Interaction of Protein Kinase C Isozymes with Rho GTPases†

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ABSTRACT: Evidence is provided for direct protein−protein interactions between protein kinase C (PKC) α, βI, βII, γ, δ, ϵ, and ζ and members of the Rho family of small GTPases. Previous investigations, based on the immunoprecipitation approach, have provided evidence consistent with a direct interaction, but this remained to be proven. In the study presented here, an in vitro assay, consisting only of purified proteins and the requisite PKC activators and cofactors, was used to determine the effects of Rho GTPases on the activities of the different PKC isoforms. It was found that the activity of PKCα was potently enhanced by RhoA and Cdc42 and to a lesser extent by Rac1, whereas the effects on the activities of PKCβI, -βII, -γ, -δ, -ϵ, and -ζ were much reduced. These results indicate a direct interaction between PKCα and each of the Rho GTPases. However, the Rho GTPase concentration dependencies for the potentiating effects on PKCα activity differed for each Rho GTPase and were in the following order: RhoA > Cdc42 > Rac1. PKCα was activated in a phorbol ester- and Ca^{2+}-dependent manner. This was reflected by a substantial decrease in the phorbol ester concentration requirements for activity in the presence of Ca^{2+}, which for each Rho GTPase was induced within a low nanomolar phorbol ester concentration range. The activity of PKCα also was found to be dependent on the nature of the GTP- or GDP-bound state of the Rho GTPases, suggesting that the interaction may be regulated by conformational changes in both PKCα and Rho GTPases. Such an interaction could result in significant cross-talk between the distinct pathways regulated by these two signaling elements.

The Rho members of the Ras superfamily of low-molecular weight GTPases each play critical regulatory roles in several key cell processes such as the cytoskeletal rearrangements underlying changes in cell shape, motility, and polarization, and are involved in the control of gene transcription through various signaling pathways (*1*−*4*). At least 14 proteins have so far been identified which are classified into the Rho, Cdc42, and Rac subfamilies, of which RhoA, Cdc42, and Rac1 are the most commonly studied examples (*3*, *5*). RhoA, which is activated by extracellular ligands such as lysophosphatidic acid (LPA), induces the assembly of contractile actin−myosin filaments (stress fibers) and associated focal adhesion complexes; Rac1 is activated by platelet-derived growth factor or insulin and induces the assembly of a meshwork of actin filaments at the cell periphery, producing lamellipodia and membrane ruffling, while Cdc42 induces actin-rich surface protrusions or filopodia.

In common with other members of the Ras superfamily of GTPases, the Rho GTPases interact with, and activate, a range of "effector proteins" which in turn bring about various cell responses. Some but not all of these interactions require the GTP-bound form of the GTPases. Examples of the growing number of effector proteins thus far identified include phospholipase D (*6*−*8*), rhophillin (*9*), diacylglycerol kinase-θ (*10*), several protein kinases, including PKN (*9*, *11*) and PRK1 and -2 (*12*, *13*), and Rho-associated kinases p160-ROCK and p150-ROK-α and -β (*4*, *14*). The conformational changes that result from GTP binding and the subsequent hydrolysis of GTP to GDP act as a "molecular switch" which is tightly regulated by association with GTPase-activating proteins (GAPs), as well as by guanine nucleotide exchange factors (GEFs) and dissociation inhibitors (GDIs), which modify the GTP and GDP binding affinities (*15*). Interaction with these factors may promote the translocation of Rho proteins to subcellular compartments, including the membrane; this is regulated by post-translational modifications of a C-terminal "CAAX" motif that include geranylgeranylation of a conserved C-terminal cysteine residue, followed by proteolytic removal of three C-terminal amino acids, catalyzed by a CAAX protease, and finally carboxymethylation, catalyzed by a prenylcysteine-dependent methyltransferase (*16*).

The protein kinase C (PKC)[1] family of serine-threonine kinases, consisting of the "conventional" (cPKC) α, βI, βII, and γ, the "novel" (nPKC) δ, ϵ, η, and θ, and the "atypical"

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; BPS, bovine brain phosphatidylserine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreitol; GDP, guanosine diphosphate; GTPγS, guanosine 5′-*O*-(thiotriphosphate); GTP, guanosine 5′-triphosphate; LUV, large unilamellar vesicles; MBP_{4-14}, myelin basic protein peptide substrate; ϵ-peptide, peptide substrate based on the cPKCϵ pseudosubstrate; cPKC, "conventional" protein kinase C; nPKC, "novel" protein kinase C; aPKC, "atypical" protein kinase C; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; Sf9, *Spodoptera frugiperda*; SD, standard deviation; TPA, 4β-12-*O*-tetradecanoylphorbol 13-acetate.

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(aPKC) ζ, ι, and λ isozymes, also act as molecular switches by catalyzing the phosphorylation of numerous target proteins, thereby regulating function (*17*−*20*). Several recent studies have demonstrated a convergence between PKC and Rho GTPase-regulated signaling pathways (*21*−*24*). Evidence that this may involve a close association between PKC and Rho GTPases was provided in a recent study (*23*), which showed that the overexpression of a constitutively active mutant of Rho (V14Rho) in Jurkat T cells resulted in an enhanced level of AP-1 activity in the presence of phorbol ester. V14Rho was found to immunoprecipitate with cPKCα, and the expression of the N-terminal domain of this isoform in T cells apparently blocked the combined effects of V14Rho and phorbol ester on AP-1 transcriptional activity. The fact that this interaction may also occur between other PKC isoforms and Rho GTPases was indicated in a recent study that showed a close association of Cdc42 with aPKCλ and -ζ, which appeared to mediate the regulation of stress fiber formation (*21*). Whereas it was suggested that one of the sites of interaction may be contained within the regulatory domain of aPKCλ, it was concluded that the interaction with Cdc42 may be indirect and involves an intermediary protein (*21*). Elsewhere, it was shown that in rat pancreatic acini, intracellular Ca^{2+} chelation eliminated, while phorbol ester increased, the amount of RhoA in detergent soluble fractions and in crude microsome preparations (*24*), providing further evidence that cPKCα may be an effector of RhoA.

Recent elegant studies have provided evidence that the homologues of mammalian PKC and RhoA, Pkc1p and Rho1p in the budding yeast *Saccharomyces cerevisiae*, respectively, may also act through a common pathway (*25*, *26*). First, it was found that replacing the *RHO1* gene, which is essential for bud formation, with the corresponding mammalian homologue gene resulted in a recessive temperature-sensitive growth phenotype, from which a dominant suppressor mutant was isolated (*26*). Support for an interaction between the gene product and Pkc1p was provided by the finding that the suppressor gene mutation occurred in *PKC1* at a position corresponding to the pseudosubstrate site of Pkc1p. Furthermore, yeast two-hybrid analysis demonstrated a direct GTP-dependent interaction between Rho1p and Pkc1p, involving a region containing the pseudosubstrate site and the C1 domain of Pkc1p. It was also suggested that the suppression of the temperature sensitivity of the *RhoA* mutant mediated by the Rho1p−Pkc1p interaction may result from an activation of the MAP kinase cascade. Elsewhere, Pkc1p was shown to co-immunoprecipitate with Rho1p from yeast lysates, and recombinant Rho1p was found to associate with Pkc1p in vitro in a GTP-dependent manner (*25*).

Whereas these studies in yeast provide strong evidence for a direct interaction between Pkc1p and Rho1p, using these observations to predict that a similar interaction may occur between mammalian PKC and Rho GTPases is difficult due to the marked differences in both the structure and enzymatic properties between yeast Pkc1p and the mammalian PKC isozymes (*27*, *28*). Studies thus far carried out in mammalian cells are consistent with an interaction between cPKCα and RhoA (*23*) and between aPKCζ and -λ and Cdc42 (*21*). However, it is difficult to distinguish direct from indirect interactions in studies which were based on measurements of the level of binding in cell lysates. A major goal of this study was to resolve whether *mammalian* PKC isozymes and Rho GTPases participate in a *direct* protein−protein interaction and to determine the consequences in terms of effects on PKC activation. This was accomplished using an in vitro assay designed to eliminate the possibility of indirect interactions by using purified recombinant proteins and the required activators and cofactors but excluding membranes. Using this approach, it was found that cPKCα was potently activated by RhoA and Cdc42 and to a lesser extent Rac1, in a GTP/GDP-, Ca^{2+}-, and phorbol ester-dependent manner. The Rho GTPases also induced cPKCβI, -βII, and -γ, nPKCδ and -ε, and aPKCζ activities, although at levels much reduced compared to those on cPKCα activity. The importance of these observations is that such PKC and Rho GTPase interactions may lead to significant cross-talk between their respective signaling pathways.

EXPERIMENTAL PROCEDURES

Materials. Adenosine 5′-triphosphate (ATP) was from Boehringer Mannheim (Indianapolis, IN). [γ-^{32}P]ATP was from New England Nuclear (Boston, MA). 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) and bovine brain phosphatidylserine (BPS) were from Avanti Polar Lipids, Inc. (Alabaster, AL). Peptide substrates were custom synthesized by the Jefferson Cancer Institute peptide synthesis facility of Thomas Jefferson University. Guanosine 5′-*O*-(thiotriphosphate) (GTPγS), guanosine 5′-diphosphate (GDP), and 4β-12-*O*-tetradecanoylphorbol 13-acetate (TPA) were obtained from Sigma (St. Louis, MO). Human RhoA, Cdc42, and Rac1 were obtained from Cytoskeleton, Inc. (Denver, CO) as purified glutathione *S*-transferase (GST) fusion proteins expressed in *Escherichia coli*. These proteins yielded homogeneous single bands on Coomassie blue-stained SDS−PAGE gels and were used without further purification. The GTPases were supplied as a mixture of GTP- and GDP-bound forms in an approximately 1:1 ratio, this being required to stabilize the recombinant proteins. All other chemicals were analytical grade and obtained from Fisher Scientific (Pittsburgh, PA).

Expression and Purification of PKC Isozymes. Recombinant PKCα, -βI, -βII, -γ, and -ε (rat brain) were prepared using the baculovirus *Spodoptera frugiperda* (Sf9) insect cell expression system (*29*) and purified to homogeneity by following published procedures (*30*). The nPKCδ, nPKCε, and aPKCζ isoforms were overexpressed in Sf9 cells as fusion proteins containing a His_6 tag attached to the C-terminus. The cloning, isolation, and purification of His_6-tagged proteins were performed using previously described methods (*30*).

Measurements of Rho GTPase-Induced PKC Activity. PKC isozyme activities were assayed by measuring the rate of phosphate incorporation into a peptide substrate. For the cPKC isoforms, a peptide corresponding to the phosphorylation site domain of myelin basic protein (QKRPSQRSKYL, MBP_{4-14}) was used as the substrate, whereas assays of nPKC and aPKCζ activity used a peptide corresponding to the pseudosubstrate region of nPKCε (ε-peptide), in which the single alanine residue was replaced with serine (*31*−*33*). For measurements of Rho GTPase-induced PKC isozyme activities, the assay (75 μL) consisted of 50 mM Tris-HCl (pH 7.40), 0.1 mM EGTA or $CaCl_2$, 50 μM MBP_{4-14} or 50 μM ε-peptide, TPA (500 nM or as indicated), GTPγS and

GDP where added, and GST fusion proteins containing either RhoA, Cdc42, or Rac1 which were present at a fixed concentration of 100 nM unless otherwise indicated. Rho proteins were made up in a buffer composed of 2 mM Tris (pH 7.6), 0.5 mM $MgCl_2$, 0.5% sucrose, and 0.1% dextran. Separate control experiments showed that the presence of these buffer components had no apparent effect on PKC activity (result not shown). For experiments in which activity was measured as a function of Ca^{2+} concentration, it was added to the assay at a level calculated to yield the required concentration when buffered by 0.1 mM EGTA (*34*). After thermal equilibration to 30 °C, assays were initiated by the simultaneous addition of the required PKC isoform (0.1 nM) along with 15 mM Mg^{2+}, 15 μM ATP, and 0.3 μCi of [γ-^{32}P]-ATP (3000 Ci/mmol) and terminated after 30 min with 100 μL of 175 mM phosphoric acid. Following this, 100 μL was transferred to P81 filter papers, which were washed three times in 75 mM phosphoric acid. The phosphorylated peptide was quantified by scintillation counting.

Measurements of Membrane-Associated PKC Isozyme Activity. The activities of PKC isoforms associated with membrane lipid vesicles were determined as described above for Rho GTPase-induced activity, except that the assay system contained large unilamellar vesicles (100 nm) composed of POPC and BPS (4:1 molar ratio) at a total concentration of 150 μM, prepared according to a previously described method (*35*).

RESULTS

Evidence for Direct Interactions between PKC Isozymes and Rho GTPases. The effects of Rho GTPases on PKC isozyme activities were determined using an assay system containing only purified proteins and the required peptide substrates, cofactors, and activators and excluding membranes. By this method, the possibility of indirect interactions between PKC isoforms and Rho GTPases was removed and any effects of Rho GTPases on PKC activities could be ascribed to *direct* protein−protein interactions.

The effects of fixed concentrations of RhoA, Cdc42, and Rac1 (100 nM) on cPKCα, -βI, -βII, and -γ, nPKCδ and -ϵ, and aPKCζ activities induced in the presence of Ca^{2+} (0.1 mM), with and without 0.5 μM TPA, are shown in Figure 1. It was found that the addition of RhoA to cPKCα in the absence of TPA resulted in only a slight increase in cPKCα activity over the "basal" level (Figure 1A). However, in the presence of TPA, cPKCα activity was enhanced ~18-fold upon addition of RhoA. In contrast, the activities of cPKCβI, -βII, and -γ, measured in the presence of TPA, were only enhanced ~2-fold over basal levels, and the activities of nPKCδ and -ϵ were unaffected under the same assay conditions. Interestingly, the level of aPKCζ activity was also potentiated ~5-fold, but in this case, the effect was independent of whether TPA was present. The presence of Cdc42 resulted in a ~14-fold enhancement of cPKCα activity, again in a TPA- and Ca^{2+}-dependent manner (Figure 1B). Like the effects of RhoA, the activities of cPKCα, -βI, -βII, and -γ and nPKCδ and -ϵ were each unaffected by Cdc42. However, the level of aPKCζ activity was also unaffected by Cdc42, which contrasts with the potentiating effect of RhoA. By contrast to the effects of RhoA and Cdc42, the presence of Rac1 resulted in a lower-magnitude

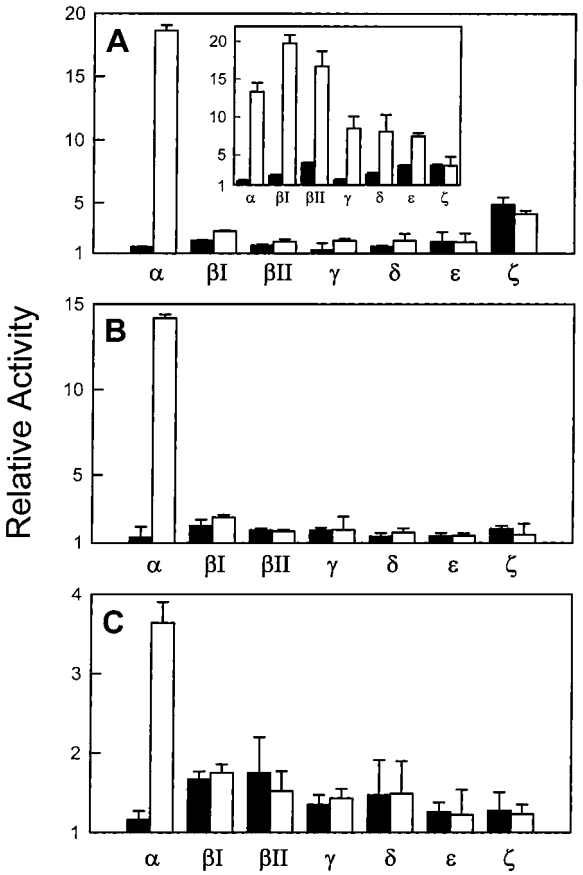

FIGURE 1: Direct effects of RhoA, Cdc42, and Rac1 on PKC isozyme activities. The activities of purified recombinant cPKCα, -βI, -βII, and -γ, nPKCδ and -ϵ, and aPKCζ isozymes were assayed in the presence of a fixed concentration (100 nM) of purified RhoA (A), Cdc42 (B), or Rac1 (C), in the presence (white bars) or absence (black bars) of 0.5 μM TPA. cPKC isoform activities were measured in the presence of 0.1 mM Ca^{2+} using MBP_{4-14} as a substrate, whereas nPKC and aPKCζ activities were determined in the absence of Ca^{2+} using ϵ-peptide as a substrate. In the inset, for comparison with Rho GTPase-induced activity, the relative activities of PKC isozymes associated with POPC/BPS lipid vesicle membranes (4:1 molar ratio), in the presence of 0.1 mM Ca^{2+} and either with (white bars) or without (black bars) 0.5 μM TPA, were measured. The Ca^{2+}- and TPA-induced specific activities of each isozyme associated with POPC/BPS vesicles in units of nanomoles per minute per milligram were 639 ± 11 (α), 767 ± 9 (βI), 699 ± 15 (βII), 520 ± 5 (γ), 512 ± 10 (δ), 724 ± 12 (ϵ), and 302 ± 7 (ζ). Relative activity is defined as the fold increase in activity induced by the presence of either Rho GTPase or POPC/BPS lipid vesicles in the presence or absence of TPA, a value of 1 corresponding to no change in activity. Data represent means of triplicate determinations (±SD). See Experimental Procedures for further details.

enhancement (~3-fold) of TPA- and Ca^{2+}-dependent cPKCα activity, while the activities of the other PKC isozymes were again unaffected (Figure 1C).

For comparison with the effects of the Rho GTPases, the increase in the activities of each PKC isozyme induced by association with membranes composed of POPC and BPS (4:1 molar ratio) were measured, again in the presence of Ca^{2+} (0.1 mM), and either with or without 0.5 μM TPA (Figure 1A, inset). The level of cPKCα activity obtained from association with vesicles in the presence of TPA and Ca^{2+} was ~15-fold greater than the basal level, which is comparable to the level of cPKCα activity induced by RhoA and Cdc42 measured under the same conditions. However, the association of the other PKC isozymes with membranes

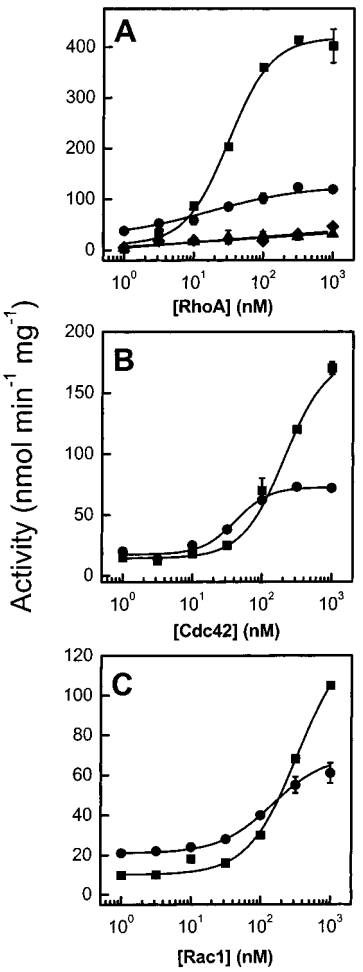

FIGURE 2: Concentration dependence of the effects of Rho GTPases on cPKCα activity. Purified, recombinant cPKCα activity was measured as a function of (A) RhoA, (B) Cdc42, and (C) Rac1 concentration, in the absence of both Ca^{2+} and TPA (▲), with 0.1 mM Ca^{2+} alone (◆), with 0.5 μM TPA alone (●), or in the presence of both Ca^{2+} and TPA (■). Data represent means of triplicate determinations (±SD). The solid curves represent fits of activity data to a modified Hill equation (*38*) using nonlinear regression analysis. See Experimental Procedures for other details.

in the presence of Ca^{2+} and TPA also resulted in a significant activity, which contrasts with the relatively low levels of activity of these isozymes induced by the presence of the different Rho proteins.

Concentration Dependence of the Effects of RhoA, Cdc42, and Rac1 on cPKCα Activity. The effects of the direct interaction between cPKCα and RhoA, Cdc42, and Rac1 on cPKCα activity were also determined as a function of the concentration of each Rho GTPase, in the presence or absence of TPA and Ca^{2+} (Figure 2). In the absence of TPA and Ca^{2+}, or with Ca^{2+} alone, no activation of cPKCα was induced by RhoA even at high levels (Figure 2A). In the presence of TPA alone, the level of cPKCα activity was increased 2-fold over the basal level by RhoA and ~3-fold by Cdc42 and Rac1, in a concentration-dependent manner. Only in the presence of *both* TPA and Ca^{2+} was a significant level of cPKCα activity obtained (Figure 2A). The low RhoA concentration corresponding to that required to induce a half-maximal level of activity obtained under these conditions implies a high-affinity and specific interaction between cPKCα and RhoA (Table 1). Further, the plateau value of

Table 1: Rho GTPase, TPA, and Ca^{2+} Concentrations Required To Induce a Half-Maximal Increase in cPKCα Activity[a]

	$[\text{Rho GTPase}]_{1/2}$[b] (nM)	$[\text{TPA}]_{1/2}$[d] (nM)	$[Ca^{2+}]_{1/2}$[e] (μM)
RhoA	21 ± 5,[c] 37 ± 2	21.4 ± 1.7	~10
Cdc42	52 ± 3,[c] 208 ± 18	7.7 ± 0.4	~5
Rac1	224 ± 21,[c] 334 ± 34	12.9 ± 0.8	~50

[a] Values of $[\text{Rho GTPase}]_{1/2}$ and $[\text{TPA}]_{1/2}$ were obtained from fits of activity data to a modified Hill equation (*36*), whereas values of $[Ca^{2+}]_{1/2}$ were estimated by inspection. The concentration of cPKCα was 0.1 nM in each experiment. Data represent means of triplicate determinations (±SD). See Experimental Procedures for other details. [b] Concentrations of Rho GTPase required to induce a half-maximal increase in activity were determined with 0.5 μM TPA in the absence of 0.1 mM Ca^{2+}. [c] Concentrations of Rho GTPase required to induce a half-maximal increase in activity were determined with 0.5 μM TPA in the presence of 0.1 mM Ca^{2+}. [d] TPA concentration required to induce a half-maximal increase in activity, determined in the presence of 0.1 mM Ca^{2+}. [e] Concentration of Ca^{2+} required to induce a half-maximal increase in activity determined with 500 nM TPA.

~400 nmol min^{-1} mg^{-1} for RhoA-induced cPKCα activity with TPA and Ca^{2+} compares with a value of 639 ± 11 nmol min^{-1} mg^{-1} measured using a similar assay system for cPKCα associated with POPC/BPS membrane lipid vesicles in the presence of TPA and Ca^{2+}.

The level of cPKCα activity induced by Cdc42 and Rac1 was also enhanced in the presence of TPA and Ca^{2+} (Figure 2B,C), but the concentrations required for half-maximal activation by Cdc42 and Rac1 were ~7 and 10-fold greater than that observed for RhoA, respectively (see Table 1). For each GTPase, the midpoints of the concentration−response curves obtained in the presence of TPA and Ca^{2+} were elevated slightly compared to those obtained in the absence of Ca^{2+}. Also, the presence of Ca^{2+} resulted in a slight reduction in the level of cPKCα activity induced by low Rho GTPase concentrations. This small "inhibitory" effect of Ca^{2+} may compete with the potentiating effect of Ca^{2+} on Rho GTPase-induced activity, which might lead to a small error in the midpoint values that might be obtained.

Phorbol Ester Concentration Dependences for Rho GTPase-Induced cPKCα Activity. The concentration−response curves obtained for TPA-induced cPKCα activity in the presence or absence of a fixed concentration of Rho GTPase (100 nM for RhoA and 500 nM for Cdc42 and Rac1) and Ca^{2+} (0.1 mM), are shown in Figure 3. Without the Rho GTPases, the level of cPKCα activity in the presence of TPA alone was marginal, independent of the presence of Ca^{2+} (Figure 3A). In the presence of RhoA, Cdc42, or Rac1, cPKCα activity induced in the absence of Ca^{2+} increased slightly as a function of TPA concentration, but failed to reach a maximal value even at the highest phorbol ester concentration that was used (10 μM). However, with the addition of Ca^{2+} (0.1 mM) there was a marked increase in the level of RhoA-, Cdc42-, and Rac1-dependent cPKCα activity which, in each case, corresponded to a large decrease in the concentration of TPA required to induce a half-maximal increase in activity, the value of which was similar for each Rho GTPase (see Table 1). The TPA concentration−response curves obtained in the presence of Rho GTPases and Ca^{2+} could be fitted with least error to an equation that assumed a single TPA-binding site (*36*).

Concentration-Dependent Effects of Ca^{2+} on Rho GTPase-Induced cPKCα Activity. The Ca^{2+} concentration depend-

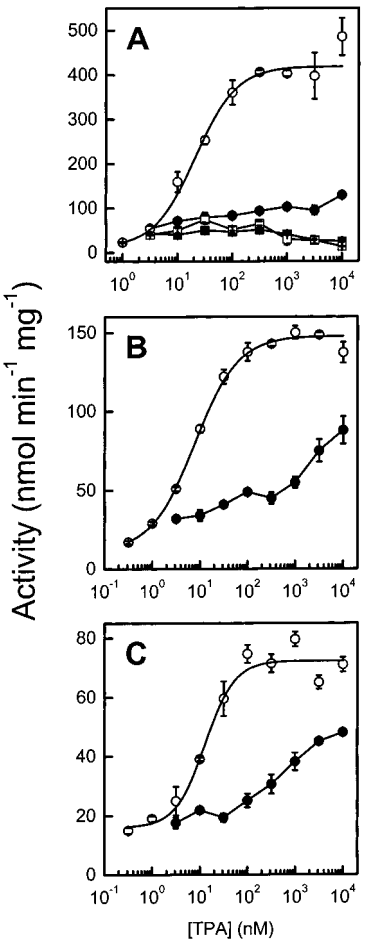

FIGURE 3: Concentration-dependent effects of TPA on RhoA, Cdc42, and Rac1-induced cPKCα activity. The activity of cPKCα was determined as a function of TPA concentration, either with (○ and ●) or without (□ and ■) 100 nM RhoA (A), 500 nM Cdc42 (B), or 500 nM Rac1 (C) and in the presence (white symbols) or absence (black symbols) of 0.1 mM Ca^{2+}. Activity data obtained in the presence of Rho GTPase and Ca^{2+} (○) were fitted to a modified Hill equation (*38*) using nonlinear regression analysis. See Experimental Procedures for other details.

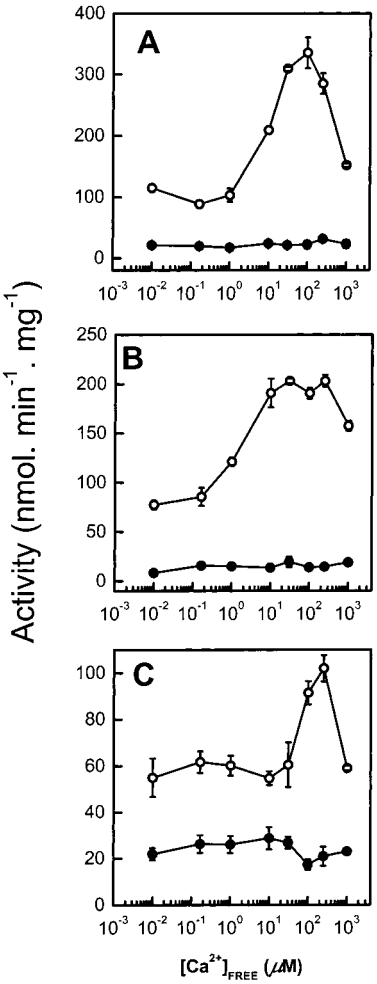

FIGURE 4: Concentration-dependent effects of Ca^{2+} on the activity of cPKCα induced by RhoA, Cdc42, and Rac1. The level of cPKCα activity was measured as a function of the level of Ca^{2+} in the presence of 100 nM RhoA (A), 500 nM Cdc42 (B), or 500 nM Rac1 (C), either with (○) or without (●) 0.5 μM TPA. Data represent means of triplicate determinations (±SD). Other details are described in Experimental Procedures.

encies of RhoA-, Cdc42-, and Rac1-induced cPKCα activity are shown in Figure 4. For each Rho GTPase, the level of cPKCα activity obtained in the absence of TPA was unaffected by Ca^{2+} within the concentration range that was used, indicating that both Ca^{2+} and phorbol ester are required to induce Rho GTPase-associated cPKCα activity. However, the presence of TPA (500 nM) was sufficient to induce a low level of Rho GTPase-associated activity at low Ca^{2+} levels, consistent with the data shown in Figure 2. Increasing the concentration of Ca^{2+} further resulted in a potentiation of the level of RhoA-, Cdc42-, and Rac1-induced cPKCα activity. The concentrations of Ca^{2+} required to induce a half-maximal increase in RhoA- and Cdc42-induced cPKCα activity estimated from the activity data were similar and were both lower than that observed for Rac1 (Table 1). Increasing the level of Ca^{2+} further resulted in a decline in the level of activity.

Effects of GTP- and GDP-Bound Forms of Rho GTPases on cPKCα Activity. A common feature of the interaction of Rho GTPases with effectors is that their activities are enhanced by GTP binding (*5*). To investigate whether this may also apply to the interaction with cPKCα, the activity of the kinase was measured in the presence of a fixed concentration of each Rho GTPase (100 nM), TPA (0.5 μM), and Ca^{2+} (0.1 mM), as a function of an increasing concentration of either GTPγS, which is inert to GTPase-catalyzed hydrolysis, or GDP (Figure 5). It was found that the level of RhoA-, Cdc42-, and Rac1-induced cPKCα activity was increased by GTPγS, while being attenuated by GDP, both effects being concentration-dependent. The inhibitory effect of GDP on RhoA-induced cPKCα activity was found to be reversible, since the addition of GTPγS to an assay containing a level of GDP sufficient to fully attenuate cPKCα activity (300 μM) resulted in a recovery of activity (Figure 5A). The possibility that the observed effects of GTPγS and GDP on cPKCα activity may have involved competition for ATP substrate binding to the kinase was ruled out by the finding that neither compound affected the activity of the enzyme measured in a Rho GTPase-independent assay that utilized BPS/POPC membranes in the presence of Ca^{2+} and TPA (results not shown).

Effects of Membrane Lipids on Rho GTPase-Induced cPKCα Activity. The conventional PKC isozymes become activated upon associating with membranes containing anionic lipids, in a phorbol ester- and Ca^{2+}-dependent manner

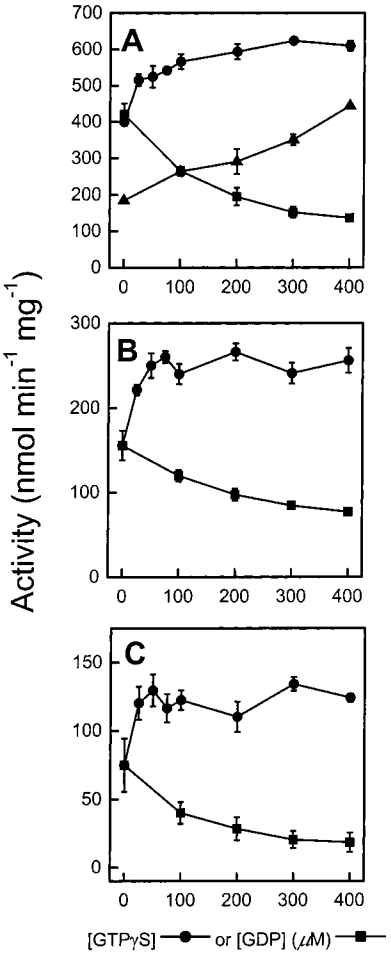

FIGURE 5: Effect of GTP and GDP on cPKCα activity induced by RhoA, Cdc42, and Rac1. The activity of cPKCα, induced in the presence of 100 nM RhoA (A), Cdc42 (B), or Rac1 (C) and fixed concentrations of Ca^{2+} (0.1 mM) and TPA (0.5 μM), was measured as a function of the concentration of either GTPγS (●) or GDP (■). In a control experiment designed to test for competition between the two ligands, the titration of GTPγS was carried out in the presence of 300 μM GDP (▲). Data represent means of triplicate determinations (±SD). Other details are described in Experimental Procedures.

(e.g., ref *37*). Rho GTPases have also been shown to translocate to membranes (*16*). To investigate the possibility that the Rho GTPases also interact with, and modulate, membrane-associated cPKCα, the effects of RhoA, Cdc42, and Rac1 on the activity of cPKCα in the presence of membranes were determined (Figure 6). It was found that the activity of membrane-bound cPKCα, measured in the presence of Ca^{2+} (0.1 mM) and TPA (0.5 μM), at concentrations shown previously to induce a maximal level of membrane-associated activity (*38*), was further elevated by each GTPase, although the magnitude of these effects was reduced compared to that observed for nonmembrane cPKCα. It should be noted that the Rho GTPase preparations used in these experiments may not interact optimally with membranes, since they are expressed in *E. coli* and were therefore not isoprenylated.

DISCUSSION

The results presented here provide evidence for a direct protein−protein interaction between certain PKC isozymes and Rho GTPases that results in kinase activation. The

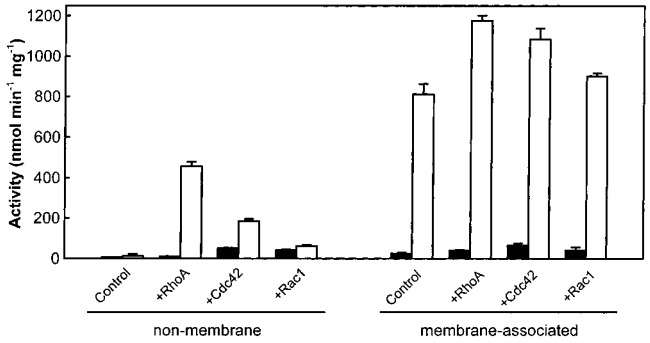

FIGURE 6: Effects of membrane association of cPKCα on the potentiation of activity by RhoA, Cdc42, and Rac1. The activity of cPKCα was measured with 0.1 mM Ca^{2+} in the absence of Rho GTPase (control) or with 100 nM RhoA, Cdc42, or Rac1, in the presence (white bars) or absence (black bars) of 0.5 μM TPA and either with or without BPS/POPC vesicles (1:4 molar ratio and 150 μM total concentration). Data represent means of triplicate determinations (±SD). Other details are described in Experimental Procedures.

potency of the effects varied according to Rho GTPase and PKC isozyme type. Thus, cPKCα was activated by Rho GTPases to a greater degree than cPKCβI, -βII, -γ, -δ, -ϵ, and -ζ, and the potencies of the effects of the different Rho GTPases on cPKCα activity were in the following order: RhoA > Cdc42 > Rac1. The activity of cPKCα was found to be dependent not only on the concentration of phorbol ester and Ca^{2+} but also on the nature of the GTP/GDP-bound state of the Rho GTPases, suggesting that the interaction may be regulated by conformational changes in *both* cPKCα and Rho GTPases.

Several previous studies have provided support for a "close association" between PKC and Rho GTPases, although, due to the complexity of the experimental systems used, it was not possible to eliminate indirect interactions involving other components. For example, it was shown that the inactivation of the Rho protein with *Clostridium difficile* toxin B-10463 resulted in an inhibition of the translocation of PKC to membranes in epithelial cells (*22*), and specific inactivation of RhoA by C3-transferase resulted in an inhibition of the translocation of aPKCζ to neutrophil membranes (*39*). Also, in another recent study, it was shown that PKCα and RhoA colocalize in smooth muscle cells by undergoing a mutual, agonist-induced translocation to caveolin-1 (*40*). Elsewhere, it has been shown that cPKCα immunoprecipitated with RhoA from the membrane fraction of Jurkat T cell lysates (*23*), although conclusive evidence in support of a direct interaction between cPKCα and RhoA was not provided since an indirect interaction involving the formation of an intermediary complex could not be ruled out. In another recent report, evidence was presented that supported a close association of Cdc42 with aPKCζ and aPKCλ in rat brain cytosol and in NIH 3T3 cell lysates, which was found to be involved in the loss of stress fibers (*21*). It was found that Cdc42 did not associate with cPKCα in this cell line, contrasting with the results obtained using T cells (*23*). Consistent with our finding that aPKCζ activity was unaffected by Cdc42, it was found that purified Cdc42 did not interact directly with purified full-length aPKCζ or with the isolated regulatory domain of this isoform. On this basis, it was concluded that the observed interaction of aPKCζ and -λ with Cdc42 in NIH 3T3 cell lysates was likely indirect

and involved an intermediary protein.

Concluding whether direct or indirect interactions occur between two proteins based on results obtained from immunoprecipitation experiments is not trivial. For example, whereas the observation that two proteins can be immunoprecipitated is consistent with a direct association between those proteins, it does not preclude the involvement of an intermediate structure. Conversely, due to the relatively crude nature of the technique, the observation that two proteins do *not* immunoprecipitate does not necessarily preclude the possibility of an interaction under different experimental conditions. Also, the method involves disruption of cell architecture and loss of compartmentalization. Therefore, to demonstrate and characterize a *direct* interaction, an assay system using purified proteins is required. In this work, direct interactions were demonstrated by virtue of an activation of PKC being obtained in an in vitro assay, in a Rho GTPase-, Ca^{2+}-, and TPA-dependent manner. Such an activation could only result from a direct physical interaction between PKC isozymes and Rho GTPases; however, we have also been able to confirm this by surface plasmon resonance and GST-pull down assays (C. J. Buzas, J. S. Buguliskis, S. J. Slater, and C. D. Stubbs, unpublished observations). The affinity constants for the interaction of Rho GTPases with PKCα, in terms of values of $[\text{Rho GTPase}]_{1/2}$ (see Table 1), are within a range of values obtained for the direct interactions of Rho GTPases with other effectors, including, for example, p190GAP, Bcr, PAK-1, WASP, and IQGAP-1 (*41*−*43*).

The results presented here showing a direct interaction between cPKCα and RhoA are consistent with those of another study showing that yeast Rho1p interacted directly with Pkc1p and that this interaction resulted in kinase activation (*25*). However, it appears that the interaction of yeast Rho1p with Pkc1p may not be equivalent to that between mammalian RhoA and cPKCα, and may not lead to equivalent effects on activity. First, our finding of an activation of cPKCα by Cdc42 contrasts with that of the previous study which found that yeast Pkc1p activity was unaffected by this Rho GTPase (*25*). Second, the results of the previous study suggested that the presence of PS alone was sufficient for a maximal level of yeast Pkc1p activity induced by Rho1p and that this activity was unaffected by the addition of Ca^{2+} or TPA. By contrast, it was observed here that mammalian, Ca^{2+}- and TPA-dependent cPKCα activity induced by association with membranes containing PS was *further* potentiated by the addition of RhoA, Cdc42, or Rac1. These differences are likely to be due to the distinct structural and enzymatic properties of yeast Pkc1p as compared to those of mammalian cPKCα. For example, although the structure of Pkc1p resembles that of the cPKC isoforms, the presence of a 348-amino acid N-terminal extension renders it considerably larger (150 for Pkc1p, cf. ∼80−85 kDa for cPKCα), and in fact, it shares only moderate sequence homology outside of the catalytic domain (*44*). Furthermore, the activity of Pkc1p appears to be independent of cofactors, including diacylglycerol and Ca^{2+}, despite the presence of both C1 and C2 domains within its structure (*27*, *28*).

The observation that Ca^{2+} and TPA together acted in a synergistic manner to increase the level of Rho GTPase-induced cPKCα activity, compared to the basal levels of activity induced by either Ca^{2+} or TPA alone, is, at first, reminiscent of the typical "cooperative" effect of Ca^{2+} and phorbol ester on the membrane-associated activities of cPKC isoforms, including cPKCα (e.g., refs *37* and *45*). For membrane-associated cPKC, this "cooperative" effect on membrane-binding affinity was suggested to be mediated by the parallel but *independent* targeting of the enzyme to the membrane by the C1 and C2 domains, rather than an allosteric interaction between the two domains. Thus, the binding of Ca^{2+} to the C2 domain induces association of cPKC with the membrane surface, resulting in a facilitated binding of phorbol ester to the C1 domain. The combined interaction then supplies the free energy required for the activating conformational change that leads to pseudosubstrate release from the active site (*37*, *46*). The mechanism by which Ca^{2+} and phorbol ester binding results in Rho GTPase-induced cPKCα activity clearly differs from the mechanism by which activation is induced by association with membranes. Whereas for membrane-associated cPKC, a decrease in the level of membrane binding and activation induced by phorbol ester binding to the C1 domain can be offset by increasing the level of Ca^{2+} binding to the C2 domain (*37*, *45*), it appears that phorbol ester binding is essential for the association of cPKCα with Rho GTPases and that Ca^{2+} binding *alone* cannot support activity. Also, whereas binding of Ca^{2+} to the C2 domain of cPKC results in an increase in the affinity of cPKC for membranes containing phorbol ester (*37*), Ca^{2+} binding does not appear to increase the affinity of the cPKCα−Rho GTPase interaction, since the concentrations of Rho GTPase required to induce a half-maximal increase in activity were found to be slightly *decreased* in the presence of Ca^{2+} (see Table 1). Rather, it appears that the synergistic potentiation of activity induced by Ca^{2+} results from an increased affinity for phorbol ester binding, and the activating conformational change resulting in Rho GTPase-induced cPKCα activity may therefore involve an allosteric interaction between phorbol ester and Ca^{2+} binding sites. The affinities for TPA binding, estimated from values of $[\text{TPA}]_{1/2}$ (see Table 1), are within a TPA concentration range commonly used in cell studies and within which TPA-induced cellular effects are often observed.

Whereas the isolated C1 domain of PKC and the intact enzyme has been previously shown to be capable of binding phorbol esters with low affinity in the absence of lipids (*47*, *48*), a *high*-affinity phorbol ester interaction appeared to require the association of the enzyme with a membrane. On the basis of a crystal structure derived for a phorbol ester-bound cysteine-rich domain, it has been suggested that the hydrophobic interior of the membrane may contribute to the high-affinity phorbol ester interaction with PKC by engaging in hydrophobic interactions with the exposed phorbol ester acyl chains which would otherwise undergo unfavorable interactions with the aqueous environment (*49*, *50*). However, it appears that phorbol esters can nevertheless interact with high affinity *in the absence of membranes* with PKC associated with Rho GTPases as shown here, and also with filamentous actin as shown previously (*36*, *51*, *52*), indicating that the hydrophobic site provided by the membrane−lipid environment is not essential. The interaction of Rho GTPases and Ca^{2+} with cPKCα may result in a structural rearrangement that leads to the exposure of a phorbol ester binding site consisting, in part, of a hydrophobic surface or groove

that interacts with the phorbol ester acyl chains and occludes them from energetically unfavorable interactions with the aqueous solvent.

PKC joins a growing number of serine-threonine kinase effectors for Rho GTPases that include the closely related PKN (*9*) and PRK2 (*13*) which interact with the GTP-bound RhoA through conserved Rho effector binding motif type 1 (REM-1), $p160^{ROCK}$ and ROKα which interact through REM-2 domains (*5*), and diacylglycerol kinase *θ* (*10*). Whereas the catalytic region of PKC is structurally homologous to both PKN and PRK2, neither the REM-1 nor REM-2 domains are present in PKC nor are other currently known consensus binding sequences, including the Cdc42/Rac interactive binding (CRIB) motif for Cdc42 and/or Rac (*53*). However, in a previous yeast two-hybrid study, it was shown that Rho1p bound to a fragment of Pkc1p containing the N-terminal pseudosubstrate and C1 domain (*26*), raising the possibility that perhaps for RhoA binding, the site(s) of interaction may reside within a corresponding region on cPKCα. Further experiments are required to determine the sites of interaction on the Rho GTPases.

The observation that the level of cPKCα activity induced by each GTP-bound Rho GTPase was enhanced by GTPγS while being inhibited by GDP, the latter being reversible by the addition of further GTPγS, is consistent with the effects on cPKCα activity being mediated by interaction with the GTP-bound rather than the GDP-bound Rho GTPase. However, in these experiments, the Rho GTPases were initially in a partially GTP-bound state, which is required to stabilize the purified recombinant proteins, and it was therefore not possible to determine whether GTP binding is an absolute requirement for cPKCα activity.

In conclusion, the results presented here show that cPKCα interacts directly with RhoA, Cdc42, and to a reduced extent Rac1, and that these interactions result in the activation of the kinase. The interaction requires both Ca^{2+} and phorbol ester and is regulated according to the GTP/GDP-bound states of the Rho GTPases. It is shown that the direct interaction between PKCα and Rho GTPases is sufficient to induce activation and that this does not require membrane association, although (maximal) membrane-associated activity induced by TPA is *further* increased in the presence of RhoA. Thus, the Rho GTPases join a class of nonmembrane targets with which PKC interacts directly, which include filamentous actin (*36*, *51*, *52*), the cytoskeletal protein, calponin (*54*), and a growing number of other proteins (*55*). To show that a direct interaction between cPKCα and Rho GTPases also occurs in the cellular environment would ultimately require the ability to isolate the interaction from the multitude of other potential protein−protein and protein−lipid interactions involving these proteins. Whereas this study provides evidence that such an interaction is possible, caution should be engendered in drawing the conclusion that an identical interaction occurs in the cell. Nevertheless, these results provide a physical basis for the apparent convergence of PKC- and Rho-mediated signaling pathways, which may have important consequences for the regulation of diverse cell functions, including cytoskeletal rearrangements.

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