Identification of the Phosphatidylserine Binding Site in the C2 Domain that Is Important for PKCα Activation and in Vivo Cell Localization†

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ABSTRACT: The C2 domain of classical PKCs binds to membranes through Ca^{2+} bridging to phosphatidylserine as recently observed through X-ray diffraction of the isolated domain. Additionally, it has been proposed that N189, T251, R216, and R249A interact directly with phosphatidylserine [Verdaguer, N., et al. (1999) *EMBO J. 18*, 6329−6338]. When these four residues were mutated to Ala to determine their role in PKC binding to phospholipid membranes, PKC activation, and in its in vivo localization, the results revealed that they were very important for the activation of full-length PKCα. N189, in particular, was involved in the activation of the enzyme after its interaction with PS, since its mutation to Ala did not decrease the level of membrane binding but did prevent full enzyme activation. On the other hand, mutations R216A, R249A, and T251A affected both membrane binding and enzyme activation, although T251A had the most drastic effect, suggesting that the protein interactions with the carbonyl groups of the phospholipid are also a key event in the activation process. Taken together, these results show that the four residues located near the calcium binding site are critical in phosphatidylserine-dependent PKCα activation, in which N189 plays an important role, triggering the enzyme activation probably by interacting with neighboring residues of the protein when lipid binding occurs. Furthermore, these results provide strong evidence for better defining one of the two phosphatidylserine isomer models proposed in the previous crystallographic report.

PKCs[1] in mammalian cells consist of at least 11 closely related isoenzymes, which, in general, contain four conserved domains named C1−C4 (*1*). PKCs consist of a single polypeptide chain that contains a regulatory region at the amino terminus and a kinase domain at the carboxyl terminus (*2*, *3*). According to their structure and cofactor regulation, PKCs can be classified into three groups. The first group, which includes the classical isoforms (α, βI, βII, and γ), can be distinguished from the other groups because its functioning is regulated by diacylglycerol (DAG) and also cooperatively by calcium and acidic phospholipids, particularly phosphatidylserine (PS). Members of the second group are the novel mammalian (δ, ϵ, η, and θ) and the yeast PKCs which are not regulated by calcium. The third group comprises the atypical PKC isoforms (ζ, λ, and μ) which are regulated neither by DAG nor by calcium (*4*, *5*).

Structurally, their regulatory domain contains two membrane-targeting motifs, namely, the C1 domain, which is present in all isozymes, including the atypical, and C2 domain, which is present in classical and novel PKCs (*3*). The C1 domain binds diacylglycerol and phorbol esters in all but the atypical PKCs. This domain is present as a tandem repeat in most PKCs and has been named as C1a and C1b (*6*). The C2 domain appears in both the classical and novel isozymes, where it is responsible for binding acidic phospholipids (in both isozymes) and Ca^{2+} (only in classical isozymes). Furthermore, the orientation of the C1 and C2 domains differs in both classes of isozyme, novel PKCs containing a C2 domain located at the amino-terminal region in the regulatory domain (also named the V1/0 region) and classical PKCs containing a C2 domain at the carboxy-terminal region of the regulatory domain and in the opposite direction (*7*).

In the classical PKCα isoenzyme, the Ca^{2+}-dependent binding to membranes shows a high specificity for 1,2-*sn*-phosphatidyl-L-serine (*8*−*10*). Thus, it has been demonstrated that amino and carboxyl groups within L-serine are required stereospecifically for activation and that interfacial conformation appears to be critical to the activation process (*8*).

To date, many important features of PS specificity have been described, although the residues which are responsible for binding PS and the subsequent activation of the enzyme have not been established. Several studies involving the full-length protein and isolated C2 domain have suggested that PS binding is mediated by the C2 domain both in vitro and in vivo (*11*−*13*). Furthermore, a crystallographic study on the isolated C2 domain of PKCα complexed to Ca^{2+} and PS has shown that Ca^{2+} bridges the protein directly to PS, and additionally, several residues in the protein, namely,

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[1] Abbreviations: CBR1−3, Ca^{2+} binding regions 1−3, respectively; DAG, diacylglycerol; DOG, 1,2-*sn*-dioleylglycerol; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine; PS, phosphatidylserine; LUV, large unilamellar vesicles.

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N189, R216, T251, and R249, seem to be involved in a direct PS interaction (*14*). Unfortunately, it has not been possible to unequivocally determine the conformation of the phospholipid, and two models were found to fit the electronic density maps. In both models, N189 interacts through its main and side chain nitrogen atoms with the oxygen atoms of the carboxyl group of the serine moiety, R216 interacts through its guanidinium group with one of the ester carbonyl groups of the phospholipid, and T251 interacts with the other carbonyl group. In addition, the fatty acyl chains of DCPS establish hydrophobic interactions with the aliphatic carbons of R249. Finally, one of the models, but not the other, shows that R249 interacts through its main chain nitrogen atom with the *sn*-1 ester oxygen atom of PS, and by means of one of its guanidinium nitrogen groups, with the *sn*-2 ester carbonyl group of DCPS. In addition, the crystallographic model proposed that K205, located in the $\beta3-\beta4$ connection of the C2 domain, is possibly involved in membrane binding (Figure 1A,B).

In this work, the four amino acids located near the calcium binding site in the C2 domain, which is supposed to interact directly with phosphatidylserine, have been mutated to alanine to study their role in the functioning of full-length PKCα. The binding and kinase activity of each mutant was measured and compared to those obtained with wild-type PKCα. Enzymatic activity was measured by using micelle and large unilamellar vesicle assays. The results obtained revealed that all four residues are important for enzymatic activation although not to the same extent. The inhibition caused by mutation was partially and progressively relieved by the addition of increasing proportions of PS to the activation mixtures, by the increasing Ca^{2+} concentrations, and by the addition of diacylglycerol. Besides, subcellular fractionation experiments have revealed that T251, R249, and R216 are also important residues for membrane translocation in vivo, suggesting that these residues in the C2 domain might play an important role in the activation mechanism of the enzyme.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids. PKCα cDNA was a gift from Y. Nishizuka and Y. Ono (Kobe University, Kobe, Japan). PKC mutants were generated by PCR mutagenesis (*15*). The mutants were obtained by using the following oligonucleotides: N189A, 5′-CGAAAGCCCAGCTGGATC; R216A, 5′-CAGTGTGGATGCGATGGT; R249A, 5′-TATCGTCGCATCCCAGTC; T251A, 5′-ATTCCGTGCCGTCCGATC; and K205A, 5′-TGCTCTCATTCGCGGGGT. Oligonucleotides, 5*Pst*I and 3*Nco*I, were used at the 5′- and 3′-ends, respectively (CTTCTGCAGCCACTGCA and TTACCATGGTGGTGCACTCCA).

Constructs, both wild-type and mutant genes, were subcloned into the mammalian expression vector pCGN (a gift from M. Tanaka, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). This vector contains the cytomegalovirus promoter and the multicloning sites that allow the expression of genes fused 3′ to the haemagglutinin (HA) epitope (*16*). All constructs were confirmed by DNA sequencing.

Cell Culture and Transfection. HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS). COS1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal calf serum (FCS). Transfection was performed according to the calcium phosphate method described by Wigler et al. (*17*).

Preparation of Protein Kinase Cα and Its Mutants. HEK293 cells were transfected with PKCα and its mutants. Cells were harvested 48 h post-transfection, pelleted, and resuspended in lysis buffer (5 mL of buffer/g of cells) containing 20 mM Tris (pH 7.5), 10 mM EGTA, 2 mM EDTA, 0.25 M sucrose, 1 mM PMSF, 10 μg/mL leupeptine, 100 μM Na_3VO_4, and 50 mM NaF. The pellet was disrupted by sonication, and the resulting lysate was centrifuged at 13000*g* for 30 min at 4 °C. The pellet was resuspended in a similar volume of lysis buffer, sonicated, and centrifuged at 13000*g* for 30 min at 4 °C. Supernatants from both centrifugations were pooled and ultracentrifuged at 100000*g* for 30 min at 4 °C. The resulting supernatant was applied to a DEAE-Sephacel column and equilibrated with buffer E [20 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, and 10 mM β-mercaptoethanol]. The bound proteins were eluted by the application of a salt gradient (0 to 1 M NaCl in buffer E) at a flow rate of 0.5 mL/min. Protein was concentrated by using a 30K Ultrafree centrifugal filter device (Millipore). The protein was then aliquoted and stored at −80 °C in the presence of 10% (v/v) glycerol and 0.05% (v/v) Triton X-100.

Preparation of Triton X-100 Micelles and Large Unilamellar Vesicles (LUV). The lipid mixtures were generated by mixing chloroform solutions of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoserine (POPS), and 1,2-*sn*-dioleylglycerol (DOG) (Avanti Polar Lipìds) and dried from the organic solvent under a stream of nitrogen and then further dried under vacuum for 90 min. Micelles were obtained by suspension of lipids in 3% Triton X-100, and further incubation at 37 °C for 5 min. Sucrose-loaded LUV were prepared as described by Rebecchi et al. (*18*).

PKC−Vesicle Binding. The binding of PKCα to phospholipid vesicles was assayed by centrifugation using sucrose-loaded LUV (0.1 μm in diameter). PKC (0.28 μg approximately) was incubated for 15 min with sucrose-loaded vesicles (1 mM) in 200 μL of 50 mM Hepes (pH 7.4), 0.3 mg/mL BSA, 200 μM $CaCl_2$, and 80 mM KCl. BSA was used to prevent protein loss due to adsorption to the tube walls. Membrane-bound protein was separated from free protein by centrifugation at 100000*g* for 1 h at 4 °C. Control experiments were performed under the same conditions but in the absence of phospholipids to eliminate the possibility of protein precipitation. The fraction of sedimented vesicles was determined by radioactivity measurements. The protein in each fraction was identified by 12.5% SDS−PAGE (*19*) analysis of the supernatant and pellet and further transferred onto nitrocellulose membranes. Immunoblot analysis of the epitope tag fused to the protein was performed by using anti-HA antibody 12CA5 and developed with chemiluminescence reagents (NEN Life Science, Inc., Boston, MA). The proteins were analyzed by densitometry.

Kinase Activity Assays. The kinase activity was assayed in vitro with purified wild-type PKCα and PKCα mutants by measuring the level of incorporation of $^{32}P_i$ into histone III-S. The reaction was started by addition of 0.2 μg of PKCα

to a 245 μL reaction mixture containing 20 mM Hepes (pH 7.4), 200 μM $CaCl_2$, 5 mM $MgCl_2$, Triton X-100 mixed micelles or LUV, 0.2 mg/mL histone III-S, and 0.02 mM ATP with [^{32}P]ATP (300 000 cpm/nmol). For the activity assays using LUV, the final concentration of lipids was 0.2 mM. In both cases, the reaction was stopped with 1 mL of 25% TCA and 1 mL of 0.05% BSA after 10 min. The reaction mixture was then stored on ice for 30 min. The proteins were collected on a 2.5 cm glassfiber filter and washed twice with 10% TCA. The level of incorporation of $^{32}P_i$ was measured by liquid scintillation counting. Basal kinase activity was measured in the presence of 0.5 mM EGTA instead of POPS, DOG, and Ca^{2+}. Additional control experiments were performed with mock cell lysates to eliminate the endogenous PKCα activity, and they represent less than 1% of the total enzyme activity measured.

Subcellular Fractionation. Cells were stimulated with either 100 or 500 nM PMA for 20 min before harvesting. After that, they were washed twice with PBS (4 °C) and further resuspended in a buffer containing 50 mM Tris (pH 7.4), 10 mM NaCl, 1 mM DTT, 1 mM Na_3VO_4, 50 mM NaF, 1 mM PMSF, 10 μg/mL soybean trypsin inhibitor, and 10 mM benzamidine. The cells were lysed by sonication. The cytosolic (s) and membrane fraction (p) were prepared and detected as described by Corbalán-García et al. (*13*).

Data Analysis. Since the origin of the sigmoidal dependence of enzyme activity on PS is not fully understood, the plots were graphically analyzed to determine the PS content which resultted in half-maximal enzyme activity ($[PS]_{1/2}$). The concentration of Ca^{2+} giving rise to half-maximal activity ($[Ca^{2+}]_{1/2}$) was determined from curve fitting of data to the Hill equation.

RESULTS

Mutagenesis Rationale. According to the structure described for the C2 domain of PKCα (14), four residues are directly involved in PS binding. As shown in Figure 1A, these residues are N189 located in CBR1, R216 located in CBR2, and T251 and R249 located in CBR3.

To assess the role of these residues in phospholipid−protein interaction, we generated single mutants of all of them in which each residue was replaced with Ala. The role of K205 was also studied by mutating it to Ala (Figure 1B). Because all five mutated residues are located in loop regions, the above mutations were not expected to cause deleterious conformational changes. All mutants were expressed in HEK293 cells as efficiently as the wild-type enzyme, suggesting similar stabilities and no important conformational changes (*12*), although we cannot rule out subtle conformational changes (Figure 1C).

Phosphatidylserine Dependence of Kinase Activities of PKCα and Phospholipid-Binding Mutants. As a first step in determining whether these mutations modulate PKCα activation by phospholipids, we examined the PS dependence of the mutants and wild-type enzyme for activation in the presence of Triton X-100 lipid mixed micelles. The enzyme activity was first measured in the presence of saturating Ca^{2+} and using increasing concentrations of POPS from 0 to 20 mol % but in the absence of DOG. Figure 2A shows that the wild-type protein reached maximal activity at 10 mol % POPS and had a $[PS]_{1/2}$ value of 5 mol %. R216A and R249A

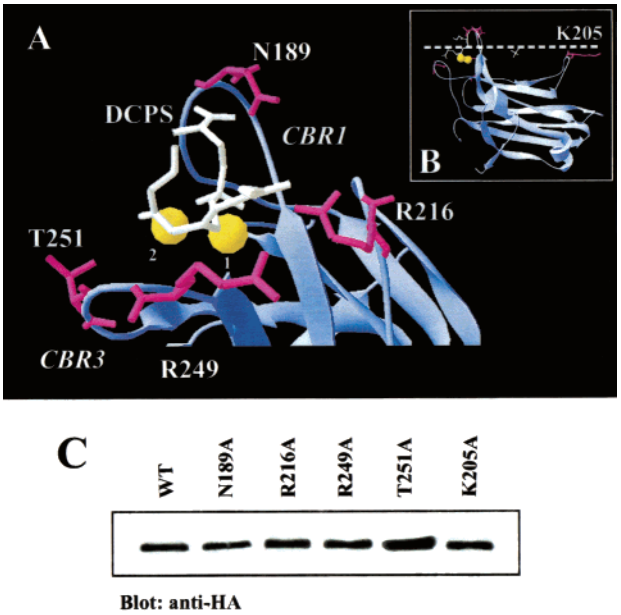

FIGURE 1: (A) Model structure of Ca^{2+} binding loops of the PKCα C2 domain. This model is based on the crystal structure of the C2 domain complexed to Ca^{2+} and DCPS (*14*). Side chains of the residues involved in lipid binding are depicted in black. DCPS is colored in pink. Ca^{2+} ions are represented in yellow. (B) Model structure of the complete C2 domain to show K205 possible localization. The program that was used was Swiss-Pdb Viewer 3.51 by Glaxo Wellcome Experimental Research (by N. Guex, A. Diemand, T. Schwede, and M. C Peitsch). (C) Expression of wild-type PKCα and PS ligand mutants in COS1 cells.

only reached 20−30% maximal activity, and T251A did not exhibit appreciable activity under these experimental conditions. The $[PS]_{1/2}$ and V_{max} values for the wild type and the mutants are summarized in Table 1. Concretely, $[PS]_{1/2}$ values in increasing order were as follows: N189A (15 mol %) < R216A = R249A ≪ T251A. These findings suggested that all the mutations, particularly T251A, decreased the capacity of PS to activate PKCα.

The inclusion of 1 mol % DOG had a marked effect on the activity of wild-type PKCα and the mutants under the conditions described above. The maximal activities of mutants R216A, R249A, and T251A increased to the same extent as that of N189A, although all exhibited slightly lower maximal activities than the wild type. Furthermore, the inclusion of 1 mol % DOG decreased the $[PS]_{1/2}$ of the wild-type protein to 4 mol % and those of the mutants to 12, 10, 10, and 13 mol % for N189A, R216A, R249A, and T251A, respectively (Figure 2B). With 5 mol % DOG included in the micelles, half-maximal enzyme activity was reached at 4 mol % POPS in the case of the wild-type protein and at 5 mol % in the case of all mutants (Table 1); although the V_{max} of the mutants was ~80%, the results suggest that increasing DOG concentrations permit a considerable decrease in $[PS]_{1/2}$ through the help of the C1 domain where DOG binds. However, the above residues are critical for the PS-dependent activation of the protein (see Table 1 as a summary).

Since the biophysical properties of the membrane are also important for activating classical PKCs (*20*−*23*), the effect of mutation on the enzyme activity was also studied by using large unilamellar vesicles (LUV). As described above, the effect of vesicles containing increasing concentrations of

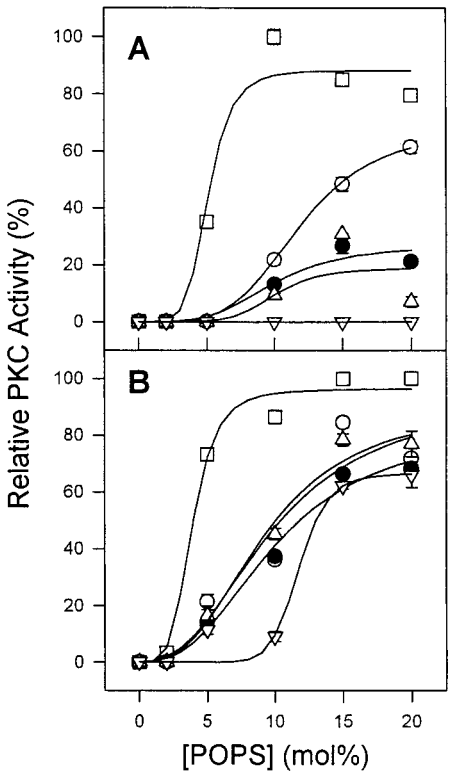

FIGURE 2: Dependence of enzymatic activity of PKCα and phospholipid-binding mutants on the PS composition in a micellar assay. Proteins include the wild type (□), N189A (○), R216A (●), R249A (△), and T251A (▽). PKC activity was measured in the absence of DOG (A) and in the presence of 1 mol % DOG (B) by using micelles composed of 0.43 mM Triton X-100 and increasing molar concentrations of POPS. Concentrations are expressed as the mole fraction of lipid with respect to the concentration of Triton X-100 in the assay. Histone was used as a substrate. Error bars indicate the standard error of the mean (SEM) for triplicate determinations. The absolute value of maximal activity was 458.9 nmol of P_i mg^{-1} min^{-1} in panel A and 776.5 nmol of P_i mg^{-1} min^{-1} in panel B.

Table 1: PS Concentration for Half-Maximal Activation of Wild-Type PKCα and C2 Domain Mutants[a]

	enzyme activity $[PS]_{1/2}$ (mol %)				
	micelles			LUV	
	0 mol % DOG	1 mol % DOG	5 mol % DOG	0 mol % DOG	1 mol % DOG
wild-type PKCα	5 (90)[b]	4 (100)	4 (100)	27 (nd)	9 (100)
N189A	15 (61)	12 (80)	5 (80)	43 (nd)	14 (70)
R216A	>20 (20)	10 (80)	5 (80)	48 (nd)	18 (70)
R249A	>20 (19)	10 (70)	5 (80)	60 (nd)	22 (59)
T251A	≫20 (nd)[c]	13 (63)	5 (80)	≫60 (nd)	40 (59)

[a] The kinase activity was assayed in vitro with purified wild-type PKCα and PKCα mutants by measuring the level of incorporation of ^{32}P into histone III-S; see Experimental Procedures for further details. $[PS]_{1/2}$ and V_{max} were graphically analyzed. [b] Percentage of Vmax is shown in braquets. [c] Not determined.

POPS from 0 to 60 mol % on the activity of wild-type PKCα and mutants was first studied in the absence of DOG and at a saturating Ca^{2+} concentration. Figure 3A shows that half-maximal enzyme activity was reached at 27 mol % POPS in the case of the wild-type protein and at 43, 48, 60, and ≫60 mol % for N189A, R216A, R249A, and T251A, respectively. These data again show that all these residues are important for enzyme activation with the order of

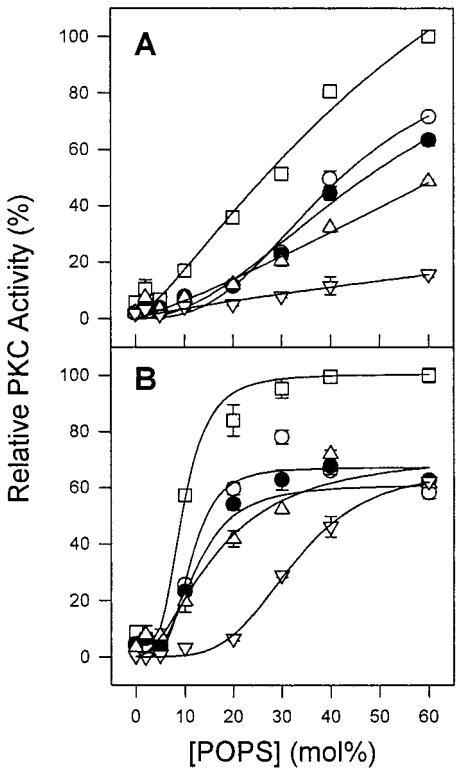

FIGURE 3: Dependence of enzymatic activity of PKCα and phospholipid-binding mutants on the PS composition in large unilamellar vesicles. Proteins include the wild type (□), N189A (○), R216A (●), R249A (△), and T251A (▽). PKC activity was measured using POPC/POPS large unilamellar vesicles (99−*X*:*X* in mole fraction) in the absence of DOG (A) and in the presence of 1 mol % DOG (B). The total lipid concentration was 0.2 mM. Histone was used as a substrate. Error bars indicate the SEM for triplicate determinations. The absolute value of maximal activity was 725 nmol of P_i mg^{-1} min^{-1} in panel A and 1160.5 nmol of P_i mg^{-1} min^{-1} in panel B.

importance being similar to that obtained for the Triton X-100 mixed micelles. When 1 mol % DOG was included in the vesicles, the $[PS]_{1/2}$, the concentration at which half-maximal enzyme activities were reached, decreased to 9 mol % POPS for the wild-type protein and 14, 18, 22, and 40 mol % POPS for N189A, R216A, R249A, and T251A, respectively, suggesting that in the case of lipid vesicles, too, DOG was able to recover the enzyme activity. In general, the data confirmed that T251 is a very important residue for the interaction of the enzyme with PS in both micelles and membrane models, while N189, R216, and R249 are also important, although to a lesser extent.

Calcium Dependence of Kinase Activities of PKCα and Phospholipid-Binding Mutants. The activity of PKCα and the mutants was also measured as a function of Ca^{2+} concentration. Figure 4 shows the calcium dependency of the five proteins in the presence of POPC/POPS/DOG (35:60:5) vesicles. The wild-type protein exhibited a $[Ca]_{1/2}$ value of 1.4 μM; mutants N189A and R249A behaved very similarly and exhibited a small increase in $[Ca]_{1/2}$, with a value of 8 μM, while R216A exhibited a $[Ca]_{1/2}$ of 26 μM. Finally, mutant T251A exhibited the most significant increase in $[Ca]_{1/2}$, which rose to 51 μM, thus demonstrating its diminished capacity to be activated by Ca^{2+} compared with wild-type protein and the other mutants. All the mutants exhibited approximately 80% of the maximal enzyme activity at saturating Ca^{2+} concentrations.

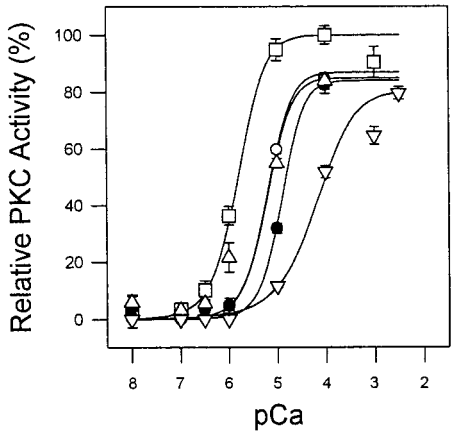

FIGURE 4: Dependence of enzymatic activity of PKCα and phospholipid-binding mutants on the Ca^{2+} concentration. Proteins include the wild type (□), N189A (○), R216A (●), R249A (△), and T251A (▽). PKC activity was measured using POPC/POPS/DOG (35:60:5) LUV and varying Ca^{2+} concentrations. The free Ca^{2+} concentration was estimated from the total Ca^{2+} and EGTA concentrations by using computer software developed by Fabiato (*34*). Histone was used as a substrate. The absolute value of maximal activity was 730 nmol of P_i mg^{-1} min^{-1}. Error bars indicate the SEM for triplicate determinations.

Table 2: Binding of Wild-Type PKCα and C2 Domain Mutants to PS-Containing Vesicles[a]

	[POPS] (10 mol %)	[POPS] (60 mol %)
wild-type PKCα/EGTA	0.88 ± 0.4	11 ± 2.7
wild-type PKCα	33 ± 4	95 ± 4.9
N189A	37 ± 3.3	86 ± 8.2
R216A	4 ± 3.6	57 ± 6.3
R249A	9 ± 1.8	59 ± 8
T251A	3 ± 2.5	43 ± 1.2
K205A	33 ± 5.3	97 ± 5.4

[a] Results are expressed as a percentage of the protein bound to the lipid vesicles after ultracentrifugation. The binding of wild-type and mutants PKCα to phospholipid vesicles was assayed by centrifugation using sucrose-loaded large unilamelar vesicles (0.1 μm in diameter).

Effect of Phospholipid-Binding Mutants on Binding to PS-Containing Vesicles. To investigate the role of PS binding sites in PKCα membrane translocation, the binding of PKCα to LUV was studied by using vesicles containing two different PS proportions, i.e., POPC/POPS/DOG molar ratios of 89:10:1 and 39:60:1. Table 2 shows that when 10 mol % POPS was included in the vesicles, the mutation of T251, R216, and R249 to Ala had a great effect on protein localization. It is interesting that mutation at N189 did not produce any significant effect on membrane binding compared to binding with the wild-type PKCα. The results obtained with R216A, R249A, and T251A correlated well with the almost total lack of enzymatic activity, which presumably stems from the inability of the protein to bind to membranes. However, N189A which only exhibited 20% of its catalytic activity at 10 mol % PS was bound to the membrane to the same extent as the wild-type protein (Figure 3B). When the PS concentration was increased to 60 mol %, N189A again exhibited a binding capacity similar to that of the wild type, and although the binding capacity of the other mutants increased considerably, those mutants were still less capable than the wild-type of binding to these vesicles. This was especially true in the case of T251A, confirming once again that this mutation is the most harmful of the four constructs being studied (Table 2).

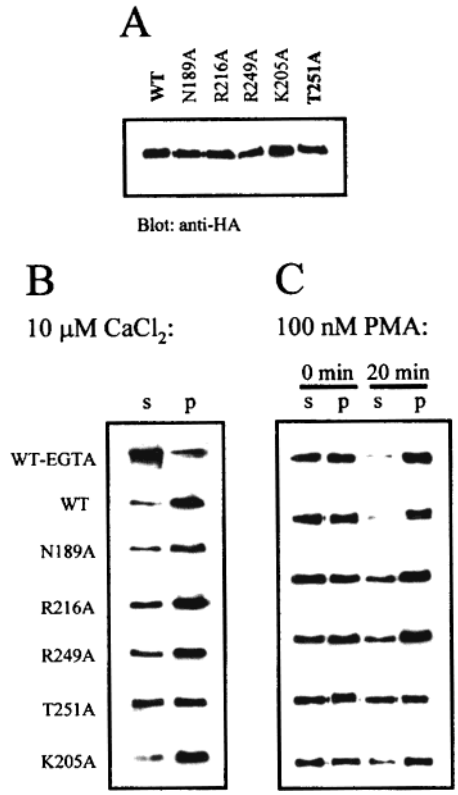

FIGURE 5: (A) Expression of wild-type PKCα and the phospholipid-binding mutants. COS1 cells were transfected with expression vectors encoding HA-tagged PKC constructs. Cell lysates were separated by SDS−PAGE [12.5% (w/v) gel] and subsequently analyzed by immunoblotting with anti-HA antibody and developed by enhanced chemiluminescence. (B) Subcellular distribution of wild-type PKCα and mutants in the presence of 10 μM $CaCl_2$. The free calcium concentration was determined as described in the legend of Figure 4. (C) Subcellular distribution of wild-type PKCα and mutants in the presence of 100 nM PMA. Lanes containing fractions corresponding to the cytosol are labeled s, and those with extracts obtained from the membrane fraction are labeled p.

Residues Involved in Phospholipid Binding Are Also Important for PKCα Translocation to the Cell Membrane in Vivo. To investigate the significance of these four residues for membrane translocation of PKCα in vivo, we transiently expressed wild-type and mutant proteins in COS1 cells. All of them were expressed at approximately the same level under the conditions that were used (Figure 5A). Subcellular fractionation of the cell lysates enabled us to obtain membrane and cytosolic fractions. Figure 5B shows that in the presence of 5 mM EGTA, the wild-type protein is mostly located in the cytosolic fraction (s) (71%). On the other hand, when 10 μM $CaCl_2$ was included in the fractionation buffer, 87% of the wild-type protein was partitioned in the membrane fraction (p). All the mutants were located in the membrane fraction (75%) except the T251A mutant that was translocated only at 57%, suggesting that this residue is very important for the Ca^{2+}-dependent membrane translocation (Table 3). To evaluate the effect of these mutations in the phorbol ester-dependent membrane translocation, the cells also were stimulated with 100 and 500 nM PMA for 20 min before harvesting. Figure 5C shows the results obtained after the subcellular fractionation with 100 nM PMA stimulation. Fifty percent of the total PKCα was constitutively located in the membrane fraction under resting conditions at time zero, while 20 min after PMA stimulation, 91% of the total

Table 3: Calcium-Dependent Subcellular Localization of Wild-Type PKCα and C2 Domain Mutants in Vivo[a]

	$[CaCl_2]$ (10 μM)		$[CaCl_2]$ (10 μM)
wild-type PKCα/EGTA	29 ± 0.2	R249A	74 ± 1.3
wild-type PKCα	87 ± 1.4	T251A	57 ± 2
N189A	75 ± 0.4	K205A	85 ± 3.2.1
R216A	74 ± 0.9		

[a] Results are expressed as a percentage of the protein found in the membrane fraction (p) of the subcellular fractionation assay. Cells were lysed by sonication in a buffer containing 50 mM Tris (pH 7.4), 10 mM NaCl, 1 mM DTT, 1 mM Na_3VO_4, 50 mM NaF, 1 mM PMSF, 10 μg/mL soybean trypsin inhibitor, and 10 mM benzamidine. The cytosolic and membrane fractions were obtained by ultracentrifugation.

Table 4: PMA-Dependent Subcellular Localization of Wild-Type PKCα and C2 Domain Mutants in Vivo[a]

	100 nM PMA		500 nM PMA	
	0 min	20 min	0 min	20 min
wild-type PKCα	50 ± 0.02	92 ± 0.71	42 ± 0.41	84 ± 0.82
N189A	51 ± 0.81	88 ± 0.41	47 ± 0.71	89 ± 1.22
R216A	40 ± 0.71	66 ± 1.22	59 ± 0.41	100 ± 0
R249A	60 ± 0.82	76 ± 0.41	59 ± 0.71	75 ± 0.41
T251A	47 ± 0.41	42 ± 0.41	45 ± 0.71	35 ± 0.41
K205A	48 ± 0.02	89 ± 0.71	49 ± 0.71	94 ± 0.02

[a] Results are expressed as a percentage of the protein found in the membrane fraction (p) of the subcellular fractionation assay as described in the footnote of Table 3.

protein was found in the membrane fraction. Similar results were obtained in the case of N189A. However, only 66 and 76% of the total protein was translocated in the case of R216A and R249A mutants, respectively (Table 4). Furthermore, the translocation of T251A to the membrane fraction was not stimulated by PMA. When the concentration of PMA used was a saturating 500 nM, all the constructs were able to translocate to the membrane fraction except R249A, which only reached 74%, and T251A, which exhibited an even lower amount of translocated protein than under resting conditions (Table 4).

These results showed that R216, R249, and, most importantly, T251 are involved in the membrane translocation of PKCα in vivo and that these residues are important for protein localization, suggesting that although the C1 domain might cooperate to overcome the lack of functionality of the C2 domain, the last one is determinant for plasma membrane translocation. At the same time, the N189A mutant did not inhibit this translocation as it did not prevent the binding of the enzyme to PS-containing vesicles.

Lys205 Is Not a Critical Residue for Membrane Translocation and Protein Activation. Residue K205 was located in the loop connecting the β3 and β4 sheets (*14*; Figure 1B), and according to the proposed structural model, it might approach the membrane surface where it would possibly interact with charged residues. To test this hypothesis, we studied the effect of the mutation of K205 to Ala (K205A) on PKCα vesicle binding. As shown in Table 2, PS-dependent binding activity was unaffected at both low and high POPS molar fractions in the vesicles. When the construct was transiently expressed in COS1 cells, which were subcellularly fractionated after stimulation by PMA or in the presence of 10 μM $CaCl_2$, the proportion of mutant protein bound to the membrane fraction was similar to that

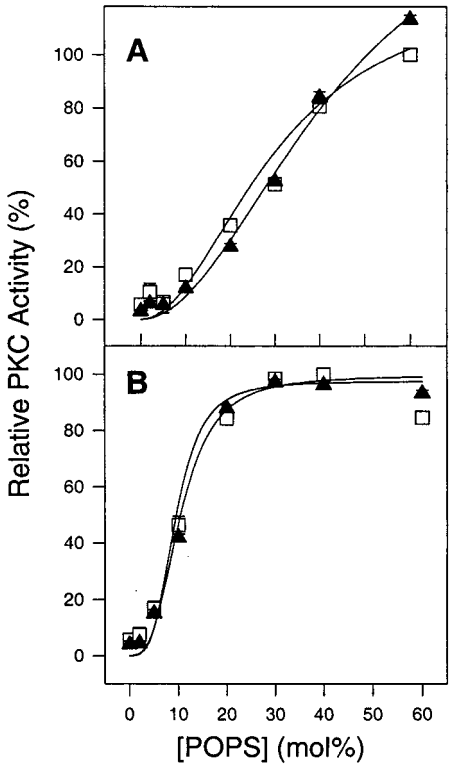

FIGURE 6: Dependence of enzymatic activity of PKCα and the phospholipid-binding mutant K205A on the PS composition in large unilamellar vesicles. Proteins include the wild type (□) and K205A (▲). PKC activity was measured using POPC/POPS (99−*X*:*X* in mole fraction) large unilamellar vesicles in the absence of DOG (A) and in the presence of 1 mol % DOG (B). The total lipid concentration was 0.2 mM. Histone was used as a substrate. Error bars indicate the SEM for triplicate determinations. The absolute value of maximal activity was 725 nmol of P_i mg^{-1} min^{-1} in panel A and 1160.5 nmol of P_i mg^{-1} min^{-1} in panel B.

of the wild-type protein (panels B and C of Figure 5 and Table 4).

As a second step in determining whether K205 could modulate the lipid-dependent activation of PKCα, we studied the PS dependence of the activation of mutant and wild-type proteins. Figure 6A shows that the PS dependence of the K205A mutant to be activated as assayed in LUV in the absence of DOG was indistinguishable from that of the wild-type enzyme. More specifically, both the mutant and wild-type enzymes were half-maximally activated by approximately 27 mol % PS. The addition of 1 mol % DOG to the vesicles decreased the concentration of POPS required for half-maximal activation, as shown in Figure 3, but did not cause any differences in the PS regulation of the mutant compared with the wild-type protein (Figure 6B). Similar results were obtained using Triton X-100 mixed micelles (data not shown). Thus, it can be concluded that K205 is not essential for PKCα membrane interaction in vivo or in vitro, and for PS-dependent enzyme activation. Although we cannot rule out the possibility that this interaction may occur in the case of the isolated domain, it seems to be irrelevant for the enzymatic activity of the full-length protein, at least under the conditions used in this work.

DISCUSSION

Conventional PKCs are activated by the Ca^{2+}-dependent translocation of proteins to membranes containing anionic

phospholipids, with preference for PS, and DAG. Structural and mutational studies have shown that the C2 domain of conventional PKCs is responsible for the Ca^{2+}-dependent translocation of the protein to membranes (*11*−*13*). It has been recently shown that the C2 domain of PKCα is responsible for its initial Ca^{2+}- and PS-dependent membrane binding, whereas the C1 domain is involved in subsequent membrane penetration and DAG binding, which eventually leads to enzyme activation (*26*, *27*).

Crystallographic studies on the C2 domain of PKCα complexed to Ca^{2+} and a soluble PS allowed for the first time the interaction of an activating phospholipid with the C2 domain of PKCα to be observed (*14*). Unfortunately, and due to problems of resolution and disorder in the phospholipid headgroup, it was not possible to obtain an unequivocal and detailed picture of the interaction of the phospholipid with the protein. Nevertheless, four protein residues, namely, N189, R216, R249, and T251, were proposed to interact directly with PS (*14*). To date, no other experimental data exist indicating clearly which residues in the domain are responsible for the interaction with PS or other anionic phospholipids. To address this issue, we individually mutated the four residues described at the C2 domain of PKCα that might be involved in the interaction with negatively charged phospholipids and hence in the activation of the enzyme. The results that were obtained demonstrate the involvement of all four residues in the phospholipid-induced enzyme activation, although not all of them appear to play the same role in the activation process.

It is very interesting to note that the N189A mutant had no effect on binding either in vitro or in vivo but inhibited enzyme activity. It has been proposed that N189 interacts with the carboxylate group of PS through backbone and side chain nitrogen in the crystallographic model. The mutation of this residue to alanine suppresses only the side chain nitrogen interaction with one of the oxygens in the carboxylate group of L-serine. When we analyzed the structure by a modeling computer program (Swiss-Pdb Viewer 3.51 by Glaxo Wellcome Experimental Research), the distances obtained from each oxygen atom of the carboxylate group of phosphatidylserine were 2.23 and 2.41 Å to the main and side chain, respectively. The theoretical mutational study (also by modeling) substituting N189 with A gave 2.21 and 2.86 Å for the distances to the main and side chain, respectively, after energy minimization, suggesting that with this mutation the distances are still compatible with interaction. From the results obtained experimentally, it is clear that this mutation did not disrupt the binding of the PS polar group with this site. However, and most interestingly, the mutant protein was bound to membranes but at the same time was inactive, suggesting that in addition to interacting with PS, the side chain of N189 was important for enzyme activation. This residue may trigger enzyme activation as a consequence of its interaction with the phospholipid. It is important to note that N189 is located in a very exposed and flexible area of the C2 domain, at the top of the CBR1 loop, and thus is very well placed to interact with other neighboring areas of the protein, for example, the C1 domain. Recent data have shown that D187 in the same loop, which coordinates Ca1, is also related to the C1 domain, since DAG was not able to translocate the mutant protein to the plasma membrane or to fully activate the enzyme (*28*). It should be mentioned in this respect that it has recently been suggested that N189 might maintain the enzyme in an inactive conformation by a specific interaction with D55 in the C1a domain (*29*), and that the interaction with the carboxylate group of PS might unleash the tethering between the C1a and the C2 domains, leading to enzyme activation. According to this model, the mutation of N189A would prevent the tethering of C1a to the C2 domain, and hence activate the enzyme independently of PS. However, this was not observed since the N189A mutant bound PS but was inactive at the same time. Another possibility would be that N189 could be important for the proper orientation of the C2 domain on the membrane surface, and this perturbation led to a decrease in enzymatic activity.

On the other hand, R216A, R249A, and T251A had a clear inhibitory effect on the binding to vesicles and biomembranes (in vivo) and on enzymatic activity. Among these mutants, the most drastic effect was demonstrated by T251A, which exhibited very low membrane binding and enzymatic activity and poor membrane translocation in vivo even at a saturating concentration of Ca^{2+} and PMA. Note that very high concentrations of PS may overcome the inhibitory effect of this mutation (Figure 2). However, the biological membrane was not able to promote a similar extent of enzyme translocation because it had an insufficiently high concentration of activating phospholipids. In the protein−lipid interaction model proposed by Verdaguer et al. (*14*), T251 would bind to the *sn*-1 carbonyl group of the phospholipid, this interaction apparently being important for the binding of PS to the C2 domain. The Ca^{2+} titration and subcellular fractionation experiments showed that this mutation particularly damaged the capacity of the enzyme to be activated by Ca^{2+}, which suggests that this residue is somehow involved in Ca^{2+} binding. However, the crystal structure described for the C2 domain of PKCα complexed to Ca^{2+} and DCPS (*14*) showed two Ca^{2+} ions coordinated by a number of amino acid residues, although T251 was not involved. It is, however, interesting to note that the crystal structure of the PKC*β* C2 domain showed that S251, which is homologous to T251, was involved in coordinating a third Ca^{2+} (*30*). It is intriguing that very similar proteins such as isozymes PKCα and PKC*β*, both of which belong to the classical family, may coordinate different numbers of Ca^{2+} ions. New experiments to clearly establish the stoichiometries of the binding of Ca^{2+} to classical PKCs and new crystallographic or NMR studies on Ca^{2+} binding are necessary to shed light on this issue to clarify whether T251 is involved in Ca^{2+} coordination. It should, nevertheless, be kept in mind that an extensive mutagenesis study of the residues involved in coordinating Ca^{2+} in the C2 domain of PKCα (*12*) showed that $[Ca]_{1/2}$ values for residues such as D193 or D254, which are known to be involved (*14*) in the coordination of Ca1 or Ca2, were approximately 310 and 550 μM under experimental conditions similar to those used in this work. These results suggest that the decrease in calcium affinity in the case of T251 (51 μM) is very small, and so if a third Ca^{2+} ion binds to this C2 domain by interacting with T251, it is not as essential for PKC activation as Ca1 and Ca2. Alternatively, the increase in $[Ca]_{1/2}$ caused by mutation of T251 may simply reflect the difficulty the phospholipid has in binding to the protein which can be overcome with an excess of calcium.

In the case of R216, mutation to Ala resulted in a highly inhibitory effect in terms of binding and kinase activity. There was good correlation between the lack of binding and lack of activity, suggesting that this mutation seriously damaged one of the key bonds which are necessary for establishing the phospholipid−protein interaction.

The case of R249 was similar to that of R216, since the lack of kinase activity correlates well with the inability of the protein to bind to membranes. The very similar effects of R216A and R249A mutants on both binding and activity fit one of the proposed structural models very well (*14*; called the *sn*-1 model in that paper), according to which the guanidinium groups of R216 and R249 interact with the *sn*-2 ester carbonyl group of PS.

It has long been established that PS is the best acidic phospholipid for activating classical PKCs (*31*, *32*). It has also been observed that the presence of DAG increased the selectivity for PS over other anionic phospholipids (*9*), which suggested that the C1 domain may cooperate with the C2 domain during PS activation. Recent observations also suggested that the concerted action of C1 and C2 domains was required for full activation of the enzyme and for its PS specificity (*28*, *29*, *33*). The in vivo results obtained in this paper further supported this hypothesis, since the mutants that were used had their C1 domain intact and nevertheless exhibited diminished translocation to the plasma membrane upon phorbol ester stimulation. Note that when plasma membrane translocation was induced by calcium, the T251A mutant was the only one that did not translocate (Figure 5B). However, when the translocation signal was PMA-dependent, the R216A, R249A, and T251A mutants have difficulty in translocating to the membrane, suggesting that C2 domain interaction with the phospholipids at the plasma membrane might play a critical and necessary step for enzyme activation, according to the model proposed by Oancea and Meyer (*27*).

In summary, we have found that the T251, R216, and R249 residues located at the C2 domain of PKCα are directly involved in binding to phosphatidylserine and in subsequent enzyme activation. Additionally, we have found that N189 may act as a trigger of enzymatic activity, since its mutation did not affect PS binding but inhibited its activation. Since DOG clearly decreased the PS concentrations needed to restore mutant activity, it can be concluded that the C1 domain cooperates with the C2 domain in enzyme activation. It is also of note that experiments carried out in vivo fully supported these observations. From these results and the previous model obtained from X-ray diffraction (*14*), we conclude that the four residues studied in this work constitute an essential part of the phospholipid binding site of PKCα in the C2 domain, allowing a first step in the enzyme activation upon phospholipid binding. Further experiments will be necessary to show if, to act as a trigger, N189 is related to other residues located either in the C2 or in the C1 domain, this last possibility being quite likely, given the cooperation observed between both domains in enzyme activation.

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