Retinoic Acid as a Modulator of the Activity of Protein Kinase Cα†

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ABSTRACT: All-*trans*-retinoic acid (atRA) is a derivative of vitamin A and possesses antitumor activity. We demonstrate that atRA is able to modulate the activity of protein kinase C α (PKCα), which is related to tumor development. In vitro, it was found that atRA activated PKCα in the presence of Ca^{2+} and in the absence of phosphatidylserine, although such activity is considerably inhibited in mutations affecting residues D246 and D248 and also residue N189, all of which are known to be essential for the interaction with Ca^{2+} and phosphatidylserine in the C2 domain. It was concluded that atRA substitutes phosphatidylserine although with lower specific activities. However, atRA had a biphasic effect on PKCα activity in the presence of activating phospholipids, such as phosphatidylserine and phosphatidylinositol 4,5-bisphosphate, yielding activation at low concentrations but inactivation at higher ones. This second inhibitory characteristic was not shown with K209 and K211 mutations (residues located in the Lys-rich cluster in the C2 domain) in PKCα. This interesting effect revealed the importance of phospholipid binding at this site to ensure maximum activity for the wild-type PKCα. The C1 domain was not related with the atRA effect on PKCα. It was concluded that whereas atRA may activate PKCα through the Ca^{2+}-phosphatidylserine-binding site of the C2 domain, it may also inhibit the activity of this enzyme when displacing the phospholipid from the Lys-rich cluster also located in the C2 domain.

Protein kinase C (PKC)[1] is a family of related protein kinases that play an important role in regulating cell growth, as they are involved in several intracellular pathways that end in transcription. PKCs include at least 11 different mammalian isoforms, which can be classified into three groups according to their structure and cofactor regulation. The first group includes the classical isoforms (α, βI, βII, and γ) that are regulated by acidic phospholipids, diacylglycerol, and phorbol esters and also by calcium. The second group corresponds to the novel isoforms (δ, ϵ, η, and θ) that are regulated by phospholipids, diacylglycerols, and phorbol esters but not by calcium. The third group comprises the atypical PKC isoforms (ξ, τ/λ, and μ) that are not regulated by diacylglycerol or by calcium (*1*−*3*). PKC isozymes participate in the processes that regulate cell signaling and which begin in cell membranes with the appearance of bioactive derivatives of phospholipids, such as diacylglycerols (*4*, *5*).

Retinoids are vitamin A derivatives capable of regulating apoptosis and cellular growth or differentiation and are used as therapeutic agents against cancer (*6*−*12*). Empirical data (see below) largely support the correlation between the reversion of malignant phenotypes induced by treatment with atRA and changes in PKC activity. By interacting with nuclear receptors, atRA may control gene expression in tumor cells, and changes in the expression of classical PKCs have been reported for a large variety of cells treated with atRA: F9 embryonal carcinoma cells (*13*), human breast carcinoma cells (*14*, 15), human pancreatic carcinoma cell lines (*16*), B16 mouse melanoma cells (*17*, *18*), HL-525 cells (*19*), B16 cells (*20*), and human neuroblastoma cells (*21*). atRA might also affect PKCα activity by altering the localization of the protein in the cell, as has been demonstrated to occur in rat splenocytes (*11*) or in human endometrial adenocarcinoma, where redistribution occurs simultaneously with cytoskeletal reorganization (*22*). The reversion of malignancy in v-Ki-*Ras*-transformed SVC1 cells by treatment with atRA has been associated with both Ras p21 downregulation and concomitant changes in the cellular localization of PKC (*23*). Finally, it is possible that atRA directly affects the activity of PKCs, although little information, either in vitro or in vivo, is available in this respect. Some authors have reported the inhibition of PKC by atRA (*24*−*26*), whereas others found it to be activated (*27*, *28*) or translocated to membranes in vivo (*29*). Even less is known concerning the possible binding of atRA to PKCs although, on the basis of sequence

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[1] Abbreviations: AtRA, all-*trans*-retinoic acid; BSA, bovine serum albumin; CBR, calcium-binding region; DEAE, diethylaminoethyl; DOG, 1,2-dioleoyl-*sn*-glycerol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; LUV, large unilamellar vesicle; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PMSF, phenylmethanesulfonyl fluoride; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; PS, phosphatidylserine; SD, standard deviation; TCA, trichloroacetic acid.

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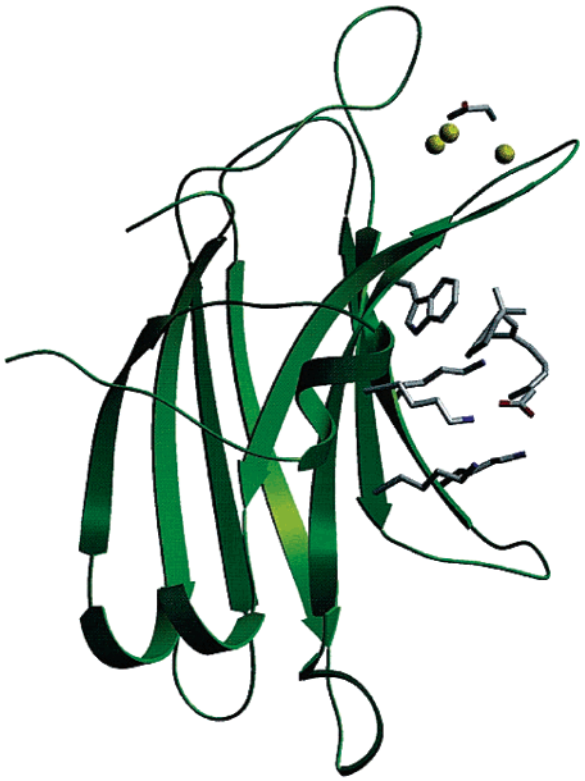

FIGURE 1: Overall structure of PKCα−C2 domain complexed with Ca^{2+} and atRA (reprinted with permission from ref *30*; copyright 2003 American Chemical Society). In the calcium-binding region, calcium ions are represented as yellow spheres, and a part of an atRA molecule is indicated as solid sticks. The atRA molecule bound to this region was only partially modeled (*30*). In the lysine-rich cluster, the atRA molecule and the interacting amino acid side chains are depicted as solid sticks.

comparisons with atRA receptors, it has been suggested that atRA might bind close to the Ca^{2+}-binding region of the protein C2 domain in classical PKCs (*26*).

Structural results (*30*) showed that atRA can interact with the C2 domain in the same two locations that had previously been reported as phospholipid-binding sites (*31*, *32*). One of the sites corresponds to the Ca^{2+}-binding region and the second to the Lys-rich cluster defined by residues at β-strands 3 and 4 (Figure 1). These observations were supported by [^{3}H]atRA-binding experiments combined with site-directed mutagenesis.

In this paper we demonstrate that atRA is a modulator of the activity of PKCα, and the mutagenesis studies confirm that this compound acts through the sites revealed by a previous X-ray diffraction study (*30*), so that atRA activates PKCα through the Ca^{2+}-binding region but it may be inhibitory when bound to the lysine-rich cluster.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS), phosphatidylinositol 4,5-bisphosphate (PIP_2), and 1,2-dioleoyl-*sn*-glycerol (DOG) were purchased from Avanti Polar Lipids Inc. (Birmingham, AL). All-*trans*-retinoic acid (atRA), histone III-S, and DEAE-Sephacel were from Sigma-Aldrich (Madrid, Spain). Anti-PKCα antibody (C-20) and anti-HA antibody (F-7) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Western lightning chemiluminiscence reagent plus was purchased from Perkin-Elmer (Boston, MA). The 30K ultrafree centrifugal filter device was purchased from Millipore Ibérica (Madrid, Spain). Purified PKCα was used as standard reference and purchased from Calbiochem (EMD Biosciences Inc., La Jolla, CA). Water was distilled two times and deionized in a Millipore system from Millipore Ibérica (Madrid, Spain).

Construction of the Expression Plasmids. Rat PKCα cDNA was a gift from Drs. Nishizuka and Ono (Kobe University, Kobe, Japan). Full-length wild-type PKCα and mutants PKCαN189A, PKCαD246N/D248N, and PKCαK-209A/K211A were prepared as previously described (*33*−*35*). Mutants PKCαW58G and PKCαF60G were generated by PCR site-directed mutagenesis using full-length PKCα and the following oligonucleotides: W58G, GTTTG-GAAAAC and 5′GTTTTCCAAACCCCCCGATGAAGTCG-GTG; F60G, 5′CTTCATCTGGGGGGGTGGAAAACAAG-GCTTC and 5′GAAGCCTTGTTTTCCACCCCCCCAGAT-GAAG.

All constructs, both wild type and mutants, were subcloned into the mammalian expression vector pCGN (a gift from Dr. Tanaka, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). This vector contains the cytomegalovirus promoter and the multicloning sites that allow the expression of the genes fused 3′ to the hemagglutinin (HA) epitope (*36*). All constructs were confirmed by DNA sequencing.

*Cell Culture, Transfection, and Purification of PKC*α. HEK293 cells were grown in Dulbecco's modified Eagle medium with 10% fetal calf serum. Transfection with wild-type and mutant DNA was performed with the Ca^{2+} phosphate method described by Wigler et al. (*37*). Protein purification was performed as previously described (*34*). Briefly, after 48 h of posttransfection growth, cells were centrifuged and resuspended in lysis buffer (5 mL of buffer/g of cells), containing 20 mM Tris-HCl, pH 7.5, 10 mM EGTA, 2 mM EDTA, 0.25 M sucrose, 1 mM PMSF, 10 μg/mL leupeptin, 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 again (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 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 from 0 to 1 M NaCl in the same buffer at a flow rate of 0.5 mL/min. The presence of PKCα in the elution fractions was checked by 12.5% SDS−PAGE and immunoblot analysis of the epitope tag fused to the protein using the anti-HA antibody (Santa Cruz Biotechnology) and developed with chemiluminiscence reagents (Perkin-Elmer). Fractions including the protein were then pooled, and the protein was concentrated using a 30K ultrafree centrifugal filter device (Millipore). Finally, PKCα was aliquoted and stored at −80 °C in the presence of 10% (v/v) glycerol and 0.05% (v/v) Triton X-100.

Preparation of Large Unilamellar Vesicles (LUVs). Large unilamellar vesicles (LUVs) were prepared using the method described by Rebecchi et al. (*38*). Dried lipid mixtures were

resuspended in 20 mM Hepes, pH 7.4, 0.2 mM EGTA, 80 mM NaCl, and 160 mM sucrose by vigorous vortexing. The lipid mixtures were then frozen and thawed at 30 °C five times, and finally they were passed 10 times through two polycarbonate filters with a 0.1 μm pore size using an extruder system (Lipex Biomembranes Inc.).

Kinase Activity Assay. The kinase activity was assayed in vitro with wild-type PKCα and mutants by measuring the level of incorporation of [^{32}P]P_i into the protein substrate (histone III-S). The reaction mixture contained 20 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2 mM LUVs, 0.2 mg/mL histone III-S, and 20 μM [γ-^{32}P]ATP (300000 cpm/nmol). AtRA was dissolved in a concentrated ethanol solution, so that the added volume was less that 2% in all cases, before being added to the reaction mixture. Samples were incubated at 25 °C for 10 min, and then the reaction was started by the addition of 0.02 μg of wild-type PKCα or mutants to a 50 μL sample. The protein concentration of the wild-type form and the mutants was normalized using densitometry after immunoblot analysis, including a known concentration of commercial purified PKCα (Calbiochem, EMD Biosciences Inc.) as reference, with anti-PKCα antibody (Santa Cruz Biotechnology) and chemiluminiscence reagents (Perkin-Elmer).

The reaction was stopped with 1 mL of ice-cold 25% trichloroacetic acid (TCA) and 1 mL of ice-cold 0.05% bovine serum albumin (BSA) after 10 min of reaction. After precipitation on ice for 30 min, protein was collected on a 2.5 cm glass fiber filter (Sartorius AG, Goettingen, Germany) and washed three times with 3 mL of ice-cold 10% TCA. The level of incorporation of ^{32}P to histone was measured by liquid scintillation counting. Basal kinase activity was measured in the presence of 0.5 mM EGTA instead of lipids and $CaCl_2$ and subtracted from the sample results. Additional control experiments were performed using mock cell lysates to eliminate the possible endogenous PKCα activity, and they represented less than 1% of the total enzyme activity measured. Data are the means of triplicate determinations (±SD).

RESULTS

Implication of the C2 Domain on Activation of PKCα by atRA. From the cocrystallization experiment, where atRA and the C2 domain from PKCα were studied at high resolution by X-ray diffraction (*30*), it was deduced that atRA bound to two sites (Figure 1). One of them was the Ca^{2+}-binding region, which includes regions CBR1 and CBR3, and the second was the lysine-rich cluster located on stands β3 and β4. With the aim of exploring whether the interaction of atRA with these two sites really affects the enzymatic activity of PKCα, key residues, which had previously been shown to be involved in the enzymatic activity, were mutated. In the Ca^{2+}-binding region, Asn189 was mutated to Ala (PKCαN189A) in CBR1 (*34*), and Asp246 and Asp248 were mutated to Asn (PKCαD246/D248N) in CBR3 (*33*). In the Lys-rich cluster, both Lys209 and Lys211 were mutated to Ala (PKCαK209/211A) (*35*).

The activity of PKCα was compared with those of mutants in the presence of different atRA concentrations (Figure 2). The activity was supported by a lipid mixture that included POPC and 1 mol % DOG, but without any phosphatidylserine. Ca^{2+} was necessary since no detectable activity was observed in its absence, i.e., in the presence of 0.5 mM EGTA (not shown). It can be deduced from Figure 2 that, under these conditions, atRA was able to activate PKCα. It can also be observed that PKCαK209A/K211A behaved very similarly to the wild type. The $[atRA]_{1/2}$ calculated from the plots for these proteins were very similar (36 μM in the case of wild-type PKCα and 33 μM in the case of PKCαK209A/K211A). This mutant is affected in the lysine-rich cluster of the C2 domain, so that the activation observed with increasing concentrations of atRA did not arise from this cluster but through the calcium-binding region of the C2 domain. On the contrary, the activity of mutants PKCαD246N/D248N and PKCαN189A, both affected in the calcium-binding region of the C2 domain, was considerably depressed. The $[atRA]_{1/2}$ calculated in the case of PKCαD246N/D248N was 107 μM, much higher than the value obtained in the case of the wild-type protein. Even more drastic was the effect of the N189A mutation, since no $[atRA]_{1/2}$ could be calculated due to the low level of protein activation. Furthermore, the maximum activity of mutants PKCαD246N/D248N and PKCαN189A decreased to 70% and 25%, respectively, compared with the wild type when the atRA concentration was 300 μM. The differences observed between the basal activity of the wild-type protein and the mutants could be due to the cooperative and sequential effect of the C1 and C2 domains in the activation of the enzyme.

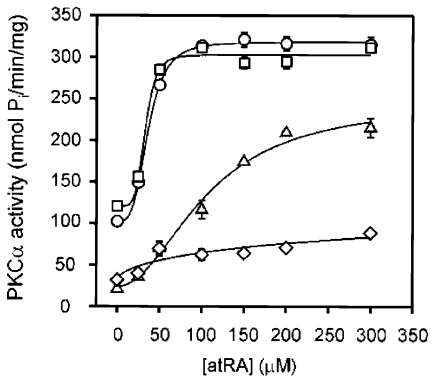

FIGURE 2: Effect of increasing atRA concentration on the catalytic activity of wild-type PKCα and C2 domain mutants. PKCα activity was measured in the presence of LUVs containing POPC/DOG (99:1, molar ratio) and with increasing concentrations of atRA. The symbols are (○) wild-type PKCα, (◇) PKCαN189A, (△) PKCαD246N/D248N, and (□) PKCαK209A/K211A. Data are the means of triplicate determinations (±SD). Since the origin of the sigmoidal dependence of enzyme activity on atRA concentration is not fully understood, the plots were analyzed graphically to determine the atRA content that resulted in half-maximal enzyme activity ($[atRA]_{1/2}$).

These results suggest that the region of the C2 domain involved in the activation of PKCα by atRA is the Ca^{2+}-binding region and not the Lys-rich cluster. Since Ca^{2+} is necessary for activation, it may be thought that atRA occupies the place of PS, as was observed in the high-resolution model obtained from X-ray diffraction experiments, where an atRA molecule was partially modeled in the vicinity of Ca1 (*30*). In this model, the acid group and few first carbon atoms of an atRA molecule were located in the area that was occupied by the Ca^{2+}/PS complex in a previous work (*31*).

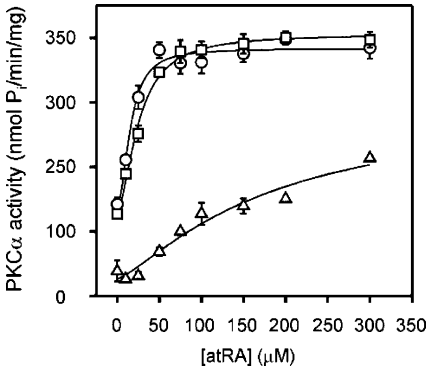

FIGURE 3: Effect of increasing atRA concentration on the catalytic activity of wild-type PKCα and C2 domain mutants. PKCα activity was measured in the presence of POPC LUVs and with increasing concentrations of atRA. The symbols are (○) wild-type PKCα, (△) PKCαD246N/D248N, and (□) PKCαK209A/K211A. Data are the means of triplicate determinations (±SD). Since the origin of the sigmoidal dependence of enzyme activity on atRA concentration is not fully understood, the plots were analyzed graphically to determine the atRA content that resulted in half-maximal enzyme activity ($[atRA]_{1/2}$).

In another experiment, in which DOG was omitted from the lipid vesicles (Figure 3), it was observed that the maximum activity was lower (160 nmol P_i/min/mg) than that observed in the presence of DOG (325 nmol of P_i min^{-1} mg^{-1}, Figure 2). However, the pattern of activation of wild-type enzyme and the mutants was very similar in the presence and in the absence of DOG. The $[atRA]_{1/2}$ values calculated in these conditions were 16 μM in the case of wild-type PKCα, 26 μM for PKCαK209A/K211A, and 179 μM for PKCαD246N/D248N, in which case the maximum activity decreased to 50% compared with the wild-type when the atRA concentration was the highest (i.e., 300 μM). This experiment showed that the presence of a small percentage of DOG did not significantly affect the behavior of the proteins toward atRA. Given the fact that the level of activity in the absence of DOG was very low, 1 mol % DOG was always used in all of the following experiments.

To explore the role of the Ca^{2+}-binding region in the activation of PKCα by atRA, several experiments were carried out in which the wild-type protein and the mutants were assayed in the presence of lipid mixtures containing increasing concentrations of POPS (Figure 4), which will mainly activate the enzyme through this site. When the proportion of POPS was 6 mol % (Figure 4A), the presence of increasing concentrations of atRA up to 50 μM produced a progressively increasing activation of wild-type PKCα. However, when atRA concentration was increased to concentrations higher than 50 μM, the activation of wild-type PKCα was reduced in parallel with the increase of atRA concentration, and when the atRA concentration was 300 μM, the enzyme showed only 60% of the maximum activity. On the other hand, the activation of mutant PKCαK209/211A was very similar to that of the wild type at concentrations lower than 50 μM atRA (Figure 4A). However, at higher concentrations, the activity of mutant PKCαK209/211A increased slightly up to 100 μM atRA, showing roughly constant activities independently of the increase of atRA concentration. However, PKCαN189 and PKCαD246N/D248N activities did not exceed 22% of the activity of the wild type, and the presence of increasing concentrations of atRA did not have any significant effect (Figure 4A).

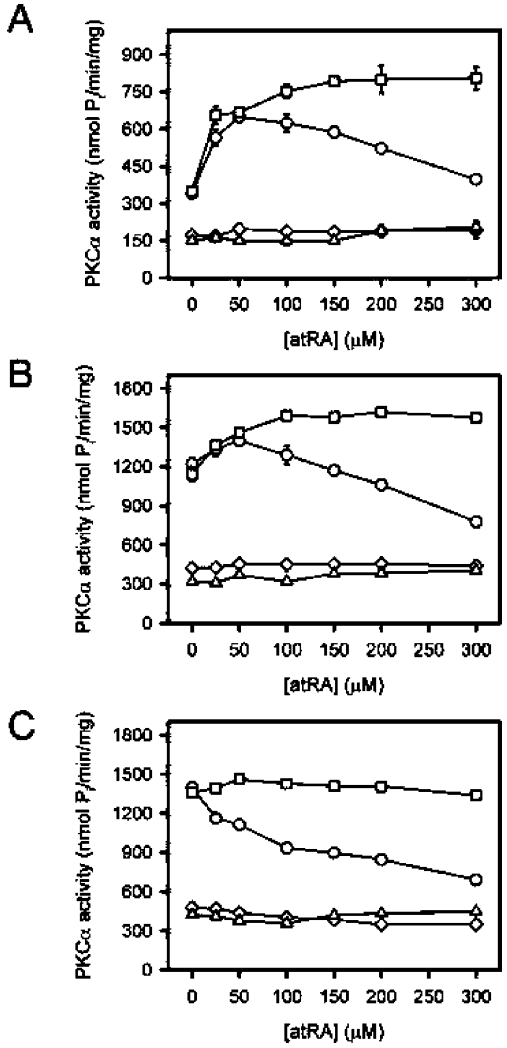

FIGURE 4: Effect of increasing atRA concentration on the catalytic activity of wild-type PKCα and C2 domain mutants mediated by POPS. PKCα activity was measured in the presence of LUVs containing POPC/POPS/DOG (99 − X:X:1, molar ratio) and with increasing concentrations of atRA. The concentrations of POPS used were 6 mol % (A), 20 mol % (B), and 50 mol % (C). The symbols are (○) wild-type PKCα, (◇) PKCαN189A, (△) PKCαD246N/D248N, and (□) PKCαK209A/K211A. Data are the means of triplicate determinations (±SD).

When the concentration of POPS was 20 mol % (Figure 4B), the activities of the wild-type protein and mutant PKCαK209/211A were very similar to those obtained with 6 mol % POPS, although in this case, the activation of wild-type PKCα fell to 55% of the maximum activity. At this POPS concentration, mutants PKCαN189A and PKCαD246N/248N did not increase their activity when atRA was increased but kept 27% of the maximum activity (Figure 4B).

Finally, when the proportion of POPS was 50 mol % (Figure 4C), wild-type PKCα showed maximum activity in the absence of atRA, and as the concentration of atRA was increased, the activity gradually decreased, so that at a concentration of 300 μM atRA, the activity was down to 50% of the maximum activity observed in the absence of atRA. On the other hand, mutant PKCαK209/K211A reached the same values of maximum activity as the wild-type PKCα, indicating that the addition of atRA had no effect (Figure

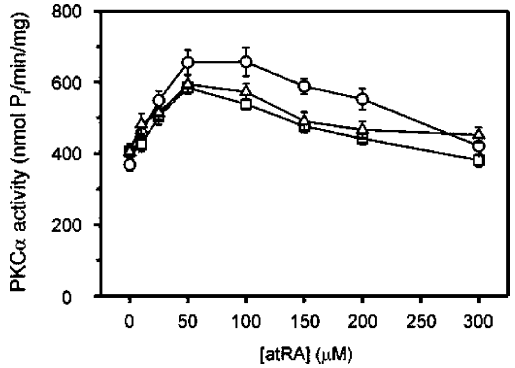

FIGURE 5: Effect of different preincubation conditions on the catalytic activity of PKCα in the presence of LUVs containing POPC/POPS/DOG (93:6:1) with increasing atRA concentrations. The symbols are (○) preincubation of LUVs with atRA (i.e., standard kinase activity assay), (□) preincubation of LUVs with PKCα, and (△) preincubation of PKCα with atRA. Data are the means of triplicate determinations (±SD).

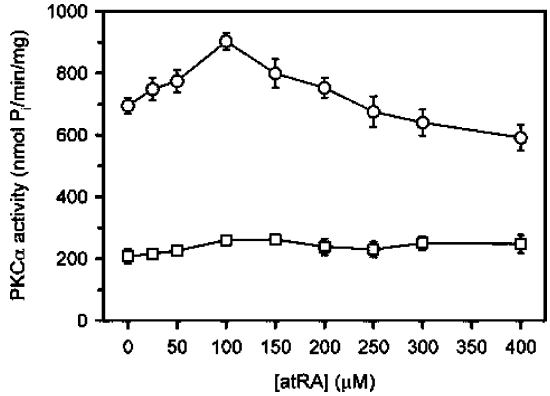

FIGURE 6: Effect of increasing atRA concentration on the catalytic activity of wild-type PKCα and a C2 domain mutant mediated by the Lys-rich cluster. PKCα activity was measured in the presence of LUVs containing POPC/PIP_2/DOG (89:10:1, molar ratio) and with increasing concentrations of atRA. The symbols are (○) wild-type PKCα and (□) PKCαK209A/K211A. Data are the means of triplicate determinations (±SD).

4C). The activity of mutants PKCαN189A and PKCαD246/248N in the presence of vesicles containing 50 mol % POPS was not affected by the presence of increasing concentrations of atRA, and the maximum activity reached by both proteins was approximately 30% that of the wild-type protein (Figure 4C), thus showing a behavior similar to that of the other POPS concentrations discussed above.

Taken altogether, these results indicate that atRA has a lower PKCα activation capacity than POPS (compare Figures 2, 3, and 4). Nevertheless, at low POPS percentages, an increase in atRA concentration produced a certain increase in activity. However, it is clear that the low activation induced by POPS in mutants PKCαN189A and PKCαD246N/D248N, which were not activated by atRA, is proof of the importance of the Ca^{2+}- and POPS-binding site in the C2 domain for the activation effect. The case of mutant PKCαK209A/K211A is interesting since it was not inhibited by atRA, suggesting that this Lys-rich cluster is somehow involved in the inhibiting effect.

At this point, it should be investigated whether the C2 domain binds primarily to free atRA or to membrane-bound atRA. AtRA must be presumably incorporated into membranes as it has a hydrophobic character. It has been demonstrated that atRA dissolved in ethanol incorporated into model membranes as previously described (*40*). Furthermore, several in vivo works show that the addition of atRA to culture cells produces different cellular effects, reflecting the penetration of atRA through the membranes (*41*−*44*). To discard the possibility that the biphasic effect observed in the activation of PKCα by atRA in the presence of low concentrations of POPS is due to a different accessibility of the protein to atRA (i.e., free atRA and membrane-bound atRA), the PKCα activation was measured under three different conditions: one of them consisted of the standard kinase activity assay (see Materials and Methods section) with the preincubation of the liposomes with atRA; the second one was preincubating PKCα with the liposomes for 10 min before starting the reaction; and the third one supposed the preincubation of PKCα with atRA for 10 min. As shown in Figure 5, the three conditions rendered very similar results with a biphasic behavior, confirming that the accessibility of PKCα to atRA was independent of the preincubation conditions used in the activity assay. Hence, the protein would bind similarly through its C2 domain to free atRA or to atRA already incorporated to the membrane.

Role of the Lys-Rich Cluster in the Interaction of PKCα with atRA. An X-ray diffraction study (*30*) revealed that atRA may interact with this region of the C2 domain and, in particular, with residues K197, K209, and K211, suggesting that this could be the way in which PKCα activity is modulated. In fact, the above experiments indicated that atRA did not inhibit protein PKCαK209/K211 when activated by POPS (see Figure 4), suggesting that atRA may inhibit the enzyme through this site. On the other hand, previous studies in our laboratory have shown that the Lys-rich cluster is the main point of interaction with PIP_2, which gives rise to PKCα activation, and that residues K209 and K211 play a fundamental role in this interaction.

To test the capacity of atRA to interact with this region of the C2 domain, the activity of PKCα was assayed in the presence of lipid vesicles containing PIP_2 (POPC/DOG/PIP_2, 89:10:1 molar ratio). Figure 6 shows that wild-type PKCα was slightly activated by increasing concentrations of atRA up to 100 μM, reaching a maximum activity of 893 nmol of P_i min^{-1} mg^{-1}, which means an increase of 26% with respect to the activity in the absence of atRA (705 nmol of P_i min^{-1} mg^{-1}). At higher concentrations of atRA, the activity decreased so that it was only 591 nmol of P_i min^{-1} mg^{-1} at 400 μM atRA. It seems that the effect of atRA on this activation is relatively similar (in the sense that it is biphasic) to those observed in panels A and B of Figure 4 for the activation in the presence of low concentrations of POPS. The slight activation observed at low atRA concentrations may be due to activation through the Ca^{2+}-binding region. The mutant protein PKCαK209/211A shows substantially lower activity in the absence of atRA (about 200 nmol of P_i min^{-1} mg^{-1}), and this activity was hardly modified by the addition of atRA up to 400 μM. The low level of activity of this mutant when the activation is carried out by PIP_2 reflects that this phospholipid operates mainly through the Lys-rich cluster. The reduced level of activity shown may depend on activation of this protein by the phospholipid through the Ca^{2+}-binding region, and the lack of effect of atRA in this

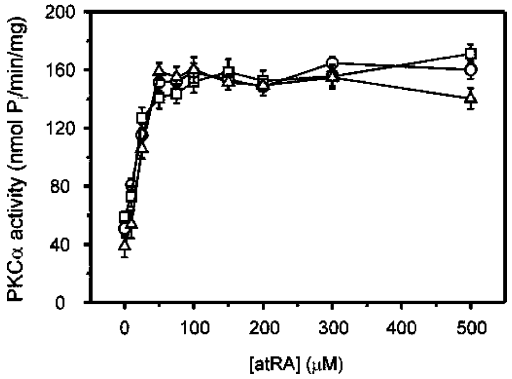

FIGURE 7: Effect of atRA on the catalytic activity of wild-type PKCα and C1 domain mutants. PKCα activity was measured in the presence of POPC LUVs and with increasing concentrations of atRA. The symbols are (○) wild-type PKCα, (□) PKCαW58G, and (△) PKCαF60G. Data are the means of triplicate determinations (±SD).

case is reminiscent of the effect seen for 50 mol % POPS (Figure 4C), indicating that, at this phospholipid concentration, atRA may not have access to this activation site.

Involvement of the C1 Domain in the PKCα Activation by atRA. To investigate the possible involvement of the C1 domain on the interaction of PKCα with atRA, key residues from the C1A domain were mutated, thus giving PKCαW58G and PKCαF60G. Both residues (W58 and F60) have been described as being essential for the activation of PKCα through the C1 domain (*39*). Enzymatic assays were carried out with lipid vesicles containing only POPC and different concentrations of atRA (from 0 to 500 μM). Figure 7 shows that the activities shown by both mutants were undistinguishable from those of the wild-type protein in the range of atRA concentrations assayed, confirming that the C1 domain was not involved in the activation of this enzyme with atRA.

DISCUSSION

The results shown in this paper confirm that atRA is able of interacting directly with PKCα, modulating its activity and thus confirming the importance of previous structural data, which showed that this compound interacted with two different sites of the C2 domain (*30*). The effect is nevertheless complex, since it may be activated in the absence of POPS and at low concentrations of this phospholipid; however, it may also act as an inhibitor at least at relatively high concentrations of POPS.

Other authors have previously found that atRA may influence the activity of PKCs, although our results differ in some aspects from these previous reports. For example, we have found that, in the presence of Ca^{2+} and in the absence of other activating phospholipids, atRA will activate PKCα, in agreement with some previous studies (*27*, *28*) but in contrast with the findings of others, in which atRA was considered solely as an inhibitor (*24*−*26*). Another important issue is to identify the part of the PKC protein which is responsible for the interaction with atRA. Radominska-Pandya et al. (*26*) compared sequences of PKCα and PKCβ with those of several known receptors of atRA and suggested a certain area situated between CBR1 and CBR3 of the C2 domain. A more recent study, which described the high-resolution structure of the C2 domain of PKCα, suggested that atRA binds to two different sites, the Ca^{2+}/PS-binding region and the Lys-rich cluster localized in the β3−β4 strands (*30*).

First of all, our results confirm that the activation caused by atRA is clearly associated to the Ca^{2+}/PS-binding region in the C2 domain, since it is dependent on the presence of Ca^{2+}, while mutants such as PKCαD246/248N, which affects Ca^{2+} binding, and PKCαN189A, which affects PS binding, inhibited PKC activity (Figure 2). Therefore, it can be concluded that the activation effect of atRA is indeed through this site of the C2 domain.

AtRA, however, has also been found to be able of inhibiting PKC activity when added in the presence of activating phospholipids, such as PS and PIP_2. The effect was not observed with mutant PKCαK209/211A, suggesting that the inhibiting effect is dependent on the Lys-rich cluster. Since, at 6 and 20 mol % POPS, atRA was activating at low concentrations and inhibitory only at higher concentrations, it seems likely that there are two simultaneous effects of atRA, one at the Ca^{2+}/PS site, which is activating, and the other at the Lys-rich cluster, which is inhibitory. At 50 mol % POPS, the effect was inhibitory even at low atRA concentrations, which may be due to the inability of atRA to bind to the activating site at this concentration of PS, although it does seem to be able to displace PS from the inactivating site, even at this high concentration of POPS. The biphasic effect observed in the case of PIP_2 seems to be similar to that seen with lower POPS concentrations.

We have previously shown that PS binds to both the Ca^{2+}-binding region and the Lys-rich cluster on the C2 domain of PKCα (*32*), but whereas it is clear that its binding to the first site is essential to activate PKC, the consequences of its binding to the second site are not clear. It has been speculated that the Lys-rich cluster may be involved in the tethering of the C2 domain to the C1A domain, so that binding of POPS will contribute to a more active conformation for PKC (*32*). If this were the case, the binding of atRA to this site may disturb the adoption of the right conformation by PKC, explaining the inhibition.

In the case of PIP_2, we have previously shown that the activation afforded by this phospholipid is mainly through the Lys-rich cluster (*35*, *45*). The presence of increasing concentrations of atRA will interfere with the accession of PIP_2 to this site, thus explaining the inhibition.

The C1 domain does not seem to be involved in the interaction of PKCα with atRA, since when atRA activates in the absence of POPS, increasing concentrations of DOG do not overcome the effect of mutating essential residues located in the Ca^{2+}-binding region, such as D246 and D248 or N189. In addition, the mutation of important residues in the C1A domain of PKCα (W58 and F60) did not alter the activating pattern shown by atRA.

In summary, it has been shown that atRA activates PKCα in the absence or at low concentrations of activating phospholipids, such as POPS or PIP_2, in a Ca^{2+}-dependent activation through the Ca^{2+}/PS-binding site of the C2 domain. On the other hand, atRA may also act as an inhibitor of PKCα done through the Lys-rich cluster, also in the C2 domain.

Our results may hence explain, at least in part, why some authors have observed activation of PKC by atRA, whereas others have reported inhibition. Great care should be taken

in this case when extrapolating results obtained with a certain range of atRA concentrations to others.

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