High-Affinity Ca^{2+}- and Substrate-Binding Sites on Protein Kinase C α As Determined by Nuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: Water proton nuclear magnetic resonance (NMR) relaxation rates were used to identify metal sites on protein kinase C (PKC) isozymes α and β using paramagnetic Gd^{3+} as a probe. The paramagnetic effect of Gd^{3+} on water proton relaxation was enhanced with PKC isozymes α and β in the presence of diheptanoylphosphatidylcholine/1,2-dioleoyl-*sn*-glycerol (PC_7/DO). The data are consistent with a single class of metal-binding sites on PKCβ and two classes of sites on PKCα: a single high-affinity site with a K_D for Gd^{3+} of 0.2 μM and a larger class of sites with a lower affinity for Gd^{3+}. Titration with Ca^{2+} abolished the observed enhancement of water proton relaxation by the PKCα·Gd^{3+} complex, consistent with displacement of Gd^{3+} by Ca^{2+}. Titrations of the PKCα·Gd^{3+} complex with $Co(NH_3)_4$ATP, a substitution-inert analogue of ATP, caused a substantial decrease in the observed water proton relaxation enhancement, consistent with formation of a ternary enzyme·metal·substrate complex with a $K_{PKC\alpha\cdot Gd\cdot[CoATP]}$ of 30–100 nM. Titration of the metal enzyme complex with a model peptide substrate derived from the pseudosubstrate sequence of PKCα caused a similar decrease in enhancement at stoichiometric concentrations consistent with the formation of a PKCα·Gd^{3+}·peptide complex with a $K_{PKC\alpha\cdot Gd\cdot[peptide]}$ of $\leq$13 nM. Titrations of the fully formed PKCα·Gd^{3+}·peptide complex with $Co(NH_3)_4$ATP caused a further decrease in enhancement consistent with formation of a quaternary complex. Control titrations in the absence of enzyme showed no significant interactions between Gd^{3+} and PC_7/DO or between Gd^{3+} and any of the substrate analogues, arguing for direct binding of Gd^{3+} to the enzyme. These results are consistent with binding of Gd^{3+} to a Ca^{2+} site on PKCα which interacts effectively with metal–nucleotide and peptide substrates located at or near the active site.

Limited information is available on the Ca^{2+}-binding properties of protein kinase C (PKC).[1] PKC isozymes α, β, and γ all require Ca^{2+} for membrane binding, kinase activity, and phorbol ester binding [reviewed in Nishizuka (1988)] but the structural and functional bases for these effects are poorly understood. Ca^{2+}-binding motifs, such as EF hands, are not readily apparent in the primary sequences of PKC isozymes. However, the C2 domain, conserved in the regulatory region of PKC isozymes α, β, and γ but absent in the Ca^{2+}-independent δ, ϵ, and ζ isozymes, has been postulated to confer Ca^{2+} binding to PKC isozymes α, β, and γ. In 1986, a Ca^{2+} bridge mechanism involving four phosphatidylserine (PS) molecules and one diacylglycerol was proposed for PKC activation (Ganong et al., 1986; Hannun et al., 1985). Direct evidence supporting this model was recently provided by Bazzi and Nelsestuen (1990). They showed, using equilibrium dialysis with ^{45}Ca, that a mixture of PKC isozymes binds Ca^{2+} in a PS-dependent manner.

Only minor differences in the Ca^{2+} requirements for activity of PKC isozymes α, β, and γ have been observed in PS vesicle systems (Jaken & Kiley, 1987; Huang et al., 1988; Sekiguchi et al., 1988). However, comparison of the activities of PKC isozymes α and β in the presence of PS/DO vesicles vs diheptanoylphosphatidylcholine (PC_7)/DO micelles revealed major differences in Ca^{2+} requirements (Walker et al., 1990). It was proposed that Ca^{2+} facilitates association of PKCβ with bilayers or vesicles but that PKCα has a Ca^{2+} requirement that is independent of phospholipid.

The difficulties associated with separation and purification of PKC isozymes in quantity have made direct metal-binding studies to evaluate the Ca^{2+} sites difficult. To overcome these limitations, we have considered the use of sensitive water proton relaxation measurements of complexes of PKC with paramagnetic probes. One such probe is Gd^{3+}, which has been used as a paramagnetic substitute for Ca^{2+} in numerous enzymes and other proteins (Stephens & Grisham, 1979; Klemens et al., 1986; Klemens & Grisham, 1988; Dwek et al., 1971; Valentine & Cottam, 1973).

Evaluation of Gd^{3+} as a Ca^{2+} substitute for PKC (Maurer et al., 1992) revealed that interactions between Gd^{3+} and PKC are complex, involving more than one class of sites. PKCβ was stimulated by Ca^{2+} or Gd^{3+} in PS/DO, whereas activity in PC_7/DO was independent of these metals. Activity of PKCα was dependent on low concentrations of Ca^{2+} in both lipid systems. Successful reduction of contaminating Ca^{2+} without the use of chelators could only be achieved in the PC_7 system. Gd^{3+} failed to substitute for Ca^{2+} in activating PKCα and only caused inhibition. On the basis of these kinase activity assays, we proposed that Gd^{3+} may bind either to a class of Ca^{2+} sites involved in enzyme–lipid interactions (PKCβ) or to a class of sites functioning at or near the active site (PKCα). In addition, results indicated that GdATP competes at a

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[1] Abbreviations: PKC, protein kinase C; PS, phosphatidylserine; DOPS, dioleoylphosphatidylserine; PC, phosphatidylcholine; PC_7, diheptanoylphosphatidylcholine; DO, 1,2-dioleoyl-*sn*-glycerol; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid.

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MgATP site. Conditions for separately evaluating the individual sites were proposed.

In the present paper, we have used Gd^{3+} as a spectroscopic probe of Ca^{2+} sites to evaluate further the differences in metal-binding properties of PKCα and PKCβ. Water proton relaxation rate measurements have provided information on the stoichiometry and affinity of Gd^{3+} binding to these two PKC isozymes in the presence of PC_7/DO. The data are consistent with the existence of a single, high-affinity Gd^{3+} site on PKCα, which is not observed in PKCβ. PKCα·Gd^{3+}·substrate complexes were characterized using suitable nucleotide [$Co(NH_3)_4ATP$] and substrate analogues. Results are consistent with the binding of Gd^{3+} to a Ca^{2+} site at or near the active site of PKCα.

EXPERIMENTAL PROCEDURES

Materials. $GdCl_3$ was from Aldrich (Gold Label) (Milwaukee, WI). Chelex-100 resin was obtained from Bio-Rad (Richmond, CA). PC_7 was purchased from Avanti Polar Lipids (Birmingham, AL) and DO was purchased from Sigma (St. Louis, MO).

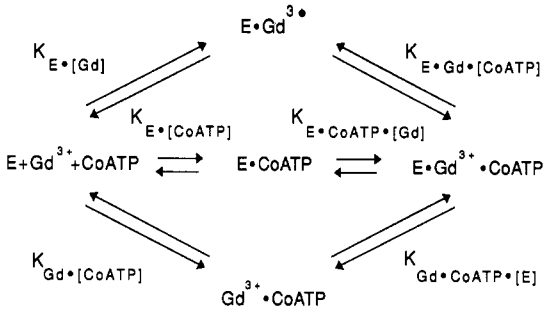
α-Peptide (RFARKGSLRQKNV) was purchased from Peptide Technologies Corp. (Washington, DC). It was made with a solid-phase peptide synthesizer using BOC chemistry and purified by reverse-phase chromatography. The peptide contained the requisite amino acids as determined by amino acid analysis and had a final purity of 92%.

Purification of PKC Isozymes. PKC was purified from rat brain by sequential chromatography on DEAE-cellulose (Whatman DE-52), phenyl-Sepharose, Q-Sepharose (Pharmacia), and phenyl-Superose (Pharmacia HR 10/10). Isozymes α and β were resolved on a hydroxyapatite column (Koken, 10 × 0.6 cm). The exact conditions for the purification are described in detail elsewhere (Maurer et al., 1992). Separated isozymes were concentrated and desalted by ultrafiltration using Amicon Centriprep-30 and Centricon-30 units. In order to stabilize the enzyme during the concentration process, 250 μM PC_7 was added to the PKCβ samples and 400 μM PC_7 was added to the PKCα samples. Lipids were prepared in the form of micelles as described elsewhere (Maurer et al., 1992). Phosphate concentrations were decreased to less than 1 μM by repetitive dilutions with 20 mM PIPES, 10% glycerol, and the appropriate concentrations of PC_7, followed by concentration via ultrafiltration. The desalted samples were concentrated 100-fold and stored at −70 °C. The concentrations of the isozyme samples were determined by phorbol ester binding (Sando & Young, 1983).

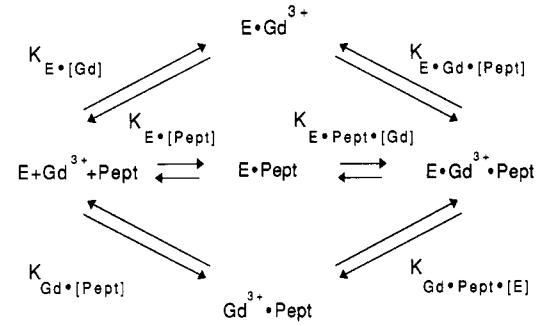
Synthesis of $Co(NH_3)_4ATP$. Substitution-inert $Co(NH_3)_4$-ATP was synthesized as a mixture of the diastereomers Δ and Λ, according to the procedure of Cornelius et al. (1977) and stored at −20 °C. Before each experiment $Co(NH_3)_4ATP$ was treated with Chelex-100 resin to remove traces of free cobalt and the sample was bubbled with O_2 in order to maintain all of the cobalt in the diamagnetic Co(III) form. Work by McClaugherty and Grisham (1982) has demonstrated negligible degradation of $Co(NH_3)_4ATP$ at pH 7.5 and 25 °C.

Kinase Activity Assays Involving $Co(NH_3)_4ATP$. Kinase activity was assessed by the ability of PKC to phosphorylate lysine-rich histone as described by Walker and Sando (1988). Reactions (75 μL) contained 20 mM PIPES (pH 7.4 at 4 °C), histone (0.2 mg/mL), 3 mM magnesium acetate, 300 μM $CaCl_2$, PKCα (1.26 nM), and [γ-^{32}P]ATP (585–780 mCi/mmol) with or without PC_7/DO (400 μM/20.4 μM). The concentration of ATP varied from 3 to 70 μM and the Co-$(NH_3)_4ATP$ concentrations tested were 0.75 and 1.50 mM.

Scheme I



Scheme II



PC_7 and DO, originally stored in chloroform, were dried down under N_2 and then resuspended in PIPES by extensive vortexing. Assays were terminated after incubation for 5 min at 30 °C by spotting 60 μL of reaction mix onto P-81 cation-exchange paper (Whatman) and removing unreacted ATP with four washes in 50 mM NaCl. Papers were dried and bound ^{32}P was quantitated by scintillation counting. All experiments were conducted in polypropylene tubes.

Solutions for NMR Experiments. All solutions were prepared with distilled, deionized water, and trace levels of contaminating metals were removed by treating the solutions with Chelex-100 resin. Stock solutions of $GdCl_3$ were prepared in 1% HCl. Additions of metal were then made into enzyme solutions with a pH of 7.45. Gd^{3+} is more soluble at pH 6.8 but PKC activity is greatly reduced in this range. Water proton relaxation rate studies of solutions containing Gd^{3+} and 20 mM PIPES show no significant loss of Gd^{3+} at the pH values used and at concentrations up to 80 μM (data not shown).

NMR Measurements. Water proton NMR measurements were performed on a variable frequency pulsed NMR spectrometer of our own design. The magnet is a Varian 4012A electromagnet with a 2100-A power supply modified for solid-state operation. The radio frequency components and the pulse programmer were designed and built by Seimco of New Kensington, PA. The frequency source is a Harris PRD7838 frequency synthesizer. All measurements were carried out at a probe temperature of approximately 21 °C.

Beckman Ultraclear centrifuge tubes (5 × 41 mm) were used as sample cells. Contaminating metal ions were removed by soaking the tubes in 2 mM EDTA, then 2 mM HCl, and finally in distilled deionized water. Samples (50 μL total) contained 2–5 μM PKC, as indicated in the figure legends.

Spin–lattice relaxation times (T_1) were measured using an inversion recovery pulse sequence $(180°-\tau-90°-5T_1)_n$. T_1 values were calculated from

$$T_1 = \tau(\text{null})/\ln 2 \qquad (1)$$

The paramagnetic contribution to the relaxation rate ($1/T_{1p}$) was calculated as the difference between the relaxation rate

of a sample in the presence ($1/T_{1(obs)}$) and absence ($1/T_{1(0)}$) of Gd^{3+}. The observed enhancement values (ϵ^*) were assessed from the ratio of $1/T_{1p}$ measured for enzyme·Gd^{3+} or enzyme·Gd^{3+}·substrate complexes ($1/T^*_{1p}$) to the $1/T_{1p}$ measured for the same metal solutions without enzyme ($1/T^0_{1p}$):

$$\epsilon^* = \frac{1/T^*_{1p}}{1/T^0_{1p}} = \frac{1/T^*_{1(obs)} - 1/T^*_{1(0)}}{1/T^0_{1(obs)} - 1/T^0_{1(0)}} \quad (2)$$

The observed enhancement (ϵ^*) values represent the weighted sum of individual enhancements for each species in solution. $\epsilon^* = \sum \chi_i \epsilon_i$ where χ_i is the mole fraction of the ith species and ϵ_i is the enhancement due to the ith species. The theoretical basis for these water proton relaxation rate equations has been reviewed by Mildvan and Engle (1972).

Computer Programs for Fitting Water Relaxation Data. For experiments involving the binding of Gd^{3+} to PKCα, best fits for enhancement plots were obtained from the computer program SigmaPlot. The equation used assumes the macromolecule contains identical independent binding sites and each site has the same microscopic ligand dissociation constant. Expressions describing the equilibrium binding of ligand are incorporated into the equation for ϵ^*.

For experiments involving the titration of PKCα·Gd^{3+} with substrates $Co(NH_3)_4$ATP and α-peptide, the REED3 program was used. REED3 is adapted from a program kindly provided by Dr. George Reed, Institute for Enzyme Research, University of Wisconsin, Madison, WI. This program uses a Marquardt algorithm for the solution of simultaneous linear equations (Reed et al., 1970). The equilibria for systems containing $Co(NH_3)_4$ATP and α-peptide are described in Schemes I and II. In the equilibrium expressions, the brackets indicate the species which dissociate. A $K_{Gd\cdot[CoATP]}$ value of 16 mM, measured previously by Gd^{3+}-EPR (Klemens, 1987), was used in the REED3 program. From water proton relaxation rate studies, the $K_{Gd\cdot[pept]}$ value was calculated to be ≥0.5 mM. For the fitting procedures involving nucleotide and peptide substrates, values of $K_{E\cdot[Gd]}$, $K_{E\cdot Gd\cdot[substrate]}$, $K_{Gd\cdot[substrate]}$, and $K_{E\cdot[substrate]}$ were varied over suitable ranges to obtain best-fit values. REED3 solves the conservation equations for metal, substrate, and enzyme and the dissociation constant equations for the six types of equilibria shown in Schemes I and II. Best-fit values describing all species in solution are then used to fit enhancement equations. For more details on the use of this program, refer to Devlin and Grisham (1990).

The METFIT program was used to curve-fit the results for the experiments involving displacement of Gd^{3+} bound to PKCα by Ca^{2+}. This program assumes that a paramagnetic metal and a diamagnetic metal compete for two classes of sites on a macromolecule and can be used to obtain best-fit values for dissociation constants for both metals at both classes of sites and water proton relaxation enhancements for the paramagnetic metal. METFIT uses a Marquardt algorithm and is similar to REED3.

RESULTS

Metal-Binding Properties of PKC Cofactors. Water proton relaxation rate measurements were used to assess binding of Gd^{3+} to the lipid system PC_7/DO and/or the PKC substrates $Co(NH_3)_4$ATP and α-peptide. Figure 1 shows the behavior of the observed enhancement of the longitudinal water proton relaxation rate when Gd^{3+} is used to titrate solutions of dioleoylphosphatidylserine (DOPS) and PC_7. No significant enhancement of water proton relaxation is observed in PC_7 micelles, demonstrating that there is no substantial binding of Gd^{3+} to PC_7 in the range of metal concentrations studied. In contrast, the addition of small amounts of Gd^{3+} to multilamellar vesicles of DOPS caused a large enhancement of water proton relaxation, consistent with the formation of a DOPS·Gd^{3+} complex. Such complexes would complicate studies of Gd^{3+} binding to PKC, and thus DOPS is an inappropriate lipid system for study of PKC·Gd^{3+} interactions. Water proton relaxation rate measurements were also performed in solutions of Gd^{3+} and PC_7/DO (400 μM/20.4 μM), $Co(NH_3)_4$ATP (5–900 μM), and α-peptide (50 μM). In all cases, no significant binding of Gd^{3+} occurred (data not shown). These results suggest that any changes in relaxation enhancement observed in solutions containing PKC isozymes will involve direct interaction between PKC isozymes and Gd^{3+}.

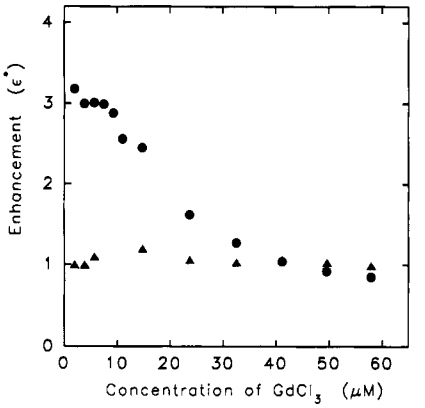


FIGURE 1: Effect of Gd^{3+} on enhancement of the water proton relaxation rate of DOPS and PC_7. Enhancement values were determined for titrations of 50 μM DOPS (●) and 400 μM PC_7 (▲) in 20 mM PIPES and 10% glycerol (pH 7.45) with the indicated concentrations of Gd^{3+}. The original sample volume was 50 μL and titrations involved 1-μL additions of metal. Results are representative of at least two separate experiments with each lipid system.

Binding of Gd^{3+} to PKCα and PKCβ. The binding of Gd^{3+} was studied in solutions containing PC_7/DO. Experiments could not be conducted in the absence of PC_7 since lipid was needed to stabilize the enzyme during the concentration and desalting procedure.

The paramagnetic effect of Gd^{3+} on water proton relaxation was enhanced in solutions containing PC_7/DO and PKCβ. As shown in Figure 2, the ϵ^* values for this isozyme remained at approximately 3 over a Gd^{3+} range of 0.5–75 μM. These titrations were not carried out further to determine stoichiometry of binding, due to a decreased solubility of Gd^{3+} in 20 mM PIPES/10% glycerol, pH 7.45, at concentrations greater than 80 μM. As a result of this inability to fully saturate the enzyme with Gd^{3+}, affinity of metal binding could also not be assessed. The results are, however, consistent with a model in which PKCβ contains a metal-binding region which attracts a large number of Gd^{3+} ions (greater than 15 per enzyme). For these water proton relaxation rate studies, PKCβ had to be carefully screened for contamination by PKCα and PKCγ. Experiments conducted with samples of PKCβ less pure than that used in Figure 2 resulted in the appearance of an apparent high-affinity high-enhancement site ($\epsilon^* = 5$) in addition to the class of sites which binds a large number of Gd^{3+} ions. This high ϵ^* site is therefore proposed to be due to the presence of contaminating PKC isozymes.

Water proton relaxation enhancement was also observed in solutions containing PC_7/DO and PKCα (Figure 3). At high concentrations of Gd^{3+}, a large class of low-enhancement

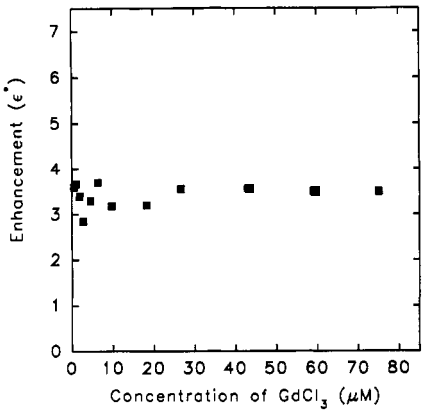


FIGURE 2: Effect of Gd^{3+} on enhancement of the water proton relaxation rate of PKCβ. Enhancement values were determined for titrations of 4 μM PKCβ in 20 mM PIPES, 10% glycerol, and 250 μM PC_7/12.5 μM DO (pH 7.45) with the indicated concentrations of Gd^{3+}. The original sample volume for PKC and lipid was 51 μL and titrations involved 1-μL additions of metal. Corrections in enhancement were made to accommodate this dilution of experimental components. Results are representative of two independent experiments.

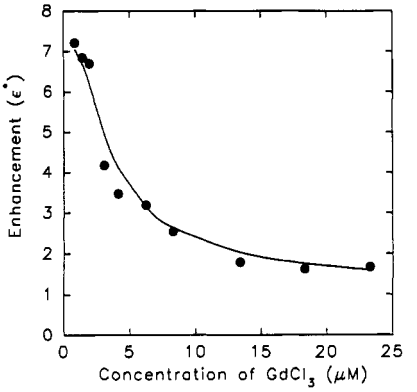


FIGURE 3: Effect of Gd^{3+} on enhancement of the water proton relaxation rate of PKCα. Enhancement values were determined for titrations of 2 μM PKCα in 20 mM PIPES, 10% glycerol, and 800 μM PC_7/20.4 μM DO (pH 7.45) with the indicated concentrations of Gd^{3+}. Titration procedures and dilution corrections similar to those of Figure 2 were employed. The best-fit curve was derived from the computer program SigmaPlot using the following parameters: number of classes of sites (n) = 1, K_D = 0.2 μM, binding site concentration (P_{tot}) = 2 μM, and bound enhancement (ϵ_b) = 8.0. Results are representative of two independent experiments.

sites was observed (data not shown). However, at low concentrations of Gd^{3+}, a much higher water proton relaxation enhancement was observed. The large observed enhancement of the water proton relaxation rate at low concentrations of Gd^{3+} is consistent with the formation of a tight binary PKCα·Gd^{3+} complex. As the concentration of Gd^{3+} is increased, the observed enhancement (ϵ^*) decreases toward unity. This is consistent either with (a) saturation of the Gd^{3+} sites on the enzyme and a consequent increase in the concentration of free Gd^{3+} or with (b) binding of Gd^{3+} to a second class of sites with a much lower affinity and a much lower enhancement. In the first case, the data could be fit by an equation of the form

$$[Gd]_T\epsilon^* = [Gd]_f\epsilon_f + [Gd]_b\epsilon_b \quad (3)$$

where the subscripts on the concentration and enhancement terms denote total Gd^{3+} (T), free Gd^{3+} (f), and bound Gd^{3+} (b). The data of Figure 3 were fit using the computer program SigmaPlot, assuming one class of metal-binding sites on the protein. The data are consistent with the presence of a single

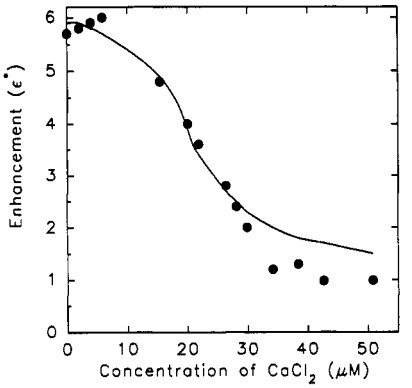


FIGURE 4: Displacement of Gd^{3+} bound to PKCα by Ca^{2+}. Solutions (51 μL) containing 3 μM PKCα, 20 mM PIPES, 10% glycerol, and 400 μM PC_7/20.4 μM DO (pH 7.45) were titrated with 1-μL additions of Gd^{3+} to a final metal concentration of 2.8 μM (volume 54 μL) followed by 1-μL additions of Ca^{2+} at the indicated concentrations. Corrections in enhancement were made to accommodate the dilution of experimental components. The data were fit with the computer program METFIT and the corresponding K_D values for Ca^{2+} and Gd^{3+} can be found in the Results section. The theoretical fit corresponded to 93% displacement of Gd^{3+} from the single high-affinity site at the end of the titration (50 μM Ca^{2+}).

high-affinity Gd^{3+} site on PKCα with a K_D of 0.2 μM and a relaxation enhancement factor of 8.0.

Displacement of Bound Gd^{3+} by Ca^{2+}. To determine whether the Gd^{3+} interaction with PKCα is at a Ca^{2+} site, a solution containing 3 μM PKCα and 2.8 μM Gd^{3+} in PC_7/DO was titrated with Ca^{2+}. As shown in Figure 4, the observed enhancement for the complex decreased from 5.9 to 1 over an added Ca^{2+} concentration range of 0–50 μM. At high concentrations of Ca^{2+}, essentially all of the paramagnetic effect of Gd^{3+} on the water proton relaxation rate is removed, suggesting a complete displacement of Gd^{3+} by Ca^{2+}. The enhancement plateau at low [Ca^{2+}], which is followed by a relatively sharp decline in enhancement as [Ca^{2+}] is increased, is inconsistent with the binding of Ca^{2+} (and Gd^{3+}) to a single class of metal-binding sites. The data of Figure 4 were fit to a model assuming two classes of binding sites for Gd^{3+} and Ca^{2+} on the enzyme. The best fit to the data consisted of a single site (to be referred to as class 1) with dissociation constants for Gd^{3+} and Ca^{2+} of K_{D1Gd} = 0.4 μM and K_{D1Ca} = 0.5 μM and a water proton relaxation enhancement factor, ϵ_{b1}, of 8.2, and a set of seven sites (to be referred to as class 2) with dissociation constants for Gd^{3+} and Ca^{2+} of K_{D2Gd} = 50 μM and K_{D2Ca} = 0.3 μM and an enhancement factor, ϵ_{b2}, of 4.0. The value of K_{D1Gd} agrees well with the best-fit value for the site observed in Figure 3. The K_D values determined for Ca^{2+} should be viewed as lower limit values, due to low levels of endogenous Ca^{2+} (estimated to be approximately 10 μM).

Effect of $Co(NH_3)_4ATP$ on the Activity of PKCα. The finding of a single, high-affinity Gd^{3+} site on PKCα, together with the observation that Gd^{3+} inhibits but does not activate PKCα in PC_7/DO (Maurer et al., 1992), raised the possibility that Ca^{2+} may be located at or near the active site. If so, it may be possible to observe interactions between Gd^{3+} and the nucleotide and protein substrates. However, the study of PKCα·Gd^{3+} interactions in the presence of nucleotides may be complicated by the high affinity of Gd^{3+} for nucleotide substrates, especially in light of our observation (Maurer et al., 1992) that Gd^{3+} may interact with PKC by binding as GdATP. Thus in order to characterize PKCα·Gd^{3+}·nucleotide interactions, we have used the substitution-inert metal·ATP complex, $Co(NH_3)_4ATP$. Since $Co(NH_3)_4ATP$ binds Gd^{3+}

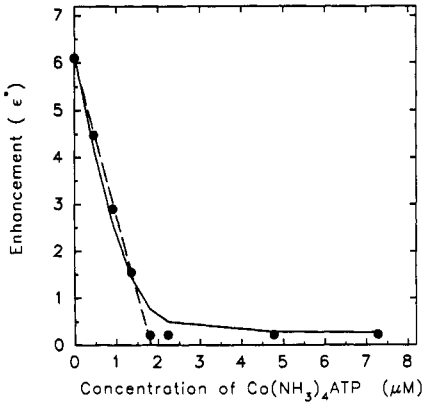


FIGURE 5: Effect of $Co(NH_3)_4ATP$ on enhancement of the water proton relaxation rate of Gd^{3+}·PKCα. Solutions (51 μL) containing 2 μM PKCα, 20 mM PIPES, 10% glycerol, and 800 μM $PC_7/20.4$ μM DO (pH 7.45) were titrated with 1-μL additions of Gd^{3+} to a final metal concentration of 1.2 μM (volume 54 μL) and then further titrated with 1-μL additions of $Co(NH_3)_4ATP$ at the indicated concentrations. Corrections in enhancement were made to accommodate the dilution of experimental components. The dashed lines emphasize the stoichiometric relationship between PKC and substrate in this titration. The solid curve shows a representative best fit to the data using values within the ranges shown in Table I. For this case, K_{EM} = 0.4 μM, K_{ES} = 100 nM, K_{MS} = 16 mM, K_{EMS} = 59 nM, ϵ_{EM} = 8, ϵ_{MS} = 1.4, and ϵ_{EMS} = 0.2. Results are representative of two independent experiments.

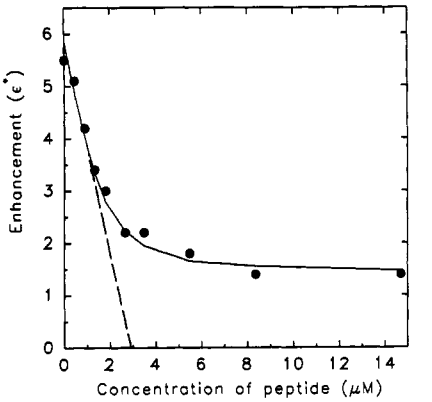


FIGURE 6: Effect of α-peptide on enhancement of the water proton relaxation rate of Gd^{3+}·PKCα. Solutions (51 μL) containing 2 μM PKCα, 20 mM PIPES, 10% glycerol, and 400 μM $PC_7/20.4$ μM DO (pH 7.45) were titrated with 1-μL additions of Gd^{3+} to a final metal concentration of 2 μM (volume = 54 μL) followed by 1-μL additions of α-peptide at the indicated concentrations. Corrections in enhancement were made to accommodate the dilution of experimental components. The dashed lines emphasize the near stoichiometric relationship between PKC, Gd^{3+}, and substrate. The solid curve shows a representative best fit to the data using values within the ranges shown in Table I. For this case, K_{EM} = 0.4 μM, K_{ES} = 16 μM, K_{MS} = 0.5 mM, K_{EMS} = 13 nM, ϵ_{EM} = 7.5, ϵ_{MS} = 1.8, and ϵ_{EMS} = 1.4. Results are representative of two independent experiments.

with very low affinity (K_D = 16 mM; Klemens, 1987), addition of this metal nucleotide to solutions of Gd^{3+} and PKCα will not interfere with binding of Gd^{3+} to the high-affinity site on PKCα. It has been shown that $Co(NH_3)_4ATP$ substitutes effectively for MgATP in a variety of enzymes and may function either as a nonhydrolyzable inhibitor or as a substrate active only through a single turnover (Cornelius et al., 1977; Li et al. 1978; Dunaway-Mariano & Cleland, 1980; Gantzer et al. 1982; Devlin & Grisham, 1990). Hexokinase is one of the few examples of an enzyme which utilizes $Co(NH_3)_4ATP$ effectively as a substrate in multiple turnovers (Cornelius & Cleland, 1978).

Steady-state kinetic studies were performed to characterize the interactions of $Co(NH_3)_4ATP$ with PKCα. Eadie–Hofstee plots (data not shown) are consistent with competitive inhibition by $Co(NH_3)_4ATP$ with respect to MgATP. In the absence of $Co(NH_3)_4ATP$, the K_m^{MgATP} was 8.5 ± 1.2 μM and the V_{max} was 0.22 ± 0.012 μmol min^{-1} mL^{-1}. In the presence of 0.75 mM $Co(NH_3)_4ATP$, the K_m^{MgATP} was 16.4 ± 1.8 μM and the V_{max} was 0.201 ± 0.012 μmol min^{-1} mL^{-1}. Similar results were also observed in three additional experiments.

Formation of Ternary Complexes of PKCα·Gd^{3+}·Substrate. Water proton relaxation rate measurements were used to characterize PKCα·Gd^{3+}·substrate complexes with $Co(NH_3)_4ATP$. When PKCα·Gd^{3+} was titrated with $Co(NH_3)_4ATP$ (Figure 5), the observed enhancement, ϵ^*, decreased from an initial value of 6 to a final limiting value of 0.2 at a PKC·$Co(NH_3)_4ATP$ ratio of approximately 1:1. Although the concentration of Gd^{3+} in Figure 5 was less than the concentration of $Co(NH_3)_4ATP$ used to make a ternary complex with PKCα, three other independent experiments show near stoichiometric interactions between enzyme, Gd^{3+}, and $Co(NH_3)_4ATP$ (for example, see Figure 7). One may therefore conclude that PKCα·Gd^{3+} can bind both stereoisomers of $Co(NH_3)_4ATP$ but it is not possible to assess whether one isomer is preferred over another. The decrease in ϵ^* in Figure 5 cannot be due to competition by $Co(NH_3)_4ATP$ for Gd^{3+} bound to PKCα because titrations of Gd^{3+} solutions with $Co(NH_3)_4ATP$ in the absence of enzyme produce no evidence of a $Co(NH_3)_4ATP$·Gd^{3+} complex. Furthermore, the low observed enhancement (ϵ^* = 0.2) at the end of this titration is inconsistent with displacement of Gd^{3+} from PKCα upon binding of $Co(NH_3)_4ATP$. Thus the decrease in ϵ^* observed in Figure 5 must result from the formation of a ternary PKCα·Gd^{3+}·$Co(NH_3)_4ATP$ complex.

Similar experiments were also conducted using a substrate peptide, α-peptide (RFARKGSLRQKNV), derived from the pseudosubstrate sequence of PKCα. An Ala to Ser substitution converts the peptide into a very effective substrate (House & Kemp, 1987). Titrations with α-peptide (Figure 6) resulted in a decrease in ϵ^* from 5.5 to 1.6. In a manner similar to the titration with $Co(NH_3)_4ATP$, most of the observed decrease in ϵ^* occurs at concentrations of α-peptide only slightly larger than that of the enzyme. As noted above, titrations in the absence of PKCα produced no evidence of a significant interaction between the α-peptide and Gd^{3+}. Thus the decrease in ϵ^* observed in Figure 6 cannot result from competition by α-peptide for Gd^{3+} bound to PKCα. The observed decrease could, however, result from (a) formation of a ternary PKCα·Gd^{3+}·α-peptide complex or (b) displacement of Gd^{3+} upon binding of α-peptide to PKCα.

These two possibilities may be distinguished by the data of Figure 7. Here the PKCα·Gd^{3+} complex was titrated with α-peptide, decreasing the observed enhancement from 5.7 to approximately 1.6, and the resulting sample was titrated with $Co(NH_3)_4ATP$. This caused a further decrease in ϵ^* to a limiting value of 0.4 at a $Co(NH_3)_4ATP$ concentration equal to that of the enzyme. These data are consistent with the formation of a ternary PKCα·Gd^{3+}·α-peptide complex, which binds $Co(NH_3)_4ATP$ to form a quaternary PKCα·Gd^{3+}·α-peptide·$Co(NH_3)_4ATP$ complex. The alternative possibility that Gd^{3+} is displaced by the peptide but forms a new complex with the enzyme upon addition of $Co(NH_3)_4ATP$ is unlikely and unnecessarily complicated. This latter possibility is likewise inconsistent with preliminary studies of the frequency dependence of water relaxation by the PKCα·Gd^{3+} complex in the presence of α-peptide (see Discussion).

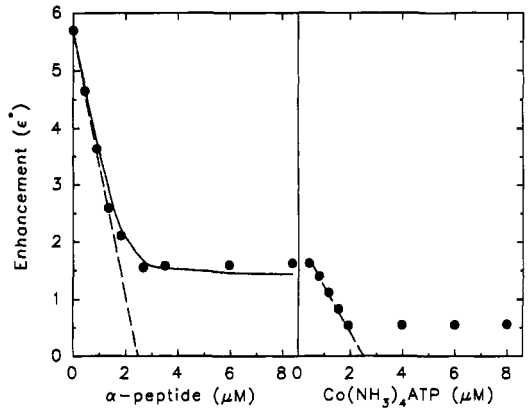


FIGURE 7: Effect of $Co(NH_3)_4ATP$ on enhancement of the water proton relaxation rate of Gd·PKCα·α-peptide. Solutions containing 3 μM PKCα, 2 μM added Gd^{3+}, 8 μM added α-peptide, 20 mM PIPES, and 400 μM PC_7/20.4 μM DO (pH 7.45) were titrated with the indicated concentrations of $Co(NH_3)_4ATP$. The original sample size of PKC and lipid was 51 μL and consecutive additions of Gd^{3+}, α-peptide, and $Co(NH_3)_4ATP$ were made. Corrections in enhancement were made to accommodate this dilution of experimental components. Dashed and solid curves are as in Figure 6. Fitting parameters for the peptide titration included K_{EM} = 0.4 μM, K_{ES} = 3 μM, K_{MS} = 0.5 mM, K_{EMS} = 1.08 nM, ϵ_{EM} = 7, ϵ_{MS} = 1.8, and ϵ_{EMS} = 1.4. Results are representative of two independent experiments. Minor differences in the curves shown in Figures 6 and 7 reflect typical variation in the observed data.

The enhancement titrations involving substrate were fit using the REED3 computer program which follows the enzyme·metal·substrate binding model described in Schemes I and II. The calculated K_D values are shown in Table I. The fitting programs assumed ternary complex enhancement factors of 0.2 for PKCα·Gd^{3+}·$Co(NH_3)_4ATP$ and 1.4 for PKCα·Gd^{3+}·α-peptide. A $K_{Gd\cdot[CoATP]}$ value of 16 mM, as measured previously by Gd^{3+}-EPR (Klemens, 1987), and a $K_{Gd\cdot[pept]}$ value of $\geq$0.5 mM, as estimated by water proton relaxation rate studies, were used in the REED3 program. For the titrations with either substrate, the best-fit value for $K_{E\cdot[Gd]}$ was 0.2–0.6 μM. The $K_{E\cdot[S]}$ value of 3–50 μM for PKCα in the presence of α-peptide (RFARKGSLRQKNV) is higher than the K_m value of 0.2 μM for a mixture of isozymes in the presence of a similar peptide (RFARKGSLRQKNVHEVKN) (House & Kemp, 1987). The higher affinity observed by House and Kemp may be due to an additional basic amino acid at the C-terminal end of the peptide, the use of PS instead of PC_7, or the mixture of isozymes employed. A peptide derived from glycogen synthase (PLSRTLSVSS) was determined to have a K_m of 32.5 $\pm$ 2.4 μM with a mixture of PKC isozymes (House et al., 1987), a value within the range we calculated for our α-peptide. The fitted K_D values indicate that the binding of Gd^{3+} to the enzyme enhances the affinity for α-peptide but has relatively little effect on the binding of $Co(NH_3)_4ATP$. $K_{E\cdot S\cdot[M]}$ values were calculated directly from the best-fit values for $K_{E\cdot[M]}$, $K_{E\cdot M\cdot[S]}$, and $K_{E\cdot[S]}$, since $K_{E\cdot S\cdot[M]} = (K_{E\cdot M}K_{E\cdot M\cdot[S]})/K_{E\cdot[S]}$. The values thus obtained are consistent with a significant increase in the affinity of PKCα for Gd^{3+} in the presence of either $Co(NH_3)_4ATP$ or the α-peptide.

DISCUSSION

Kinase activity studies (Maurer et al., 1992) suggest that Gd^{3+} can serve as a probe for more than one class of Ca^{2+} sites on PKC and conditions for separately evaluating the individual sites have been proposed. In this report, we used the defined conditions to explore differences in the direct binding of paramagnetic Gd^{3+} to two PKC isozymes as measured with water proton relaxation rate NMR. The paramagnetic effect of Gd^{3+} on water proton relaxation was enhanced in solutions of PKCα and PKCβ in the presence of PC_7/DO. The data are consistent with a single class of metal-binding sites on PKCβ (Figure 2) and two classes of sites on PKCα: a single high-affinity site with a K_D for Gd^{3+} of 0.2 μM (Figure 3) and a larger class of sites with a lower affinity for Gd^{3+}. Displacement of Gd^{3+} from PKCα by a titration with Ca^{2+} argues that Gd^{3+} occupies Ca^{2+} sites on this isozyme (Figure 4). Titrations of PKCα·Gd^{3+} with $Co(NH_3)_4ATP$ (Figure 5) and α-peptide (Figure 6) caused substantial decreases in water proton relaxation rate enhancement and are consistent with the formation of ternary enzyme·metal·substrate complexes with $K_{PKC\alpha\cdot Gd\cdot[substrate]}$ values of 30–100 nM for $Co(NH_3)_4ATP$ and $\leq$13 nM for α-peptide. Titration of the PKCα·Gd^{3+}·peptide complex with $Co(NH_3)_4ATP$ provided evidence for a quaternary complex.

The interactions of Gd^{3+} with nucleotide and peptide substrates support the prediction, arising from kinase activity studies (Maurer et al., 1992), that the high-affinity Gd^{3+} site is near the active site of PKCα. Water proton relaxation measurements of ternary enzyme·metal·substrate complexes were made possible by using substitution-inert $Co(NH_3)_4$-ATP, which serves as a competitive inhibitor with respect to MgATP, and by using α-peptide, a model peptide based on the pseudosubstrate domain of PKCα, which serves as an effective phosphorylatable substrate. Upon introduction of these substrates, the observed relaxation enhancement of the PKCα·Gd^{3+} complex dropped rapidly, consistent with formation of ternary enzyme·metal·substrate complexes. The decrease in observed enhancement could result from a decrease in the number of fast-exchanging water molecules on the enzyme-bound Gd^{3+}, from a change in τ_c, the dipolar correlation time, or from a change in $1/\tau_m$, the exchange rate for water molecules interacting with the enzyme-bound Gd^{3+} (Mildvan & Engle, 1972). These three possibilities may be distinguished by a study of the frequency dependence of water relaxation. For many such complexes, the dipolar correlation time, τ_c, may be determined from a plot of T_{1p} vs ω_I^2, where ω_I is the nuclear Larmour frequency (Peacocke et al., 1969). With a value of τ_c determined in this way, the number of fast-exchanging water molecules in the complex may also be determined (Mildvan & Engle, 1972). If the paramagnetic contribution to the relaxation rate, $1/T_{1p}$, is exchange-limited, then $1/T_{1p}$ should be frequency independent. Preliminary measurements of the PKCα·Gd^{3+} complex in the presence and absence of α-peptide and $Co(NH_3)_4ATP$ demonstrate that $1/T_{1p}$ is strongly frequency dependent in all cases. Such an observation of frequency dependence would normally be consistent with the formation of ternary PKCα·Gd^{3+}·α-peptide and PKCα·Gd^{3+}·$Co(NH_3)_4ATP$ complexes and would also demonstrate that $1/T_{1p}$ is not exchange-limited. (On the other hand, the low enhancements observed for the ternary complexes may indicate that any water ligands remaining in the first coordination sphere of Gd^{3+} in the ternary complexes are not in rapid exchange with bulk solvent. Frequency dependences of $1/T_1$ can also occur for outer-sphere protons which may have relatively long residence times near the bound Gd^{3+}). With the small amounts of pure PKCα isozyme available in these studies, $1/T_{1p}$ could not be determined accurately at frequencies above 40 MHz, and accurate estimates of τ_c, the dipolar correlation time, could thus not be made. (We estimate that accurate determinations of τ_c

Table I: Binding of Substrates to PKCα[a]

substrate	$K_{E\cdot[M]}$	$K_{E\cdot[S]}$	$K_{M\cdot[S]}$	$K_{E\cdot M\cdot[S]}$	$K_{E\cdot S\cdot[M]}$
CoATP	0.2–0.6 μM	600–100 nM[b]	16 mM	30–100 nM	15–150 nM
α-peptide	0.2–0.4 μM[c]	50–3 μM	>0.5 mM	$\leq$13 nM	$\leq$2 nM

[a] K_D values were determined using the REED3 program for titrations of PKC$\alpha\cdot Gd^{3+}$ with the appropriate substrate (E = PKCα, M = Gd^{3+}, S = substrate). For definitions of the individual K_D values, refer to Schemes I and II. [b] This table is written to emphasize that the highest K_{ES} values go together with the lowest K_{EMS} values. [c] These K_D values were determined for two separate experiments which individually contained best fits for K_{EMS} with smaller ranges. Reasonable fits could also be obtained for K_{EM} values of 0.5–0.6 μM; however, the resulting K_{EMS} values did not have ranges which overlapped well between experiments (K_{EMS} $\leq$120 nM).

would require 5–10 times more PKCα than is currently available).

It is unlikely that variations in τ_c upon formation of the ternary PKC$\alpha\cdot Gd^{3+}\cdot$substrate complexes could be large enough to account for the observed changes in ϵ^*. On the other hand, changes in the number of fast-exchangeable water molecules on enzyme-bound Gd^{3+} could readily account for the changes in ϵ^* observed in Figures 5–7. This could result from displacement of coordinated waters upon substrate binding or from the trapping or freezing of one or more waters at the active site upon substrate binding. Such changes would be consistent with an enzyme·metal·substrate complex, in which the metal acts to at least some extent as a bridge between enzyme and substrate. In such cases, the binding of either metal or substrate to the enzyme should enhance the binding of the other species. This is the behavior observed in Table I for α-peptide where the best-fit values for $K_{E\cdot M\cdot[S]}$ are smaller than the best-fit values for $K_{E\cdot S}$ and where the best-fit values for $K_{E\cdot S\cdot[M]}$ are significantly smaller than the best-fit values for $K_{E\cdot M}$. This effect is not as apparent with $Co(NH_3)_4ATP$, which already has such a high affinity for the enzyme. The enhancement values of less than 1 found at the end of the $Co(NH_3)_4ATP$ titration, however, suggest that very few if any positions are available on Gd^{3+} for water ligand exchange after this substrate binds.

Our results indicate that PKC$\alpha\cdot Gd^{3+}$ can bind α-peptide without the presence of a nucleotide. This contrasts with the kinetic studies of Leventhal and Bertics (1991). The discrepancy may reflect different enzyme preparations—purified PKCα in our studies versus mixed isozymes used by Leventhal and Bertics. Another possibility is that Gd^{3+} binding may promote a more stable conformation for peptide binding than does Ca^{2+}. On the other hand, the kinase mechanism may be more random than predicted by Leventhal and Bertics.

From an analysis of the primary sequences of PKCα and PKCβ (Coussens et al., 1986), it is possible to postulate a location for the Ca^{2+}-binding sites on PKC. The greatest difference between PKCα and PKCβ lies in the V3 region which forms the "hinge" linking the regulatory and catalytic domains. The initial amino acid sequence (292–303) in the V3 region of PKCα contains more basic residues and also more closely resembles a portion of an EF-hand motif than does the corresponding sequence found in PKCβ (Coussens et al., 1986). Thus V3 may comprise at least part of the high-affinity Gd^{3+} binding site seen on PKCα. Access of the V3 region to the active site is supported by work of Flint et al. (1990) showing that a residue in the V3 region of PKCβ can be autophosphorylated by an intrapeptide mechanism. The sites on PKCβ which attract a large number of Gd^{3+} ions may be located in the conserved C2 region found in the regulatory domain of the Ca^{2+}-dependent isozymes α, β, and γ (Coussens et al., 1986) but absent in the Ca^{2+}-independent isozymes δ, ϵ, and ζ [reviewed in Nishizuka (1988)]. Preliminary studies with baculovirus-expressed PKCα indicate that, at Gd^{3+} concentrations greater than those used in Figure 3, PKCα also contains a large number of metal sites with ϵ^* similar to that shown with PKCβ (work in progress). Since multiple metal sites were observed, even with a system overexpressing a single PKC isozyme, it is proposed that these multiple sites are inherent to PKC and are not due to the presence of other metal-binding contaminants.

The Ca^{2+} competition data of Figure 4 are consistent with the binding of Gd^{3+} and Ca^{2+} to two classes of metal-binding sites on PKCα. The best fit to the data is achieved by assuming that Gd^{3+} binds more tightly than Ca^{2+} to the single high-affinity (class 1) site but less tightly than Ca^{2+} to the seven class 2 binding sites. In this best-fit model, Gd^{3+} can effectively discriminate between the two classes of sites, but Ca^{2+} does not. This is a similar result to that observed in numerous studies of the binding of Ca^{2+} and lanthanide ions to calbindin (Chiba et al., 1984; O'Neil et al., 1984; Szebenyi et al., 1981; Vogel et al., 1985) and calmodulin (Wang et al., 1984). Ca^{2+} binds more tightly than Gd^{3+} to the N-terminal pseudo-EF site of calbindin, whereas the reverse is true at the C-terminal EF site. Similarly, Ca^{2+} and lanthanides exhibit opposite preferences for the four sites of calmodulin. Ca^{2+} binds more strongly to sites III and IV, whereas Tb^{3+} and Dy^{3+} prefer sites I and II.

Bazzi and Nelsestuen (1990) reported that when $^{45}Ca^{2+}$ and a mixture of isozymes were subjected to equilibrium dialysis, PKC bound at least eight Ca^{2+} ions in the presence of a PS/PC lipid system. Our results suggested that the Gd^{3+}-binding sites seen with PKCβ in PC_7/DO may be the same class of sites as seen by Bazzi and Nelsestuen with $^{45}Ca^{2+}$. It is likely that a high-affinity Ca^{2+} site on PKCα would not have been observed in the equilibrium dialysis studies, because, as discussed above, Ca^{2+} may not discriminate between the two types of sites. It is also possible that PKCα represented only a small fraction of the mixed isozyme preparation used in their studies. Kinase activity studies (Maurer et al., 1992) indicated that PKCβ phosphorylates histone in a Ca^{2+}-dependent manner in PS/DO and in a Ca^{2+}-independent manner in PC_7/DO. It is possible that Gd^{3+} can still bind to Ca^{2+} sites on PKCβ even though PC_7/DO has promoted an enzyme conformation which allows for kinase activity in the absence of metal. In contrast, these Gd^{3+}/Ca^{2+} sites may be essential for creating the appropriate interactions between PKCβ and PS/DO. Water proton relaxation rate experiments with Gd^{3+} were not conducted in the presence of PS because formation of PS·Gd^{3+} complexes is difficult to distinguish from Gd^{3+}·PKC·PS binding.

In summary, these studies confirm that there are major differences in the binding of Gd^{3+} to PKCα and PKCβ. PKCβ is proposed to have a metal-binding region which attracts a large number of Gd^{3+} ions. Preliminary evidence suggests PKCα also possesses these sites and in addition contains one high-affinity Gd^{3+} binding site which interacts effectively with peptide and metal–nucleotide substrates at the active site. This difference in metal-binding properties may reflect distinct

reaction mechanisms for PKCα and PKCβ. Eventual high-resolution NMR studies to map the structures of the catalytic sites of PKC will aid in the development of specific reagents for activating or inhibiting individual isozymes.

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