Biochemistry

 Volume 39, Number 2 January 18, 2000

Articles

Interaction of Protein Kinase C with Filamentous Actin: Isozyme Specificity Resulting from Divergent Phorbol Ester and Calcium Dependencies†

Simon J. Slater, Shawn K. Milano, Brigid A. Stagliano, Kevin J. Gergich, Jeffery P. Curry, Frank J. Taddeo, and Christopher D. Stubbs*

Department of Pathology, Cell Biology and Anatomy, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

Received July 19, 1999; Revised Manuscript Received October 12, 1999

ABSTRACT: The mechanism of activation of protein kinase C isoforms by filamentous actin (F-actin) was investigated with respect to isozyme specificity and phorbol ester and Ca^{2+} dependencies. It was found that the "conventional" (cPKC), α, βI, βII, and γ, "novel" (nPKC) δ and ϵ, and "atypical" (aPKC) ζ isoforms were each activated by F-actin with varying potencies. The level of activity along with the affinity for binding to F-actin was further potentiated by the phorbol ester *4*β-12-*O*-tetradecanoylphorbol 13-acetate (TPA), the potency of which again varied for each isoform. By contrast to the other cPKC isoforms, the level of cPKC-γ activity was unaffected by TPA, as was also the case for aPKC-ζ. It was found that whereas in the absence of F-actin the soluble form of cPKC-βI contained two phorbol ester binding sites of low and high affinity, respectively, as previously reported for cPKC-α [Slater et al. (1998) *J. Biol. Chem. 273*, 23160−23168], the F-actin-bound form of the isozyme contained only a single site of relatively low affinity. The level of TPA required to induce cPKC-α, -βI, and -βII activity and the binding of these isozymes to F-actin was reduced in the presence of Ca^{2+}. By contrast, the activity of cPKC-γ was unaffected by Ca^{2+}, as were the activities of nPKC-δ and -ϵ and aPKC-ζ, as expected. Thus, the interaction with F-actin appears to be a general property of each of the seven PKC isozymes tested. However, isoform specificity may, in part, be directed by differences in the phorbol ester and Ca^{2+} dependences, which, with the notable exception of cPKC-γ, appear to resemble those observed for the activation of each isoform by membrane association. The observation that cPKC isoforms may translocate to F-actin as well as the membrane as a response to an elevation of Ca^{2+} levels may allow for the functional coupling of fluctuations of intracellular Ca^{2+} levels through cPKC to F-actin cytoskeleton-mediated processes.

The 12 isozymes that constitute the protein kinase C (PKC)[1] family each occupy a central position in signal transduction pathways that regulate both normal and aberrant cellular functions, including differentiation, proliferation, secretion, and metabolism (*1*−*6*). In general, PKC isozymes can be distinguished on the basis of the presence or absence of structural motifs that direct cofactor requirements for membrane association and activation (*7*). Thus, the "conventional" α, βI, βII, and γ (cPKC) isozymes contain a conserved C1 domain that harbors the phorbol ester and

† This work was supported by U.S. Public Health Service Grant: AA08022, AA07215, AA07186, and AA07465.

* Corresponding author: Room 271 JAH, Department of Pathology and Cell Biology, Thomas Jefferson University, Philadelphia, PA 19107. Tel (215) 955-5019; Fax (215) 923-2218; email: Chris.Stubbs@mail.tju.edu.

[1] Abbreviations: ATP, adenosine 5′-triphosphate; BPS, bovine brain phosphatidylserine; DAG, 1,2-dioleoylglycerol; Me_2SO, dimethyl sulfoxide; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; F-actin, filamentous actin; MARCKS, myristoylated alanine-rich C-kinase substrate; MBP_{4-14}, myelin basic protein peptide substrate; PDBu, phorbol 12,13-dibutyrate; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; cPKC, "conventional" protein kinase C; nPKC, "novel" protein kinase C; aPKC, "atypical" protein kinase C; RET, resonance energy transfer; SAPD, sapintoxin D; TPA, 4β-12-*O*-tetradecanoylphorbol 13-acetate; 4α-TPA, 4α-12-*O*-tetradecanoylphorbol 13-acetate.

10.1021/bi9916527 CCC: $19.00
Published on Web 12/17/1999

diacylglycerol binding site(s) and a C2 domain that binds two Ca^{2+} ions. The "novel" δ, ϵ, η, and θ (nPKC) isoforms contain a C1 domain but lack a functional C2 domain, resulting in the membrane association and activation of these isoforms being Ca^{2+}-independent. In the case of "atypical" PKC-ζ and -ι/λ (aPKC), neither of these regions are functional and these isoforms are therefore unresponsive to Ca^{2+} and phorbol esters or diacylglycerols.

It is becoming clear that each PKC isoform may specifically regulate distinct cellular functions (*8*). The divergent roles of cPKC, nPKC, and aPKC isoforms in the regulation of cellular processes may, in part, be explained by differing cofactor and activator requirements. However, the apparent functional specificity may also result from the targeting of each isoform to different subcellular compartments, resulting in the localization of each isoform with the requisite substrates (*9*−*11*). For example, it has been shown that treatment of NIH 3T3 cells with phorbol ester results in the accumulation of cPKC-α in the endoplasmic reticulum, whereas cPKC-βII localizes with the filamentous actin (F-actin) cytoskeleton and cPKC-γ associates with the Golgi organelles (*12*). Intracellular localization of a PKC isoform may result from interaction with specific PKC-binding proteins. These include substrates that bind to the enzyme in a phospholipid-dependent manner (*11, 13*) and others termed RACKs (receptors for activated C-kinase) that interact specifically with the activated form of single PKC species through a site distinct from that involved in catalysis (*9*). For example, RACK1 appears to interact specifically with three exposed β-strands of the C2 domain of cPKC-βI and -βII, resulting in an increased level of activity (*14, 15*). A further class of PKC binding proteins termed receptors for inactive C-kinase (RICKs) have been suggested to bind the inactive or partially active form of PKC isozymes. Whereas an isozyme-specific RICK remains to be discovered, it has been suggested that such a protein may target the inactive form of nPKC-ϵ to the Golgi apparatus, involving a binding site located within the C1 domain (*16, 17*). The pleckstrin homology domain of Bruton tyrosine kinase (Btk) has also been suggested to interact with the C1 domain of inactive PKC, although this interaction was not reported to be isozyme-selective (*18*). Also, two members of the family of anchoring proteins for cAMP-dependent protein kinase, AKAP-79 (*19, 20*) and gravin (*21*), have been shown to bind and inactivate PKC isoforms in a Ca^{2+}/calmodulin- and phosphatidylserine-dependent manner.

It has been widely shown by immunocytochemical and subcellular fractionation studies that individual PKC isoforms may colocalize with components of the cytoskeleton in both resting and stimulated cells (*22*). Formative work by Jaken and co-workers using an overlay assay revealed several cytoskeletal proteins that bound PKC isoforms in a phosphatidylserine- (PS-) dependent manner (*11, 23, 24*). For example, the focal adhesion related proteins, talin, vinculin, and the F-actin cross-linking protein myristoylated arginine-rich C-kinase substrate (MARCKS) are all substrates for cPKC-α (*24, 25*), and the cross-linking proteins ankyrin and spectrin are substrates for cPKC-β (*26*).

There is growing evidence that the F-actin component of the cytoskeleton may be an important target in the subcellular localization of several PKC isoforms (*12, 25, 27*−*35*). Recently, it was shown that a key event in the regulation of glutamate exocytosis from mossy fiber nerve terminals may be mediated by a specific interaction of nPKC-ϵ with F-actin (*30*). In vitro experiments indicated that recombinant nPKC-ϵ bound to purified F-actin in a phorbol ester-dependent manner through a motif located between the first and second cysteine-rich regions of the C1 domain that is unique to this isoform (*33*). Whereas the same study provided evidence that other PKC isoforms lacking this motif may not interact with F-actin, another laboratory reported that cPKC-βII, despite lacking the motif present in PKC-ϵ, may also interact with F-actin through a unique sequence located within the V5 domain (*31*). The association of PKC isoforms with the F-actin cytoskeleton also appears to differ according to the cell line studied. For example, it has been shown that phorbol ester treatment results in a translocation of cPKC-βII but *not* cPKC-βI to the actin cytoskeleton of the T-lymphoblastoid cell line MOLT-4 and also NIH-3T3 cells (*31*), whereas elsewhere cPKC-βII was not found to associate with the actin cytoskeleton in COS-7 cells (*36*). Therefore, the question whether the direct interaction of PKC with F-actin is specific to a particular isoform and whether the presence of a putative actin-binding signal within the structure of that isoform is both necessary and sufficient to confer an ability to interact with F-actin has remained unknown.

In the present study, the isoform specificity of the interaction of PKC with F-actin was investigated by comparison of the requirements of each isoform for phorbol ester and Ca^{2+}. It was found that under, standardized experimental conditions, each of the seven PKC isoforms tested was activated by interaction with F-actin. However, it was found that the isozymes differed markedly with respect to the effects of phorbol esters and Ca^{2+} on F-actin binding and activation. Thus, the interaction of cPKC-α, -βI, and -βII with F-actin and the level of activity were each potentiated by phorbol ester and Ca^{2+}, whereas nPKC-δ and -ϵ were phorbol ester-dependent but Ca^{2+}-independent and aPKC-ζ required neither phorbol ester nor Ca^{2+}. By contrast with the other cPKC isoforms, F-actin-associated cPKC-γ activity was unaffected by phorbol ester and Ca^{2+}. Thus, whereas the interaction with F-actin appears to be a general property of each PKC isoform, it is possible that the targeting of a particular isoform within each class to the F-actin cytoskeleton may, in part, be directed by differences in activator and cofactor requirements.

MATERIALS AND METHODS

Materials. Peptide substrates were custom-synthesized by the Jefferson Cancer Institute peptide synthesis facility of Thomas Jefferson University. Sapintoxin D (SAPD) was from Calbiochem (La Jolla, CA) or Alexis Biochemicals (San Diego, CA). Phorbol 12,13-dibutyrate, 4β-12-*O*-tetradecanoylphorbol 13-acetate (TPA) and 4α-12-*O*-tetradecanoylphorbol 13-acetate (4α-TPA) were obtained from Sigma (St. Louis, MO). Adenosine 5′ triphosphate (ATP) was from Boehringer Mannheim (Indianapolis, IN) and [γ-^{32}P]ATP was from New England Nuclear (Boston, MA). Pyrenyl G-actin was supplied by Cytoskeleton, Inc. (Denver, Co). All other chemicals were of analytical grade and were obtained from Fisher Scientific (Pittsburgh, PA).

Expression and Purification of PKC Isozymes. Recombinant PKC-α,-βI, -βII, -γ, and -ϵ (rat brain) were prepared

by use of the baculovirus *Spodoptera frugiperda* (Sf9) insect cell expression system (*37*) and purified to homogeneity according to previously described procedures (*38*). To aid isolation and purification, the isoforms nPKC-δ and aPKC-ζ 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 aPKC-ζ was performed by a previously described method (*39*) and essentially similar methods were used for the preparation of $(His)_6$-tagged nPKC-δ with some modifications. Briefly, an antisense oligonucleotide primer (TCGCGATTCCAGGAATTGTCATATTTGGG) containing the last 27 bases of a published coding sequence (*40*) was designed to replace the stop codon with an in-frame *Nru*I blunt site (underlined). The sense primer (GGATCCAAT**ATG**GCACCGTTCCTGCG) contained the first 17 nucleotides including the start codon (boldface type) and a *Bam*HI restriction site (underlined). By use of these primers, full length nPKC-δ cDNA lacking the stop codon was amplified by PCR with *Pfu* DNA polymerase (Stratagene, La Jolla, CA) and previously cloned intact nPKC-δ cDNA as a template (F. J. Taddeo, M. D. Yeager, and C. D. Stubbs, manuscript in preparation). The PCR product was gel-purified and subcloned into pCR Blunt with a zero blunt cloning kit (Invitrogen, Carlsbad, CA). After confirming the sequence was confirmed by dideoxy sequencing, the full-length nPKC-δ cDNA was excised from pCR Blunt with *Eco*RI and *Nru*I and subcloned into the *Eco*RI/*Eco*47III site of the vector pGEX 5x-2/$(His)_6$, constructed as described previously (*39*), which resulted in the attachment of the coding sequence for $(His)_6$ terminated with a stop codon at the 3′ end of the nPKC-δ cDNA. The last 1740 base pairs of nPKC-δ including the $(His)_6$ sequence were excised from pGEX 5x-2/$(His)_6$ by using *Eco*4711 and *Bsa*A1 and ligated into the *Eco*47III/*Stu*I site of a pFastBac transfer vector containing a nPKC-δ insert, constructed as previously described (*39*).

Preparation of F-Actin. Rabbit skeletal muscle actin was purified according to a previously described procedure (*41*). Briefly, 10 g of rabbit skeletal muscle acetone powder (Sigma, St. Louis, MO) was extracted with six 100 mL aliquots of buffer G [2 mM Tris-HCl, pH 8.0, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, and 0.005% (w/v) NaN_3], which favors the formation of globular actin (G-actin). Each extraction involved stirring at 0 °C for 20 min followed by squeezing through several layers of cheesecloth. The combined extracts were centrifuged (100 000*g* for 90 min at 4 °C) and the supernatant was concentrated 10-fold by using a Mini-Ultrasette fitted with a 10K molecular weight cutoff membrane (Filtron Tech. Corp., Northborough, MA). The resultant G-actin was polymerized to form F-actin in buffer F [2 mM Tris-HCl, pH 8.0, 0.2 mM $CaCl_2$, 0.5 mM β-mercaptoethanol, 50 mM KCl, 1 mM ATP, 2 mM $MgCl_2$, and 0.005% (w/v) NaN_3] for 2 days at 4 °C. The resultant F-actin suspension was then adjusted to 600 mM in KCl and centrifuged at 150000*g* for 90 min at 4 °C. The F-actin pellet was depolymerized in buffer G to form G-actin, which was then dialyzed against the same buffer for 3 days at 4 °C. The G-actin was then repolymerized to form F-actin by adding KCl, $MgCl_2$, and ATP to yield final concentrations of 50 mM, 2 mM, and 1 mM, respectively. Prior to use, the F-actin was repelleted by centrifugation at 150000g for 90 min at 4 °C and resuspended in a buffer containing 2 mM Tris-HCl, pH 8.0, 0.5 mM β-mercaptoethanol, 50 mM KCl, and 2 mM $MgCl_2$.

To verify that the F-actin remained fully polymerized over the time period of the activity and binding measurements, the extent of polymerization was determined from measurements of the enhanced fluorescence intensity that occurs due to excimer formation when pyrene-labeled G-actin is polymerized (*42*). Briefly, pyrenyl G-actin (0.1 mg mL^{-1}) and unlabeled G-actin (1 mg mL^{-1}) were copolymerized in buffer F. An aliquot of the resultant pyrenyl F-actin was then diluted to a final concentration of 60 μg mL^{-1} in a buffer system identical to that used for activity and binding assays in a quartz cuvette and the fluorescence intensity at 407 nm resulting from excitation at 350 nm was measured as a function of time on a PTI Alphascan spectrofluorometer (Photon Technology International, Inc., South Brunswick, NJ). In a separate experiment, a solution containing pyrenyl G-actin (0.1 mg mL^{-1}) and unlabeled G-actin (1 mg mL^{-1}) was added to an assay buffer containing all components used for the measurements of activity and binding except for Mg^{2+} to give final concentrations of 6 and 60 μg mL^{-1}, respectively. The increase in fluorescence intensity resulting from polymerization to pyrenyl F-actin, initiated by the addition of Mg^{2+} at a level the same as that used in activity and binding experiments (15 mM), was monitored as a function of time.

PKC Activity Associated with F-Actin. The phosphotransferase activity of each PKC isoform was 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 aPKC-ζ activity used a peptide corresponding to the pseudosubstrate region of nPKC-ϵ (ϵ-peptide), in which the single alanine residue was replaced by serine (*43*−*45*). 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 ϵ-peptide, 60 μg mL^{-1} F-actin, and TPA (500 nM), as indicated. After thermal equilibration to 30 °C, assays were initiated by the simultaneous addition of the required PKC isoform (0.3 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. Phosphorylated peptide was determined by scintillation counting.

Binding of PKC Isoforms to F-Actin. The association of PKC isoforms with F-actin was measured on the basis of separation of F-actin-bound from free enzyme by centrifugation. Briefly, the binding assay (200 μL) consisted of 50 mM Tris-HCl (pH 7.40), 15 mM $MgCl_2$, 15 μM ATP, 60 μg mL^{-1} F-actin, and the required PKC isoform (5 nM). Where binding was measured as a function of Ca^{2+} concentration, it was added to the binding assay at a level calculated to yield the required concentration when buffered by 0.1 mM EGTA (*46*). After equilibration for 30 min at 30 °C, the F-actin was pelleted along with bound PKC by centrifugation at 150000*g* for 60 min at 4 °C. The amount of PKC left in the supernatant was quantified by transferring a 10 μL aliquot into a standard lipid vesicle activity assay, which has been described previously (*47*). Briefly, the assay (75 μL)

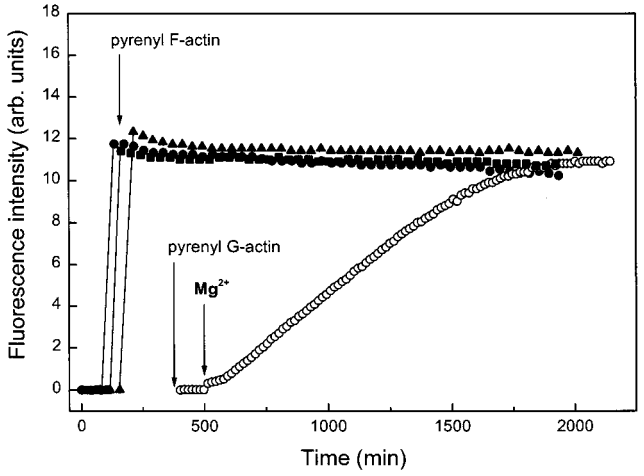

FIGURE 1: Determination of the polymerization state of F-actin under the assay conditions used for measurements of activity and binding. Pyrenyl F-actin, initially prepared by copolymerization of pyrenyl G-actin and unlabeled G-actin (1:10 mol/mol) was added to an assay system identical to those used for assays of activity and binding, as described under Materials and Methods. The pyrene emission fluorescence intensity at 407 nm upon excitation at 350 nm, which resulted from pyrene excimer formation, was measured as a function of time, either in the absence of TPA and Ca^{2+} (●), in the presence of TPA and Ca^{2+} (▲) or with 1 μM phalloidin (■). In a separate experiment, a mixture of pyrenyl G-actin and unlabeled G-actin (1:10 mol/mol) was added to an identical assay system (containing both TPA and Ca^{2+}) but lacking Mg^{2+}. Polymerization of pyrenyl G-actin, initiated by addition of Mg^{2+} (15 mM), was monitored as a function of time by measuring the corresponding increase in pyrene emission fluorescence intensity due to excimer formation (○).

consisted of 0.1 mM $CaCl_2$, 50 μM MBP_{4-14} or ϵ-peptide, large unilamellar vesicles (LUV), 15 mM Mg^{2+}, 15 μM ATP, and 0.3 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) in 50 mM Tris-HCl (pH 7.40). LUV consisting of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and bovine brain phosphatidylserine (BPS) at a 4:1 molar ratio (150 μM) and 0.3 mol % TPA of 100 nm diameter were prepared as described elsewhere (*48*). Assays were performed at 30 °C as described for measurements of F-actin-induced activity.

Determination of SAPD Binding. Phorbol ester binding to cPKC-βI was quantified on the basis of resonance energy transfer (RET) from cPKC-βI tryptophans to the 2-(*N*-methylamino)benzoyl fluorophore of the phorbol ester, SAPD (*38*) Briefly, the fluorescence intensities at the emission maxima of cPKC-βI tryptophans and SAPD (340 and 425 nm, respectively), obtained upon excitation of the tryptophan fluorophore at 290 nm, were determined a PTI Alphascan spectrofluorometer. The assay consisted of 50 mM Tris/HCl (pH 7.40), 0.1 mM EGTA or 0.1 mM $CaCl_2$, 60 μg mL^{-1} F-actin, 15 mM $MgCl_2$, 15 μM ATP, and cPKC-βI (0.1 μM) in a total volume of 2 mL. After incubation for 10 min, SAPD was titrated from Me_2SO stock solutions to give the required concentration. The contribution of RET to the observed signal was isolated by first correcting the observed fluorescence intensities for volume changes incurred in the titration, and second normalizing the intensities for the contribution from the direct excitation of the SAPD fluorophore, according to RET = $(F_{i,+PKC} - F_{i,-PKC}) - (F_{0,+PKC} - F_{0,-PKC})$, where $F_{i,+PKC}$ and $F_{i,-PKC}$ are the fluorescence intensities measured after each SAPD addition, in the presence and absence of cPKC-βI, respectively, and $F_{0,+PKC}$ and $F_{0,-PKC}$ are the fluorescence intensities measured in the absence of SAPD in the presence and absence of cPKC-βI, respectively.

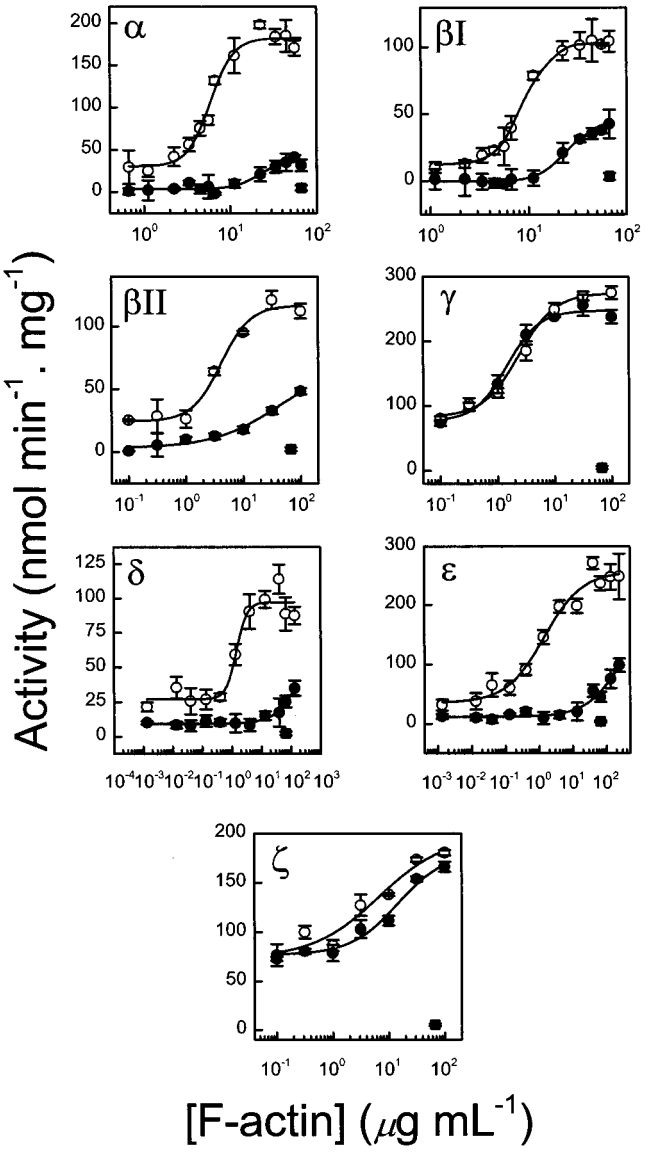

FIGURE 2: Concentration-dependent effects of F-actin. The specific activities of purified recombinant PKC-α, -βI, -βII, -δ, -ϵ, and -ζ were determined as a function of purified F-actin concentration, either in the absence (●) or presence (○) of TPA (1 μM). To verify that F-actin was not a phosphate acceptor for each isoform, activity was measured in the presence of TPA and a fixed concentration of F-actin (60 μg mL^{-1}) but in the absence of either MBP_{4-14} for cPKC or peptide ϵ for nPKC and aPKC (■). Data represent means of triplicate determinations (± SD). The solid curves represent fits of activity data to a modified Hill equation (*51*) by nonlinear regression analysis. See Table 1 for values of $[actin]_{1/2}$ and *n*. Other details are described under Materials and Methods.

RESULTS

To directly determine isozyme-specific effects of phorbol ester and Ca^{2+} on F-actin-induced activity, in vitro activity and binding assays containing purified recombinant PKC isozymes, purified F-actin, a peptide substrate (MBP_{4-14} for cPKC and ϵ-peptide for nPKC and aPKC), and the requisite cofactors and activators were used. The polymerization state of actin is sensitive to ionic strength and also to the presence of Ca^{2+} and Mg^{2+}, both of which affect PKC activity.

Table 1: Summary of the Calculated Values of $[\text{F-actin}]_{1/2}$, $[\text{TPA}]_{1/2}$, and $[Ca^{2+}]_{1/2}$ and for the Corresponding Hill Coefficients (*n*) Determined for F-Actin[a]

isozyme	$[\text{F-actin}]_{1/2}$[b] (μg mL^{-1})	*n*	$[\text{TPA}]_{1/2}$[c] (nM)	*n*	$[Ca^{2+}]_{1/2}$[c] (μM)	*n*
α	5.9 ± 0.6	1.1 ± 0.7	nd[d]	nd	20 ± 50.6 (29 ± 4)	1.8 ± 0.7 (2.1 ± 0.7)
βI	7.1 ± 0.2	1.4 ± 0.3	595 ± 54 (920 ± 495) 80 ± 15[e] (116 ± 57)	1.4 ± 0.2 (1.3 ± 0.3) 1.1 ± 0.2[e] (0.7 ± 0.1)[e]	25 ± 2.1 (32 ± 6)	1.2 ± 0.2) (1.4 ± 0.3)
βII	6.1 ± 0.9	1.7 ± 0.5	517 ± 82 105 ± 20[e]	1.5 ± 0.3 1.1 ± 0.2[e]	nd	nd
γ	1.4 ± 0.2[f] 1.5 ± 0.3	1.9 ± 1.2[f] 1.3 ± 0.8	ne[g]	ne	ne	ne
δ	1.4 ± 0.3	2.4 ± 1.5	34 ± 9 (21 ± 8)	1.2 ± 0.3 (0.9 ± 0.2)	ne	ne
ϵ	1.4 ± 0.5	0.8 ± 0.2	3.8 ± 0.7 (2.8 ± 1.7) 653 ± 56[h]	1.5 ± 0.5 (1.2 ± 0.6) 1.1 ± 0.6[h]	ne	ne
ζ	7 ± 8[f] 14 ± 8	0.8 ± 0.6[f] 1.0 ± 0.4	ne	ne	ne	ne

[a] TPA and Ca^{2+} concentration dependences for activation and binding are shown in parentheses. [b] Values of $[\text{F-actin}]_{1/2}$ and *n* were calculated by fitting F-actin concentration−response curves obtained in the presence of TPA (1 μM) to a modified Hill equation. [c] Values of $[\text{TPA}]_{1/2}$ and $[Ca^{2+}]_{1/2}$ were calculated in a similar manner from curves obtained in the presence of a fixed concentration of F-actin (60 μg mL^{-1}). [d] Not determined. [e] Determined in the presence of 0.1 mM Ca^{2+}. [f] Determined in the absence of TPA. [g] No effect. [h] Values of $[\text{PDBU}]_{1/2}$ obtained from the PDBu concentration−response curve for F-actin-induced activity.

Therefore, initial experiments were undertaken to verify that the F-actin preparation used was stable under the assay conditions used (Figure 1). To achieve this, pyrenyl G-actin was initially polymerized to form pyrene-labeled F-actin, which leads to an increase in fluorescence intensity due to the close proximity of pyrene fluorophores and the resultant formation of pyrene excimers (*42*). This fluorescence signal was found to be a constant function of time under all assay conditions used and was close in level to that observed in the presence of an excess concentration of phalloidin (1 μM), which fully stabilizes the filamentous form of actin (*49*). Polymerization of pyrenyl G-actin induced under PKC assay conditions by 15 mM Mg^{2+} was observed as an increase in fluorescence intensity which reached a maximum level that was similar to that observed in the presence of phalloidin (Figure 1).

Concentration Dependent Effects of F-Actin on PKC Activity. The F-actin concentration−response curves for the activation of PKC-α, -βI, -βII, -γ, -δ, -ϵ, and -ζ in the presence and absence of TPA are shown in Figure 2. It was found that the activities of each of the PKC isoforms, determined in the absence of phospholipid under standard conditions, was enhanced in the presence of F-actin. For cPKC-α, -βI, and -βII, increasing the level of F-actin in the absence of TPA resulted in a relatively small increase in the level of activity, which failed to reach saturation at the highest concentration of F-actin used. However, the addition of TPA (1 μM) resulted in a marked decrease in the concentration of F-actin required to induce activity of these PKC isoforms and also a slight increase in the level of activity obtained at low F-actin concentrations. Fitting the activity data for cPKC-α, -βI, and -βII to the Hill equation yielded values of F-actin concentration required to induce a half-maximal increase in the level of activity ($[\text{F-actin}]_{1/2}$) and Hill coefficients (*n*) that were similar for each of these isoforms (see Table 1). Importantly, the level of F-actin required to induce maximal cPKC-γ activity in the absence of TPA was less than that required for the activation of cPKC-α, -βI, and -βII. Thus, the value of $[\text{F-actin}]_{1/2}$ for F-actin-induced cPKC-γ activity determined in the *absence* of TPA was ∼5-fold less than those determined for cPKC-α, -βI, and -βII in the *presence* of TPA (Table 1). By contrast with the other cPKC isoforms, the concentration dependence of F-actin-induced cPKC-γ activity was unaffected by TPA, suggesting that the interaction of this isoform with F-actin alone may be sufficient to induce a maximal level of activity.

The activities of both nPKC-δ and -ϵ were also enhanced by F-actin, TPA addition resulting in a large decrease in the concentration of F-actin required for maximal activation. The values of $[\text{F-actin}]_{1/2}$ obtained for the nPKC isoforms in the presence of TPA were ∼5-fold less than those determined for the cPKC-α, -βI, and -βII, and were similar to the value obtained for cPKC-γ (Table 1). The values of $[\text{F-actin}]_{1/2}$ obtained for aPKC-ζ was similar to that obtained for cPKC-α, -βI, and -βII but was unaffected by the presence of TPA, which is consistent with the reported inability of this isoform to bind phorbol esters (*50*). The values of *n* determined from data obtained in the presence of TPA were ∼1 for cPKC-α, -βI, -βII, -γ, -δ, -ϵ, and -ζ, indicating a lack of cooperativity in the interaction of all of these isoforms with F-actin (Table 1). Finally, the level of activity of each isoform determined in the presence of a maximally activating concentration of F-actin, but in the absence of peptide substrates, was negligible under all assay conditions used, ruling out the possibility that the observed activity was due to F-actin itself being a phosphate acceptor (Figure 2, ■).

Phorbol Ester Concentration Dependence of F-Actin Association and Activation. The concentration-dependence curves shown in Figure 3A for the enhancement of F-actin-induced PKC isozyme activity by TPA were determined in the presence of a fixed concentration of F-actin with and without Ca^{2+} (0.1 mM). The data reveal marked differences in the potencies of the enhancing effect of TPA on F-actin-induced activity of the PKC isoforms. Thus, in the absence of Ca^{2+}, a similar concentration of TPA was required to induce a half-maximal increase in activity ($[\text{TPA}]_{1/2}$) for cPKC-βI and -βII (Table 1), whereas a maximal level of cPKC-α activity was not attained, even at a very high concentration of the phorbol ester (10 μM). Whereas this result suggests a relatively low-affinity interaction of TPA with cPKC-α, it was also found that the level of activity was unaffected by the "inactive" epimer of TPA, 4α-TPA (Figure 3A, □), indicating that the effect of TPA was stereospecific. By contrast with the other cPKC isoforms,

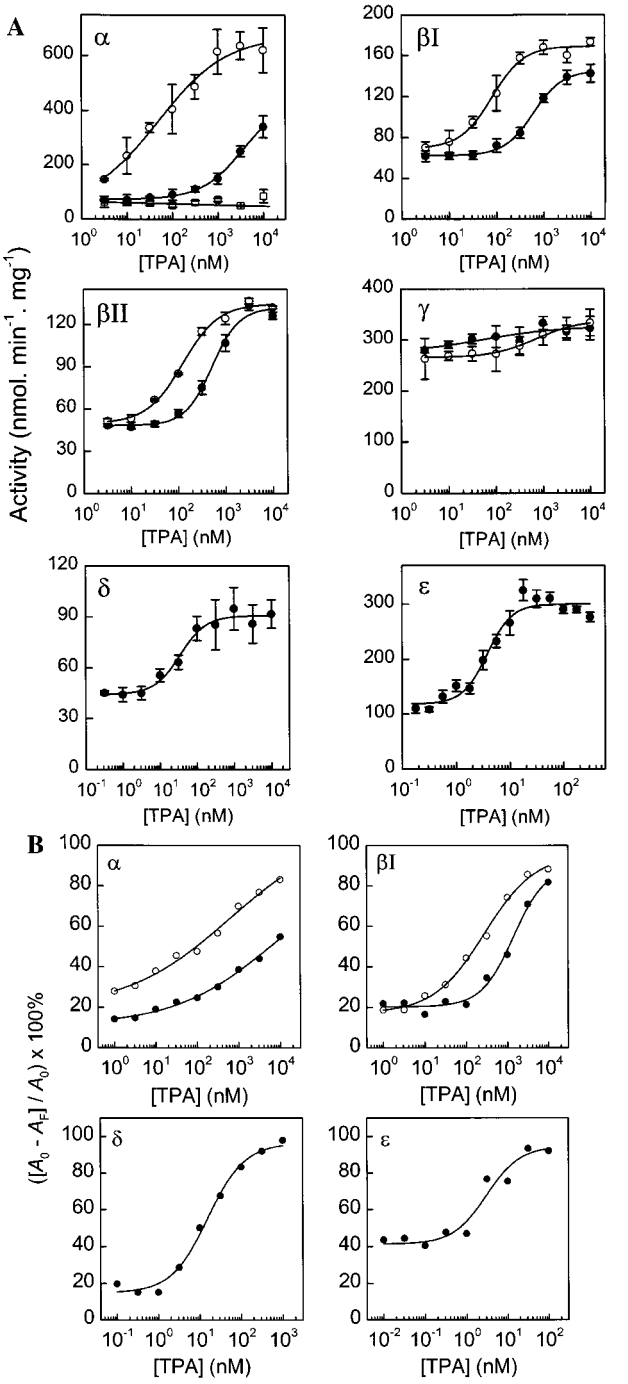

FIGURE 3: Phorbol ester concentration-dependent effects on F-actin-induced PKC isoform activities and F-actin binding. (A) PKC-α, -β$_I$, -β$_{II}$, -δ, -ϵ, and -ζ activities were determined in the presence of a fixed level of F-actin (60 μg mL^{-1}) as a function of TPA concentration, either in the absence (●) or presence (○) of 100 μM Ca^{2+} for the cPKC isoforms. Also shown for cPKC-α is the concentration dependence for the effects of 4α-TPA on F-actin induced activity (□). (B) The level of binding of cPKC-α and -β$_I$ and nPKC-δ and -ϵ to F-actin was measured under identical conditions used above for activity determinations as a function of TPA concentration, either in the absence (●) or presence (○) of 100 μM Ca^{2+} for the cPKC isoforms. Data were normalized according to $([A_0 - A_F]/A_0) \times 100$, where A_0 is the activity of each isoform obtained under identical assay conditions but in the absence of F-actin and A_F is the activities of free PKC remaining in the supernatant after centrifugal separation of F-actin. Data are representative of triplicate determinations and the solid curves represent fits of data to a modified Hill equation (*51*) by nonlinear regression analysis. See Table 1 for calculated values of $[TPA]_{1/2}$ and *n* and Materials and Methods for further details.

the activity of cPKC-γ induced by F-actin was found to be unaffected within a TPA concentration range spanning 4 orders of magnitude, again suggesting that interaction with F-actin alone is sufficient to induce maximal activation of this isoform. The TPA concentration range required to induce nPKC activity was much reduced compared to that required for cPKC-α, -β$_I$, and -β$_{II}$ activity. Thus, the values of $[TPA]_{1/2}$ for nPKC-δ and -ϵ were ∼10-fold and 500-fold less than those determined for cPKC-β$_I$ and -β$_{II}$, respectively, indicating that these isoforms may contain a relatively high-affinity phorbol ester binding site when F-actin-associated (Table 1). The values of *n* calculated for each isoform were each ∼1, suggesting a lack of cooperativity in F-actin-induced activity with respect to phorbol ester binding.

Since the cPKC isoforms each contain Ca^{2+}-binding sites within a common C2 domain that mediates in the association of these isoforms with membranes and in the conformational changes that lead to activation (*51*), the question arises whether activity induced by F-actin may also be Ca^{2+}-dependent. The presence of Ca^{2+} (0.1 mM) resulted in a marked decrease in the TPA concentration requirements for cPKC-α, -β$_I$, and -β$_{II}$ activity induced by F-actin, as shown in Figure 3A. Thus, for cPKC-β$_I$ and -β$_{II}$, the effect of Ca^{2+} was to decrease the value of $[TPA]_{1/2}$ for TPA-induced activation by ∼1 order of magnitude, whereas the maximum level of TPA-induced activity was unaffected (Table 1). The effect of Ca^{2+} may therefore be to reduce the concentration of TPA required to induce binding to F-actin and/or to induce an activating conformational change. By contrast with cPKC-α, -β$_I$, and -β$_{II}$, the level of cPKC-γ activity induced by F-actin was unaffected by the presence of Ca^{2+} (Figure 3A). The values of *n* obtained for the interaction of TPA with cPKC-β$_I$ and -β$_{II}$ and nPKC-δ and -ϵ were each ∼1 in both the presence and absence of Ca^{2+}, again suggesting a lack of cooperativity in F-actin binding with respect to TPA binding (Table 1).

The concentration-dependent effects of TPA on the binding of PKC isoforms to F-actin, determined under conditions identical to those used for assays of activity in the presence and absence of Ca^{2+}, are shown in Figure 3B. The values of $[TPA]_{1/2}$ determined from the concentration−response curves for TPA-induced binding of PKC-α, -β$_I$, -δ, and -ϵ to F-actin were found to correspond closely with those obtained for TPA-induced activity (Table 1). In the case of the cPKC isoforms, the presence of Ca^{2+} (0.1 mM) resulted in a decrease in the TPA concentration requirement for binding, which again corresponded closely to the effects of Ca^{2+} on the TPA concentration dependencies for F-actin-induced activity (Table 1). These results indicate that the TPA- and Ca^{2+}-induced association of these isoforms with F-actin results in an activating conformational change. To confirm that PKC isoforms interact directly with F-actin in a phorbol ester- and Ca^{2+}-dependent manner, the "amount" of PKC isoforms remaining in the supernatant after centrifugation was measured by SDS−PAGE followed by Western blots instead of specific activities. Consistent with the results shown in Figure 3B, it was found that for both cPKC-α and cPKC-β$_I$ the presence of TPA led to a decrease in the density of the band corresponding to each isoform relative to that obtained in the presence of F-actin alone, and that this effect was enhanced by Ca^{2+} (results not shown).

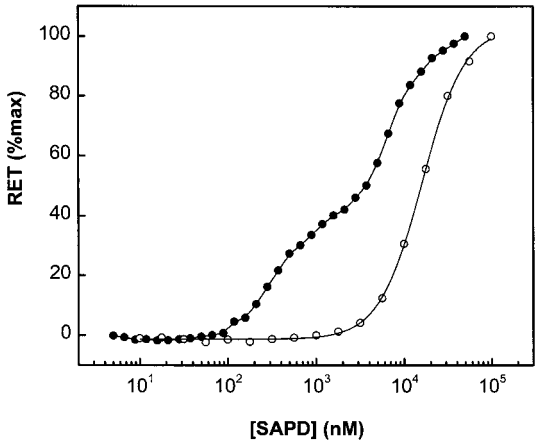

FIGURE 4: Effect of F-actin on the binding of phorbol ester to cPKC-βII. Binding of the fluorescent phorbol ester, SAPD was quantified by measuring RET between the tryptophans of cPKC-βII and the SAPD fluorophore (*38*), as a function of SAPD concentration, either in the absence (solid symbols) or presence (open symbols) of F-actin (60 μg mL^{-1}). Data were normalized to the maximal level of RET obtained in the presence and absence of F-actin, respectively, and are representative of triplicate determinations. Other details are as described under Materials and Methods.

Phorbol Ester Binding to cPKC-βI Associated with F-Actin. The effects of the interaction of PKC with F-actin on phorbol ester binding was determined by a previously described assay based on measurements of fluorescence resonance energy transfer between the tryptophans of this isozyme and the fluorophore of the phorbol ester SAPD (*38*). Since initial experiments indicated that cPKC-βI and -βII were the only isoforms tested that were significantly activated by SAPD within the concentration range used for binding assays (results not shown), cPKC-βI was chosen as a representative isoform for this measurement. The binding isotherm obtained for the interaction of SAPD with cPKC-βI in the absence of F-actin was found to be "dual sigmoidal" (Figure 4). This indicates the existence of two phorbol ester binding sites of low and high affinity, respectively, on the soluble form of cPKC-βI, as reported previously for both the soluble and membrane-associated forms of cPKC-α (*38*, *39*). The affinities of both these interactions was markedly reduced by the inclusion of F-actin in the binding assay and resulted in a monophasic binding isotherm, consistent with the presence of a single phorbol ester binding site on the F-actin associated form of cPKC-βI.

Ca^{2+} Concentration Dependence of F-Actin Association and Activation. The F-actin-induced activities of cPKC-α and -βI, chosen as representative isoforms, were measured in the presence (○) and absence (●) of a fixed concentration of TPA (1 μM) and F-actin, as shown in Figure 5A. It was found that in the absence of TPA the activities of cPKC-α and -βI induced by F-actin were slightly enhanced by high levels of Ca^{2+}; however, the presence of TPA resulted in a marked decrease in the Ca^{2+}-concentration requirements for F-actin-induced activation of both cPKC isoforms. The Ca^{2+} concentrations required for a half-maximal increase in activity ($[Ca^{2+}]_{1/2}$) and *n*, obtained for cPKC-α and -βI from fits of the corresponding Ca^{2+} concentration−response data, were found to be similar (Table 1). By contrast with the cPKC isoforms, the level of nPKC-δ and -ϵ activity induced by F-actin, measured either in the presence or absence of TPA, was independent of Ca^{2+} concentration, in keeping with a lack of a functional Ca^{2+} binding site within the C2 domains of these isoforms (*52*).

Measurements of the Ca^{2+} dependence of the association of PKC-α, -βI, -δ, and -ϵ with F-actin under conditions identical to those used in activity assays yielded concentration−response curves that were similar to those obtained for Ca^{2+}-induced activity (Figure 5B). Thus, for the cPKC

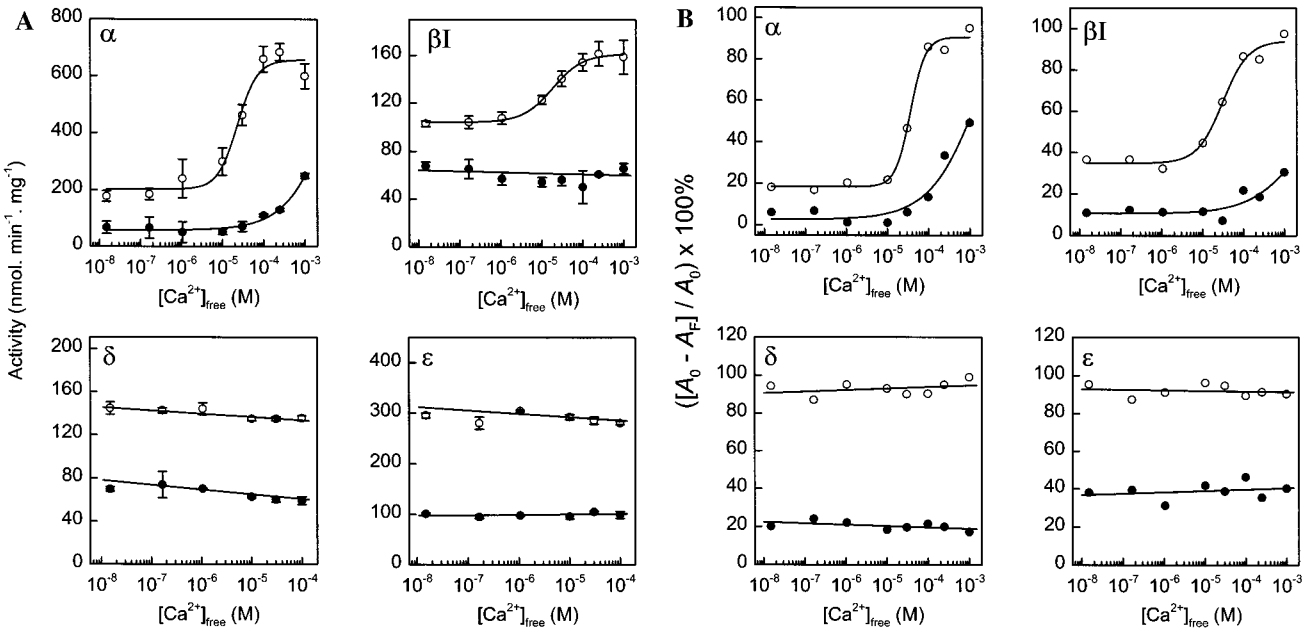

FIGURE 5: Concentration dependence of the effects of Ca^{2+} on PKC isoform activity induced by F-actin. (A) PKC-α, -βI, -δ, and -ϵ activities were measured in the presence of a fixed level of F-actin (60 μg mL^{-1}) as a function of Ca^{2+} concentration, either in the presence (○) or absence (●) of TPA (1 μM). (B) The level of binding of cPKC-α and -βI and nPKC-δ and -ϵ to F-actin was measured under the same conditions used above for activity determinations as a function of Ca^{2+} concentration, either in the absence (●) or presence (○) of TPA (1 μM). Data were normalized as described in the legend to Figure 3B and are representative of triplicate determinations. The solid curves represent fits of data to a modified Hill equation (*51*) by nonlinear regression analysis, which yielded values of $[Ca^{2+}]_{1/2}$ and *n* listed in Table 1. See Materials and Methods for other details.

isoforms a low level of binding was observed at high Ca^{2+} concentrations in the absence of TPA and the Ca^{2+} concentration requirements for this interaction were reduced markedly in the presence of TPA (Table 1), whereas the interaction of the nPKC isoforms with F-actin was independent of Ca^{2+}-concentration.

DISCUSSION

In this study the isoform specificity and cofactor requirements of the interaction of PKC with F-actin were determined. The results indicate that the seven PKC isoforms bound to F-actin with varying affinities and that these interactions resulted in an enhanced level of isozyme activity. The interaction of cPKC-α, -βI, and -βII isoforms with F-actin and the ensuing level of activity were significantly enhanced by either phorbol ester or Ca^{2+} alone and synergistically enhanced by these activators in combination, whereas a maximal level of binding and activation of the nPKC isoforms required only phorbol ester, and aPKC-ζ required neither phorbol ester nor Ca^{2+}. By contrast with the other cPKC isoforms, cPKC-γ required neither phorbol ester nor Ca^{2+} for maximal F-actin induced activity.

Several recent studies have shown that individual isoforms colocalize with the F-actin cytoskeleton as a response to an external stimuli, which is likely to be an important event in the subcellular compartmentalization of the enzyme and the isozyme-specific regulation of associated cellular processes (*12*, *25*, *27*−*34*). The question whether there is PKC isoform selectivity at the level of an interaction with the actin filament itself has been examined in two previous studies. In one of these studies it was shown that cPKC-βII could be coimmunoprecipitated with F-actin from MOLT-4 cells, whereas cPKC-βI apparently failed to do so despite the fact that these splice variants differ only in the carboxyl-terminal 50 amino acids (*31*). This isozyme-specific interaction of cPKC-βII with F-actin was also demonstrated in an in vitro assay using purified PKC isoforms and F-actin. This difference from the results of the present study, which showed that both cPKC-βI and cPKC-βII were activated by F-actin in a phorbol ester- and Ca^{2+}-dependent manner, may arise from differences in experimental conditions. For example, the activity and binding assays used in the previous study contained lipid vesicles composed of PS and diacylglycerol, which were excluded from assays in the present study. This would tend to complicate the interpretation of results since the PKC isoforms would be expected to interact with both vesicles and F-actin. Also, it has been shown that F-actin itself may interact with negatively charged phospholipids such as PS, which may also interfere with the interaction of PKC isoforms with F-actin (*53*). Consistent with this, we have found that the level of activity of nPKC-ε induced in the presence of F-actin together with phospholipid vesicles containing PS was dramatically reduced compared to that induced by either F-actin or vesicles alone (F. J. Taddeo, S. J. Slater, and C. D. Stubbs, unpublished observation). A further complication arises from the finding that F-actin itself may be a substrate for PKC in the presence of PS, which may therefore compete for peptide−substrate phosphorylation (*31*, *54*).

Recently, evidence for the existence of an actin-binding motif within the C1 domain of nPKC-ε that is unique to this isoform was presented, which was suggested to mediate in a phorbol ester-dependent interaction with F-actin specific to this isoform (*30*, *33*). The binding of other PKC isoforms to F-actin was found to occur to a negligible extent; an observation that is at variance with the findings of the present study, which indicate that each isoform may interact with F-actin and also those that report binding of cPKC-βII (*31*) and aPKC-ζ (*29*). These observed differences in the effects of F-actin on PKC isoform activities again likely result from differences in the assay conditions used. For example, the previous study distinguished nPKC-ε from the other isoforms on the basis that this isoform was the only one to bind F-actin as a response to phorbol 12,13-dibutyrate (PDBu). The data shown here (see Table 1) indicate that replacing PDBu with TPA results in a >100-fold decrease in the phorbol ester concentration requirements for nPKC-ε activity, consistent with the phorbol ester binding site on the F-actin-associated enzyme being hydrophobic (*39*). The contrasting observations that F-actin appears to bind only nPKC-ε in the presence of PDBu (*33*), while binding multiple isoforms in the presence of TPA as shown in the present study, may therefore reflect the reduced affinity of each PKC isoform for PDBu binding compared to TPA. Furthermore, the present results indicate that the differences in observations may also reflect the increased binding affinity of nPKC-ε for F-actin and TPA compared to the other PKC isoforms, as indicated by the relatively low values of $[\text{F-actin}]_{1/2}$ and $[\text{TPA}]_{1/2}$ (Table 1).

From the SAPD binding isotherms shown in Figure 4 for cPKC-βI, the interaction with F-actin results in a marked decrease in phorbol ester binding affinity relative to that observed for the free isozyme. In addition, the observation that the SAPD binding isotherm obtained for cPKC-βI in the presence of F-actin was monophasic, contrasting with the "dual sigmoidal" curve obtained in the absence of F-actin, suggesting that only one of the two phorbol ester binding sites present on the soluble form of this isozyme is retained/utilized upon interaction with F-actin. Supporting this was the observation that the TPA concentration−response curve for F-actin-induced cPKC-βI activity was also monophasic, as was also the case for cPKC-βII and nPKC-δ and -ε, suggesting that the activation of each of these isoforms is also mediated by a single phorbol ester binding site. Further, the observation that the values of $[\text{TPA}]_{1/2}$ obtained for TPA-induced activation of nPKC-δ and -ε were much lower relative to those obtained for cPKC-βI and -βII isoforms suggests that this site on the F-actin-associated nPKC isoforms is of relatively high affinity. The lack of a phorbol ester dependence of F-actin-associated cPKC-γ activity suggests that either phorbol esters bind "nonproductively" in a manner that does not result in an activating conformational change or that interaction with the site(s) may be hindered by the interaction with F-actin. Thus, in the case of cPKC-γ it appears that F-actin binding alone is sufficient to induce optimal activation, as for aPKC-ζ.

The C2 domain appears to play an important role in the interaction of cPKC-α, -βI and -βII with F-actin. The observation that the Ca^{2+} concentration requirements for F-actin-induced activity of cPKC-α, -βI, and -βII obtained in the presence of TPA were similar to those previously observed for activity induced by association with membranes containing TPA (*51*), suggests that similar Ca^{2+}-binding site(s) within the C2 domains of these isoforms may be

involved in both cases. Also, the interaction of Ca^{2+} with the C2 domains and TPA with the C1 domains of cPKC-α, -βI, and -βII appears to result in a synergistic enhancement of the level of F-actin binding and activity, which is reminiscent of the "cooperative" effect of these two domains on membrane-binding affinity and associated activity (*4*, *7*, *55*). By contrast, it would appear that interaction with the C2 domain of cPKC-γ is not necessary for the F-actin-induced activity of this isoform, even though this domain is required for recruiting the isozyme to the membrane.

It was shown previously that the association of cPKC with membranes and the ensuing activation may be accompanied by distinct Ca^{2+}-induced conformational changes, the former resulting in the exposure of the V3 hinge region and the carboxyl-terminal end of the PKC molecule and the latter in the removal of the pseudosubstrate from the substrate binding site (*51*, *56*, *57*). More recently, evidence was presented that cPKC-α in particular may associate with membranes at Ca^{2+} levels markedly lower than those required for activation, whereas the cPKC-βI, -βII, and -γ isoforms appeared to share similar Ca^{2+} requirements for membrane association and activation (*51*). By contrast, the present observation that the levels of TPA and Ca^{2+} required to induce binding of cPKC-α to F-actin were similar to those required to induce activity suggests that in this case the activating conformational change and F-actin association occur within similar phorbol ester and Ca^{2+} concentration ranges. Whether this conformational change also results in altered proteolytic sensitivity of each of the PKC isoforms remains to be determined, although evidence that this may occur was provided by recent studies, which showed that the association of nPKC-ε and cPKC-βII with F-actin resulted in a decreased susceptibility to proteolysis (*31*, *33*).

In conclusion, the results indicate that the interaction with F-actin may be a general property of all PKC isoforms. The specificity of this interaction for a particular isoform may be determined by the presence or absence of functional C1 and C2 domains in each particular isozyme type and the differing affinities of the respective phorbol ester- and Ca^{2+}-binding sites, in addition to the specific F-actin binding motifs in nPKC-ε and cPKC-βII (*30*, *58*). The similar Ca^{2+}- and phorbol ester dependencies of the interaction of PKC isoforms with F-actin to those observed for membrane-associated PKC suggests that the interaction with F-actin serves partly to localize the enzyme and to facilitate the activating conformational change. In this regard, it has been shown that the surface of the F-actin contains regions of high negative charge with which PKC isoforms may associate (e.g., ref *59*), probably with some degree of specificity as indicated by divergent cofactor and activator requirements. Finally, the observation that the cPKC isoforms may translocate to F-actin as well as the membrane as a response to a Ca^{2+} transient may allow for the functional coupling of fluctuations of intracellular Ca^{2+} levels to the PKC-mediated remodeling of the F-actin cytoskeleton and as such may have an important role in, for example, secretion, cell morphology changes and motility.

REFERENCES

1. Vaughan, P. F., Walker, J. H., and Peers, C. (1998) *Mol. Neurobiol. 18*, 125−155.
2. Toker, A. (1998) *Frontiers Biosci. 3*, D1134−1147.
3. Mellor, H., and Parker, P. J. (1998) *Biochem. J. 332*, 281−292.
4. Newton, A. C. (1997) *Curr. Opin. Cell Biol. 9*, 161−167.
5. Blobe, G. C., Stribling, S., Obeid, L. M., and Hannun, Y. A. (1996) *Cancer Surveys 27*, 213−248.
6. Nishizuka, Y. (1995) *FASEB J. 9*, 484−496.
7. Newton, A. C., and Johnson, J. E. (1998) *Biochim. Biophys. Acta 1376*, 155−172.
8. Hofmann, J. (1997) *FASEB J. 11*, 649−669.
9. Mochly-Rosen, D. (1995) *Science 268*, 247−251.
10. Dekker, L. V., and Parker, P. J. (1994) *Trends Biochem. Sci. 19*, 73−77.
11. Jaken, S. (1996) *Curr. Opin. Cell Biol. 8*, 168−173.
12. Goodnight, J. A., Mischak, H., Kolch, W., and Mushinski, J. F. (1995) *J. Biol. Chem. 270*, 9991−10001.
13. Hyatt, S. L., Liao, L., Chapline, C., and Jaken, S. (1994) *Biochemistry 33*, 1223−1228.
14. Mochly-Rosen, D., Khaner, H., and Lopez, J. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 3997−4000.
15. Mochly-Rosen, D., Khaner, H., Lopez, J., and Smith, B. L. (1991) *J. Biol. Chem. 266*, 14866−14868.
16. Lehel, C., Olah, Z., Iakab, G., Szallasi, Z., Petrovics, G., Harta, G., Blumberg, P. M., and Anderson, W. B. (1995) *J. Biol. Chem. 270*, 19651−19658.
17. Lehel, C., Olah, Z., Jakab, G., and Anderson, W. B. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 1406−1410.
18. Yau, L., Suzuki, H., Ozawa, K., Deng, J., Lehel, C., Fukamachi, H., Anderson, W., Kawakami, Y., and Kawakami, T. (1997) *J. Biol. Chem. 272*, 13033−13039.
19. Klaunck, T. M., Faux, M. C., Labudda, K., Langeberg, L. K., Jaken, S., and Scott, J. D. (1996) *Science 271*, 1589−1592.
20. Faux, M. C., and Scott, J. D. (1997) *J. Biol. Chem. 272*, 17038−17044.
21. Nauert, J. B., Klauck, T. M., Langeberg, L. K., and Scott, J. D. (1997) *Curr. Biol. 7*, 52−62.
22. Keenan, C., and Kelleher, D. (1998) *Cell. Signal. 10*, 225−232.
23. Chapline, C., Ramsey, K., Klauck, T., and Jaken, S. (1993) *J. Biol. Chem. 268*, 6858−6861.
24. Jaken, S., Leach, K., and Klauck, T. (1989) *J. Cell Biol. 109*, 697−704.
25. Allen, L. H., and Aderem, A. (1995) *J. Exp. Med. 182*, 829−840.
26. Gregario, C. C., Repasky, E. A., Fowler, V. M., and Black, J. D. (1994) *J. Cell Biol. 125*, 345−358.
27. Zalewski, P. D., Forbes, I. J., Giannakis, C., Cowled, P. A., and Betts, W. H. (1990) *FEBS Lett. 273*, 131−134.
28. Zalewski, P. D., Forbes, I. J., Giannakis, C., and Betts, W. H. (1991) *Biochem. Int. 24*, 1103−1110.
29. Gomez, J., Martinez de Aragon, A., Bonay, P., Pitton, C., Garcia, A., Silva, A., Fresno, M., Alvarez, F., and Rebollo, A. (1995) *Eur. J. Immunol. 25*, 2673−2678.
30. Prekeris, R., Mayhew, M. W., Cooper, J. B., and Terrian, D. M. (1996) *J. Cell Biol. 132*, 77−90.
31. Blobe, G. C., Stribling, D. S., Fabbro, D., Stabel, S., and Hannun, Y. A. (1996) *J. Biol. Chem. 271*, 15823−15830.
32. Hu, Y. L., and Chien, S. (1997) *J. Histochem. Cytochem. 45*, 237−249.
33. Prekeris, R., Hernandez, R. M., Mayhew, M. W., White, M. K., and Terrian, D. M. (1998) *J. Biol. Chem. 273*, 26790−26798.
34. Nakhost, A., Forscher, P., and Sossin, W. (1998) *J. Neurochem. 71*, 1221−1231.
35. Niggli, V., Djafarzadeh, S., and Keller, H. (1999) *Exp. Cell Res. 250*, 558−568.
36. Edwards, A. S., Faux, M. C., Scott, J. D., and Newton, A. C. (1999) *J. Biol. Chem. 274*, 6461−6468.
37. Stabel, S., Schaap, D., and Parker, P. J. (1991) *Methods Enzymol. 200*, 670−673.
38. Slater, S. J., Ho, C., Kelly, M. B., Larkin, J. D., Taddeo, F. J., Yeager, M. D., and Stubbs, C. D. (1996) *J. Biol. Chem. 271*, 4627−4631.

39. Slater, S. J., Taddeo, F. J., Mazurek, A., Stagliano, B., Milano, S., Kelly, M. B., and Stubbs, C. D. (1998) *J. Biol. Chem. 273*, 23160−23168.
40. Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K., and Nishizuka, Y. (1988) *J. Biol. Chem. 263*, 6927−6932.
41. Pardee, J. D., and Spudich, J. A. (1982) *Methods Enzymol. 85*, 164−181.
42. Kouyama, T., and Mihashi, K. (1981) *Eur. J. Biochem. 114*, 33−38.
43. Ohno, S., Akita, Y., Konno, Y., Imajoh, S., and Suzuki, K. (1988) *Cell 53*, 731−741.
44. House, C., and Kemp, B. E. (1987) *Science 238*, 1726−1728.
45. Saido, T. C., Mizuno, K., Konno, Y., Osada, S. I., Ohno, S., and Suzuki, K. (1992) *Biochemistry 31*, 482−490.
46. Fabriato, A., and Fabriato, F. (1979) *J. Physiol. (London) 75*, 463−505.
47. Slater, S. J., Kelly, M. B., Taddeo, F. J., Rubin, E., and Stubbs, C. D. (1994) *J. Biol. Chem. 269*, 17160−17165.
48. MacDonald, R. C., MacDonald, R. I., Menco, B. P., Takeshita, K., Subbarao, N. K., and Hu, L. R. (1991) *Biochim. Biophys. Acta 1061*, 297−303.
49. Estes, J. E., Seldon, L. A., and Gershman, L. C. (1981) *Biochemistry 20*, 708−712.
50. Ways, D. K., Cook, P. P., Webster, C., and Parker, P. J. (1992) *J. Biol. Chem. 267*, 4799−4805.
51. Slater, S. J., Milano, S. K., Stagliano, B. A., Gergich, K. J., Ho, C., Mazurek, A., Taddeo, F. J., Kelly, M. B., Yeager, M. D., and Stubbs, C. D. (1999) *Biochemistry 38*, 3804−3815.
52. Newton, A. C. (1995) *J. Biol. Chem. 270*, 28495−28498.
53. Bouchard, M., Pare, C., Dutasta, J.-P., Chauvat, J.-P., Gicquaud, C., and Auger, M. (1998) *Biochemistry 37*, 3149−3155.
54. Reiss, N., Oplatka, A., Hermon, J., and Naor, Z. (1996) *Biochem. Mol. Biol. Int. 40*, 1191−1200.
55. Mosior, M., and Newton, A. C. (1996) *Biochemistry 35*, 1612−1623.
56. Orr, J. W., and Newton, A. C. (1994) *J. Biol. Chem. 269*, 8383−8387.
57. Keranen, L. M., and Newton, A. C. (1997) *J. Biol. Chem. 272*, 25959−25967.
58. Blobe, G. C., Stribling, D. S., Fabbro, D., Stabel, S., and Hannun, Y. A. (1996) *J. Biol. Chem. 271*, 15823−15830.
59. Yamamoto, T., Nakayama, S., Kobayashi, N., Munekata, E., and Ando, T. (1994) *J. Mol. Biol. 214*, 714−731.

BI9916527