing them to film. In order to compare the specificity of different antibodies, a blot was often stripped of previously bound antibody and probed with a different primary antibody by incubating the blot in a solution containing 62.5 mM Tris, pH 6.8, 2% SDS, and 100 mM β-mercaptoethanol at 60 °C for 1 h. The blot was then washed in TTBS, and the Western blotting process was repeated.

Phosphorylation/Dephosphorylation. Dephosphorylation of membrane proteins was accomplished by incubating membranes with protein phosphatase 2A (0.006 unit/μg of protein substrate) for 10 min at 30 °C in 10 mM Tes buffer at pH 7.5 containing 100 mM KCl, 1 mM EDTA, and 1 mM DTT. Endogenous and cAMP-dependent phosphorylation of internal membrane proteins was achieved by incubating membranes (1.7 mg/mL protein) in dephosphorylation buffer with 50 mM K_2HPO_4 added. Phosphorylation was initiated by the addition of 100 mM $MgCl_2$, 100 μM ATP, and 0.6 μCi of [γ-^{32}P]ATP per microgram of protein. Endogenous phosphorylation was achieved by incubating the membranes for 10 min at 30 °C, while PKA-dependent phosphorylation was achieved by incubation with 1.0 unit of the catalytic subunit of cAMP-dependent protein kinase per microgram of protein substrate for 30 s at 30 °C. Sequential phosphorylation of dephosphorylated $InsP_3R$ by endogenous kinase followed by PKA was accomplished by incubation at 30 °C for 8 min with 100 μM ATP followed by 2 min with PKA. Reactions were stopped by the addition 100 mM EDTA. Phosphorylated proteins were detected by autoradiographic exposure to X-ray film, or by exposure to a storage phosphor screen followed by phosphorimaging using a Molecular Dynamics Model PSF phosphorimager with ImageQuant version 3.3 software.

Immunoprecipitation. Phosphorylated membranes (0.3 mg) were centrifuged at 100000*g* and resuspended in solubilization buffer (30 mM Tris, pH 7.5, 150 mM NaCl, 0.4% Triton X-100, 10 mM EGTA, 1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL antipain, and 20 μg/mL pepstatin A) to a protein concentration of 1.2 mg/mL. The mixture was incubated on ice for 30 min, and the Triton-insoluble material was removed by centrifugation at 8500*g* for 10 min. Rabbit serum (1 μL) containing anti-$InsP_3R1$ antibodies was added to the solubilized material followed by mixing for 2 h at 4 °C. Immobilized protein A (50 μL) was washed twice with solubilization buffer, added to the antibody−protein complex, and incubated for 90 min at 4 °C. The immunoprecipitation complex was washed twice with solubilization buffer and once with solubilization buffer without Triton X-100, and the immunoprecipitate was eluted from the protein A by incubation in Laemmli sample dilution buffer containing 10 mM EGTA for 20 min at 37 °C. The immunoprecipitate was then electrophoresed on a 5% SDS−polyacrylamide gel, transferred to PVDF, and exposed to a

storage phosphor screen. Phosphorylation was quantitated from each immunoprecipitate's phosphorimage signal area. The areas of the peaks corresponding to $InsP_3R$ polypeptide were determined by densitometric scanning of the corresponding Western blot. Densitometric scanning was performed using a Bio-Rad Model 620 video densitometer. The relative percent $[^{32}P]P_i$ incorporation was calculated by normalizing the phosphorimage signal area to the area under each signal's protein mass peak.

Measurement of $InsP_3$-Mediated Ca^{2+} Release Rates. Ca^{2+} loading was achieved by incubating membrane vesicles in 10 mM Tes buffer at pH 7.5 containing 100 mM KCl, 100 μM ATP, 10 mM $MgCl_2$, 100 μM $CaCl_2$, and 50 mM KH_2PO_4 for 10 min at 30 °C. The Ca^{2+}-loaded vesicles were centrifuged at 100000*g*, resuspended, and dephosphorylated as described above. The phosphatase was inhibited by the addition of 100 nM okadaic acid, further Ca^{2+} uptake was inhibited by the addition of 200 nM thapsigargin, and aliquots of the dephosphorylated vesicles were phosphorylated as described above prior to fluorescence measurement. Ca^{2+} release rates were determined as previously described using 5 μM rhod-2 as the fluorophore (Quinton & Dean, 1992) with 0.1 mg of platelet internal membrane protein in 1 mL at 30 °C. $InsP_3$ was then added to a concentration of 20 μM to initiate Ca^{2+} release from vesicles. $InsP_3$-insensitive Ca^{2+} stores were emptied by the addition of 10 μM A23187. Rates of Ca^{2+} release were determined from first-order plots of the fluorescence data using the expression:

$$\ln [1 - (F_{obs} - F_{min})/(F_{max} - F_{min})]$$

where F_{obs} is the observed fluorescence at a time between 0 and that required for maximum $InsP_3$-mediated Ca^{2+} release, F_{min} is the fluorescence at the point of $InsP_3$ addition, and F_{max} is the maximum level of fluorescence (resulting from the release of $InsP_3$-sensitive and -insensitive Ca^{2+} stores). Zero time was at the point of $InsP_3$ addition. Rates were obtained by multiplying the rate constant derived from the first-order plot by the total amount of Ca^{2+} released ($InsP_3$ plus A23187).

RESULTS

Identification of the Platelet $InsP_3$ Receptor. Previously we have shown that polyclonal antibodies, which we raised against a peptide representing a portion of the N-terminal region of type 1 $InsP_3$ receptor, recognize $InsP_3R$ within platelet internal membranes (Dean & Quinton, 1995). Western blotting with polyclonal antibodies known to react specifically with the C-terminal region of $InsP_3R1$ (Mignery et al., 1989) reveals that a protein is recognized with the same molecular mass in both platelets and brain as that which is recognized by our anti-N-terminal peptide antibodies, as demonstrated by blotting and reprobing with both antibodies upon the same membrane (Figure 1). This result confirms that platelet internal membranes contain $InsP_3R1$. The amount of the unknown 120 kDa protein recognized by the anti-N-terminal antibodies in the rat brain homogenate is variable depending upon the preparation of rat brain used.

A recent review (Authi, 1993) suggested that the phosphorylated 260 kDa band which we claimed to be $InsP_3R$ (Quinton & Dean, 1992) could be actin binding protein (ABP), an abundant protein in platelets with a molecular mass close to 260 kDa known to be a major substrate for

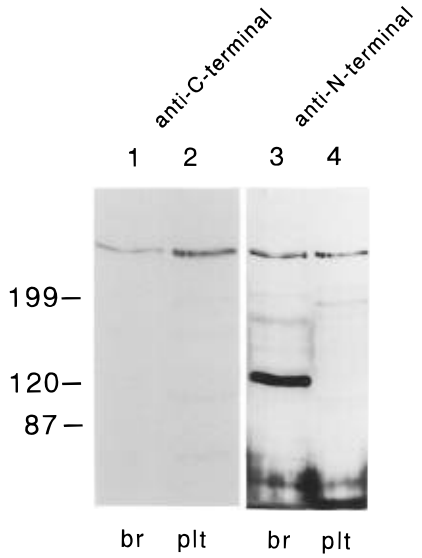

FIGURE 1: Immunoblotting of rat brain microsomes and platelet internal membranes for $InsP_3$ receptor. Rat brain microsomal protein (br, 100 μg, lanes 1 and 3) and platelet internal membrane protein (plt, 50 μg, lanes 2 and 4) were blotted and probed with anti-C-terminal $InsP_3R1$ or anti-N-terminal $InsP_3R$ antibodies. Numbers to the left indicate molecular mass markers in kDa. Both immunoblots were performed with the same membrane; i.e., the left blot (lanes 1 and 2) was stripped of bound antibody (see Experimental Procedures) and reprobed to give the right blot (lanes 3 and 4).

PKA. Thus, it became imperative not only to show that $InsP_3R$ could be phosphorylated but also to distinguish $InsP_3R$ from ABP. A monoclonal anti-ABP antibody (G-2) was used to distinguish between these two proteins (Brown & Binder, 1992, 1993). Western blots were made from SDS−polyacrylamide gels containing platelet internal membrane proteins. The blots were first probed with the anti-$InsP_3R$ peptide antibody, followed by an antibody-stripping procedure, and then reprobed with the anti-ABP antibody. The results presented in Figure 2 show that the anti-$InsP_3R$ peptide antibody detected a single high molecular mass protein corresponding to the mass of $InsP_3R$, while the anti-ABP antibody (G-2) detected two proteins, one at 280 kDa (the intact ABP protein) and one at 200 kDa, which is a commonly encountered proteolytic fragment of ABP [see Fox et al. (1983)] with which G-2 is reactive. These results demonstrate that $InsP_3R$ can be readily distinguished from ABP in platelet membranes.

$InsP_3R$ Phosphorylation. Our studies have previously shown that treatment of Ca^{2+}-loaded vesicles with PKA results in approximately 20% inhibition of the $InsP_3$-mediated release rate relative to the rates for untreated vesicles (Quinton & Dean, 1992). However, this degree of inhibition seemed low in view of the strong inhibitory effects upon platelet activation by adenylate cyclase-activating hormones. Since phosphorylation of internal membrane proteins by membrane-bound kinases occurs in the absence of added PKA (Quinton & Dean, 1992), this endogenous phosphorylation could play a role in the observed effects of phosphorylation upon $InsP_3$-mediated Ca^{2+} release. It should be pointed out that any phosphorylation occurring after purification of platelet internal membranes is catalyzed by membrane-bound kinases since the purification process separates the internal membranes from the cytosol and plasma membrane (Dean, 1988).

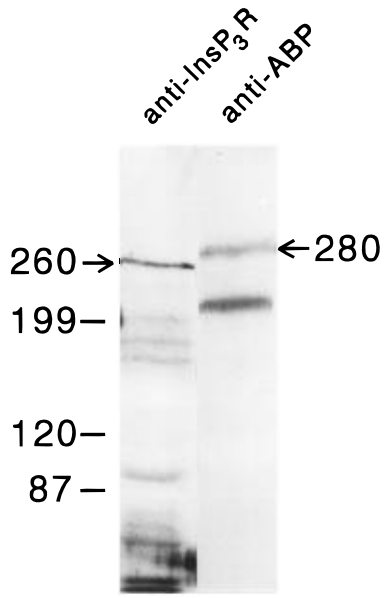

FIGURE 2: Immunoblotting of platelet internal membranes for $InsP_3R$ and actin binding protein. Platelet internal membrane protein (50 μg) was probed with either anti-N-terminal $InsP_3R$ or anti-ABP antibodies. Numbers to the left indicate molecular mass markers in kDa. Both immunoblots were performed with the same membrane; i.e., the blot from the left lane was stripped of bound antibody (see Experimental Procedures) and reprobed to give the blot in the right lane.

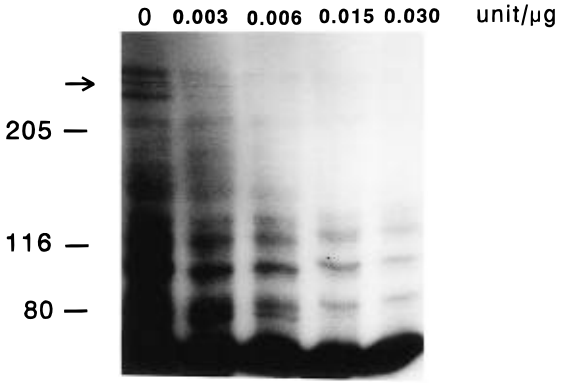

FIGURE 3: Effect of protein phosphatase 2A upon endogenously phosphorylated platelet membranes. Platelet internal membranes were phosphorylated with [γ-^{32}P]ATP and then treated with the indicated amounts of protein phosphatase 2A for 10 min. The autoradiograph was obtained as described under Experimental Procedures. Optimum dephosphorylation was obtained with 0.2 unit of phosphatase (0.006 unit/μg of protein). The arrow denotes the migration of the 260 kDa $InsP_3R$. Molecular mass standards are listed in kDa.

In order to study the effects of $InsP_3R$ phosphorylation, it was necessary to define conditions where endogenous and cAMP-dependent phosphorylation could be studied both separately and sequentially. To accomplish this goal, a procedure was developed to remove phosphates resulting from endogenous phosphorylation of $InsP_3R$. Platelet internal membrane vesicles were first phosphorylated endogenously in the presence of [γ-^{32}P]ATP and treated with various amounts of protein phosphatase 2A for 10 min. Phosphorylation was monitored by autoradiography to determine the amount of phosphatase necessary to dephosphorylate the membrane proteins. As shown by the data in Figure 3, we determined that the higher molecular mass proteins (>200 kDa) could be dephosphorylated using 0.006 unit of phosphatase/μg of protein.

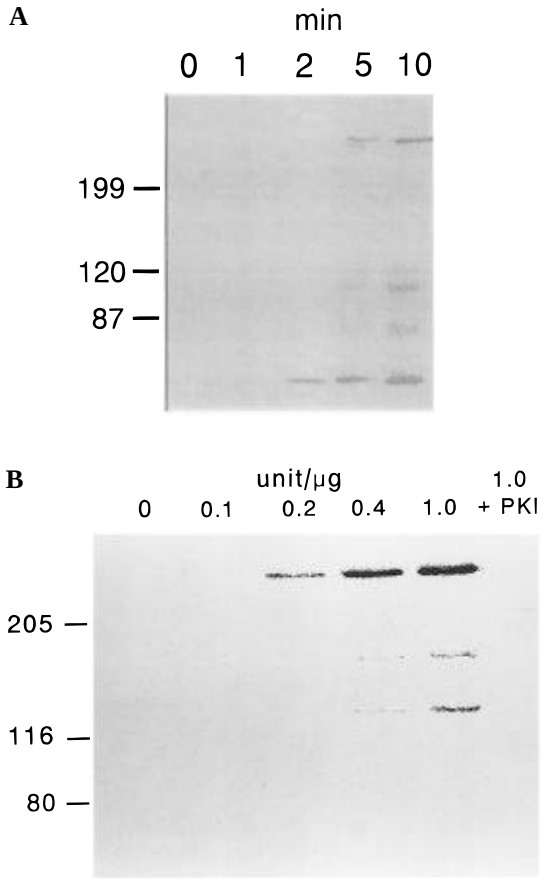

FIGURE 4: Endogenous and PKA-dependent $InsP_3R$ phosphorylation. Platelet internal membranes were dephosphorylated using the optimal conditions determined from the data in Figure 3. Phosphorylation was carried out as described under Experimental Procedures, and phosphorylated $InsP_3R$ was immunoprecipitated after solubilization with Triton X-100. (A) Phosphorimage showing the time course of endogenous phosphorylation of $InsP_3R$. Dephosphorylated $InsP_3R$ was phosphorylated by endogenous kinases for the indicated times. (B) Phosphorimage showing PKA-dependent phosphorylation of $InsP_3R$. Membranes were treated with the indicated amounts of PKA for 30 s at 30 °C in the absence or presence of 0.1 μg of cAMP-dependent protein kinase inhibitor peptide (PKI).

To determine the time course of endogenous phosphorylation, *dephosphorylated* membrane vesicles were incubated with [γ-^{32}P]ATP for the indicated times (Figure 4A), and $InsP_3R$ was then immunoprecipitated with polyclonal anti-C-terminal $InsP_3R1$ antibodies following solubilization with Triton X-100. The immunoprecipitates were subjected to SDS gel electrophoresis, and [^{32}P]PO_4 was quantitated by phosphorimaging. Maximal endogenous phosphorylation of $InsP_3R$ occurred between 5 and 10 min after addition of ATP as shown in Figure 4A.

Having established the time course of endogenous phosphorylation, we then endeavored to identify conditions where PKA-dependent phosphorylation on $InsP_3R$ could be completed before significant endogenous phosphorylation occurred. This was accomplished by determining the amount of exogenous PKA that would yield maximal phosphorylation within a 30 s time period, a time when little endogenous phosphorylation has occurred (Figure 4A). Using the same procedure of phosphorylation with [γ-^{32}P]ATP followed by immunoprecipitation, it was observed that 1 unit of PKA/μg of platelet membrane protein yielded maximal phosphorylation in 30 s (Figure 4B). This phosphorylation could be

Table 1: Quantitation of Kinase-Mediated Phosphate Incorporation[a]

condition	relative $[^{32}P]P_i$ incorporation[b] expt 1	expt 2
endogenous phosphorylation	83460	75880
PKA phosphorylation	275020	209420
endogenous plus PKA phosphorylation[c]	183060	164620

[a] Experiments were performed as described under Experimental Procedures. [b] Values were calculated by dividing the phosphorimage signal area by the area under the corresponding protein peak of a Western blot (see Experimental Procedures). [c] Endogenous phosphorylation was allowed to proceed for 8 min followed by 2 min of PKA-dependent phosphorylation.

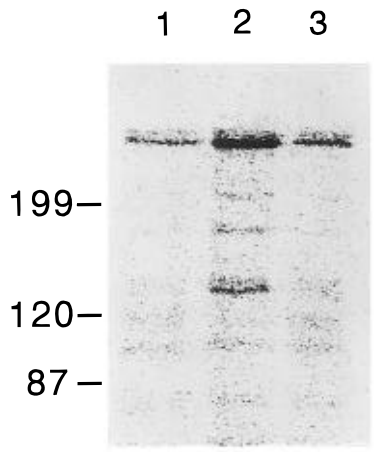

FIGURE 5: $InsP_3R$ phosphorylation by endogenous kinases and PKA. Phosphorylations and immunoprecipitations were performed as described under Experimental Procedures. Lane 1, endogenous phosphorylation (10 min); lane 2, PKA-dependent phosphorylation (1 unit of PKA/μg of total protein for 30 s); lane 3, endogenous phosphorylation (8 min) followed by PKA-dependent phosphorylation (2 min).

completely inhibited by addition of PKA inhibitor peptide (PKI), indicating that this phosphorylation is specifically catalyzed by PKA (Figure 4B). Densitometric scanning of the respective Western blots revealed that each lane contained approximately equal amounts of $InsP_3R$ (data not shown).

Sequential Phosphorylation of $InsP_3R$. Using the conditions established for each type of phosphorylation, platelet internal membrane proteins were dephosphorylated and then were rephosphorylated by one of three treatments: endogenous phosphorylation, PKA-dependent phosphorylation, or endogenous phosphorylation followed by PKA-dependent phosphorylation as described under Experimental Procedures. The phosphorylated membrane proteins were then solubilized and immunoprecipitated in the same manner as described for Figure 4. To quantitate the relative level of phosphorylation, the ^{32}P signal obtained by phosphorimaging was divided by the relative amount of $InsP_3R$ polypeptide on the blots as determined by Western blotting and densitometric scanning. The results from two separate immunoprecipitations are shown in Table 1. Figure 5 shows that the dephosphorylated $InsP_3R$ is directly phosphorylated by endogenous kinases and by PKA, where PKA-dependent phosphorylation resulted in over 3-fold more $[^{32}P]P_i$ incorporation than endogenous phosphorylation (Table 1). The extent of PKA-dependent phosphorylation was decreased by approximately 60% when $InsP_3R$ was first phosphorylated by endogenous kinase, as calculated using the average incorporation from the two experiments in Table 1. These

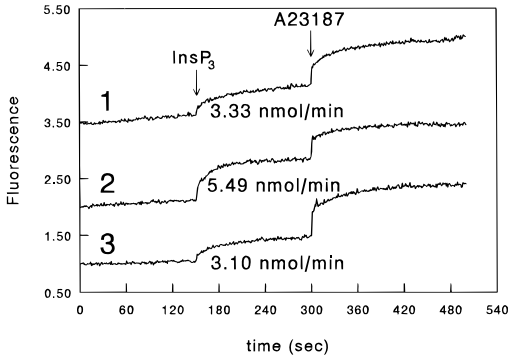

FIGURE 6: Effects of endogenous and cAMP-dependent phosphorylation on $InsP_3$-mediated Ca^{2+} release. Rhod-2 (5 μM) was used to monitor changes in Ca^{2+}. $InsP_3$ (20 μM) and A23187 (10μM) were added at the times indicated by the arrows. Trace 1, dephosphorylated platelet internal membrane vesicles; trace 2, endogenously phosphorylated membrane vesicles; trace 3, endogenous followed by PKA-dependent phosphorylation of membrane vesicles. The upper and lower traces are offset from the middle trace by 1 fluorescence unit to enable comparison of the three traces. All traces were generated using the same membrane preparation in each assay.

Table 2: Effect of Phosphorylation State on $InsP_3$-Mediated Ca^{2+} Release[a]

condition	Ca^{2+} release (nmol/min)[b]
dephosphorylation	2.91 ± 0.38 ($n = 7$)
endogenous phosphorylation	6.01 ± 0.76 ($n = 7$)
endogenous plus PKA phosphorylation[c]	2.91 ± 0.65 ($n = 4$)
PKA phosphorylation[d]	2.73 ± 0.84 ($n = 3$)

[a] Experiments were performed as described under Experimental Procedures. [b] Data are expressed as the mean $\pm$ standard deviation. [c] PKA-dependent phosphorylation was initiated by the addition of 1 unit of catalytic subunit of cAMP-dependent protein kinase per microgram of membrane protein after 8 min of endogenous phosphorylation. [d] PKA-dependent phosphorylation (30 s) of dephosphorylated membranes.

data establish that the $InsP_3R$ is capable of being phosphorylated by cAMP-dependent protein kinase and that this PKA-dependent phosphorylation is subject to regulation by prior endogenous phosphorylation.

Effect of Phosphorylation on $InsP_3$-Mediated Ca^{2+} Release. The difference in time courses between PKA-dependent and endogenous phosphorylation (10 min for endogenous phosphorylation versus 30 s for PKA-dependent phosphorylation) allowed for separate studies on the effect of each type of phosphorylation on $InsP_3$-mediated Ca^{2+} release. Platelet internal membrane vesicles were loaded with Ca^{2+} by the action of endogenous Ca^{2+}-ATPase. Rhod-2, a membrane-impermeable Ca^{2+} fluorophore, was employed to detect Ca^{2+} release from the vesicles. Using the $InsP_3$-mediated Ca^{2+} release rate from dephosphorylated membrane vesicles as a control value (Figure 6, trace 1, and Table 2), conditions favoring endogenous phosphorylation resulted in a 2-fold increase in the rate of Ca^{2+} release relative to the control (Table 2), as well as an increase in the extent of Ca^{2+} release (Figure 6, trace 2). When the endogenously phosphorylated vesicles were additionally phosphorylated by PKA, the $InsP_3$-mediated Ca^{2+} release rate and extent returned to levels comparable to the dephosphorylated control values (Figure 6, trace 3, and Table 2). The release rates resulting from PKA-dependent phosphorylation alone upon dephosphorylated vesicles differed little from the control values (Table 2). The fluorescence tracings also demonstrate

that the vesicles from each treatment contained approximately the same amount of sequestered Ca^{2+}, as shown by the total amount of Ca^{2+} released from each vesicle (the sum of $InsP_3$- and A23187-releasable Ca^{2+}). Therefore, the changes in the rate and extent of $InsP_3$-mediated Ca^{2+} release are not due to differences in the Ca^{2+} loading but to changes occurring at the level of the $InsP_3$ receptor itself.

DISCUSSION

The main conclusion of this paper is that the platelet $InsP_3R$ is capable of being phosphorylated by cAMP-dependent protein kinase, which has the effect of decreasing the rate of $InsP_3$-mediated Ca^{2+} release from platelet internal Ca^{2+} stores. In addition, the results of these experiments suggest that conditions within the platelet leading to phosphorylation by endogenous kinases allow $InsP_3R$ to release Ca^{2+} at a higher rate as compared to the dephosphorylated receptor. Also, the effects of exogenously added PKA suggest that those agents that increase cytosolic cAMP levels cause a further increase in $InsP_3$ receptor phosphorylation which inhibits $InsP_3$-mediated Ca^{2+} release and thus inhibits platelet activation. It is interesting to note that PKA-dependent phosphorylation alone had little effect, if any, on the Ca^{2+} release rates from dephosphorylated membranes. These results are consistent with our previous studies in which PKA-dependent phosphorylation was shown to cause a small decrease in the rate and extent of $InsP_3$-mediated Ca^{2+} release (Quinton & Dean, 1992). By taking into account the endogenous phosphorylation occurring within the internal membranes, we have a clearer picture of the effects of increases in platelet cytosolic cAMP.

We have demonstrated direct phosphorylation of $InsP_3R$ by internal membrane-associated kinases as well as by PKA using immunoprecipitation. PKA is 3-fold more effective in catalyzing the incorporation of P_i (mass of phosphate/mass of $InsP_3R$ polypeptide) into the dephosphorylated $InsP_3R$ than the endogenous kinase. Comparing the effects of PKA-dependent phosphorylation versus endogenous followed by PKA-dependent phosphorylation reveals that $InsP_3R$ exhibits a 60% decrease in cAMP-dependent incorporation of $[^{32}P]P_i$. These results imply that prior endogenous phosphorylation induces a state of the receptor where Ca^{2+} is released at a higher rate and PKA phosphorylation sites are less available for cAMP-dependent phosphorylation. While PKA-dependent phosphorylation of the $InsP_3$ receptor is not dependent upon prior endogenous phosphorylation [known as hierarchal phosphorylation; see Roach (1991)], it appears that cAMP-dependent inhibition of $InsP_3R$-mediated Ca^{2+} release is dependent upon a particular sequence of phosphorylation events. To our knowledge, this is the first instance in which an $InsP_3$ receptor has been shown to demonstrate this effect.

Phosphorylation by both endogenous kinases and PKA affects the $InsP_3$-mediated Ca^{2+} release rate. The Ca^{2+} release experiments show that these changes in Ca^{2+} release rates are not due to differences in vesicular Ca^{2+} content or inadequate $InsP_3$ concentrations. The changes in the rate and extent of $InsP_3$-mediated Ca^{2+} release were seen without significant differences in the total Ca^{2+} load upon differing phosphorylation states. Also, we have addressed the concern of receptor affinity for $InsP_3$ by using a very high concentration of $InsP_3$ (20 μM). Reported K_Ds of $InsP_3R$ for $InsP_3$ have ranged from 2 nM to 23 nM (Matter et al., 1993; Chadwick et al., 1990; Nunn & Taylor, 1990; Theibert et al., 1990; Challis et al., 1991) with one reported value as high as 100 nM (Supattapone et al., 1988a,b). Those laboratories that have studied the effect of cAMP-dependent phosphorylation on the $InsP_3$ receptor's affinity for $InsP_3$ report that there is no significant change in the K_D upon phosphorylation (Supattapone et al., 1988a; Burgess et al., 1991). Therefore, 20 μM $InsP_3$ should saturate both the phosphorylated and the unphosphorylated forms of the $InsP_3$ receptor.

Figure 1 shows that $InsP_3R1$ is present within the platelet's internal membrane system; however, it is not known whether this represents the $InsP_3R$ subtype 1a or 1b, or possibly both. Subtypes 1a and 1b differ by the presence (1a) or absence (1b) of a 40 amino acid insert between the consensus PKA phosphorylation sites (Nakagawa et al., 1991). Danoff et al. (1991) have shown by phosphopeptide mapping that the subtype 1b $InsP_3R$ (minus the 40 amino acid insert), which is preferentially expressed in peripheral tissues, is phosphorylated at one of the two consensus PKA sites while the subtype 1a receptor (including the insert), preferentially expressed in neuronal tissues, is phosphorylated at both PKA sites. This suggests that the conformation of $InsP_3R$ influences the accessibility of kinases to their respective sites (Ferris et al., 1992b).

Based upon the facts that the subtype 1a $InsP_3R$ has predominantly been found in neuronal tissues and that all human peripheral tissues investigated for $InsP_3R$ contain subtype 1b (Yamada et al., 1994), it seems probable that platelet internal membranes contain the subtype 1b $InsP_3R$. In addition, Bourguignon et al. (1993) have reported the presence of an $InsP_3R$ in the platelet plasma membrane. Similarly, one laboratory has published data indicating the presence of $InsP_3$ binding in the plasma membrane of liver (Feng & Klaus-Friedmann, 1993), and others have shown $InsP_3R1$ to be present in T-lymphocyte plasma membranes (Khan et al., 1992; Bourguignon et al., 1993; Bourguignon & Jin, 1995) and caveolae of smooth muscle (Fujimoto et al., 1992). While we believe that the platelet plasma membrane does not contain $InsP_3R1$ (Dean & Quinton, 1995), it is possible that it could contain either $InsP_3R2$ or $InsP_3R3$. Other tissues are known to express multiple isoforms of $InsP_3R$ (Newton et al., 1994; Bush et al., 1994), but this has yet to be shown in platelets.

A recent review of $InsP_3$-mediated Ca^{2+} signaling (Furuichi & Mikoshiba, 1995) lists Ca^{2+}−calmodulin-dependent kinase, protein kinase C, and PKA as those kinases whose activities have been identified to phosphorylate $InsP_3R$; all of these kinases are present in platelets (Dean & Adunyah, 1989; Hawiger et al., 1994). For any of these kinases to be considered as the endogenous kinases whose activities are present within our internal membranes, they must be membrane-bound forms of the kinases. Platelet internal membranes which have been stripped of calmodulin show calmodulin-dependent kinase activity with the addition of exogenous calmodulin (Dean & Adunyah, 1989), and certain isoforms of this kinase can be bound to membranes by anchoring proteins (McNeill et al., 1994). Protein kinase C is known to be associated with the platelet plasma membrane during its activation but does not associate with the internal membranes (Nishizuka, 1984). PKA could be ruled out as a candidate since this paper has shown that the actions of

PKA and the endogenous kinases have differing consequences. There are also studies reporting that $InsP_3R$ can phosphorylate itself (Ferris et al., 1992) and that there are at least three potential ATP binding sites within its amino acid sequence (Yamada et al., 1994).

The main effect of cAMP on platelet Ca^{2+} metabolism appears to be a reduction of the cytosolic Ca^{2+} concentration. The Ca^{2+}-ATPases within the dense tubular system (Tao et al., 1992b) and the plasma membrane (Johansson et al., 1992) both exhibit increased activities when cytosolic cAMP is elevated. From the data presented in this paper, it seems reasonable to suggest that cAMP-dependent phosphorylation of $InsP_3R$ would also result in a relative decrease in cytosolic Ca^{2+} during platelet activation by inhibiting its release from internal stores. However, although the elevation of cytosolic Ca^{2+} induced by most platelet agonists is inhibited by cAMP, this does not appear to be the case of collagen-mediated platelet activation (Smith et al., 1992, 1993). This may be explained by the observations that there is a greater dependence on external Ca^{2+} for collagen-mediated platelet activation and that $InsP_3$-sensitive Ca^{2+} stores do not appear to have as important a role as in activation by other agonists. Thus, cAMP appears to regulate all of the well-characterized proteins involved in Ca^{2+} metabolism in the platelet.

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