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Identification, activity, and structural studies of peptides incorporating the phorbol ester-binding domain of protein kinase C

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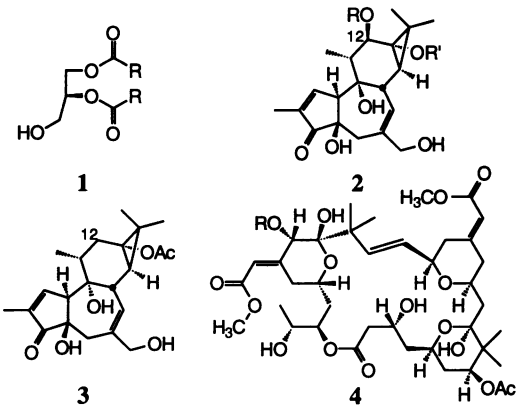
ABSTRACT **The family of homologous enzymes known as protein kinase C (PKC) has been the object of intense interest because of its crucial role in cellular signal transduction. Although considerable information about the activation of PKC has been gained through structure–activity, molecular modeling, and synthetic studies of both natural and designed activators, information about the structure of PKC itself has been limited by its large size and requirement for phospholipid cofactors. Additionally, difficulties in the purification of truncated mutants of PKC have thus far prevented their analysis by nuclear magnetic resonance (NMR) or x-ray crystallographic methods. We describe the identification, synthesis, ligand-binding analysis, cofactor requirements, and preliminary NMR evaluation of two subdomains (peptides B and C) of the regulatory domain of PKC-γ. Peptides B and C bind [^{3}H]phorbol 12,13-dibutyrate with good affinity (K_d = 6.4 μM and 414 nM, respectively) in the presence of phosphatidylserine. In comparison, the binding affinity of [^{3}H]phorbol 12,13-dibutyrate for PKC was found to be 2.6 nM. Like PKC itself, these peptides also recognize other PKC activators, including dioctanoylglycerol and teleocidin B-4, and exhibit an ability to differentiate phorbol ester from its C-4 epimer. NMR studies of PKC subdomains are also described, indicating that both peptides B and C are well behaved in solution and do not exhibit any concentration-dependent changes. Finally, these studies reveal that peptide B becomes conformationally ordered only in the presence of phospholipid, suggesting that the regulatory domain of PKC itself might be organized for activation only when associated with the lipid bilayer, where its activator (diacylglycerol) is encountered.**

Since the identification of its pivotal role in cellular signal transduction (1), the family of enzymes known as protein kinase C (PKC) has served as a key lead in the elucidation of normal and abnormal cellular processes. Under normal circumstances, PKC is activated by the binding of 1,2-diacyl-*sn*-glycerol (DAG, structure **1**) (2–4), a second messenger released in response to the binding of extracellular agents to cell surface receptors. Differing significantly in structure from DAG, plant-derived phorbol esters (structure **2**) and a diverse range of other agents (5) also bind to and activate PKC in competition with DAG but, unlike DAG, initiate a series of abnormal events associated with tumor promotion. In contrast, prostratin (structure **3**), which differs from the phorbol esters only as a result of an ester deletion at the C-12 position, is not a potent promoter but exhibits cytoprotective effects in cells infected with the human immunodeficiency virus (6), a finding of potential consequence in AIDS therapy. Representative of a third structural class of activators, the marine-derived macrocyclic lactone bryostatin (structure **4**), now in clinical trials, binds to PKC in competition with phorbol esters but blocks a subset of phorbol ester-induced responses including tumor promotion (7–12). Efforts to understand the structural basis for these contrasting activities, an issue of primary importance in the identification of tumor promoters and in the development of cancer prevention protocols and of medicinal leads, have focused on synthetic (13, 14) and computational (2, 15–19) studies of PKC activators. These studies have provided invaluable information on the common structural characteristics of activators, but delineation of the structural relationship between these activators and PKC, and consequently between normal and abnormal PKC activation, has been hampered by the lack of detailed structural information about the activator binding site on PKC. The size and physical properties of PKC along with its requirement for phospholipid cofactors in the conformation required for phorbol ester/DAG binding (20–22) have thus far prevented structural analysis of PKC itself by crystallographic or NMR methods.



To date, 11 isozymes of PKC have been identified (23–26). The four primary subspecies of PKC (α, βI, βII, and γ) are large, single-chain polypeptides with four homologous (C_1–C_4) and five variable (V_1–V_5) regions (Fig. 1). Proteolysis of PKC by trypsin (27) or calpain (28) in the V_3 region produces two peptides, one of which, the "catalytic domain," contains the apparatus for protein phosphorylation, and the other of which, the "regulatory domain," retains phorbol ester/DAG-binding ability. This observation provided the first evidence that the phorbol ester/DAG-binding ability of PKC could be retained in a truncated subunit, although this subunit of ≈36 kDa is still too large for NMR analysis. At the outset of this work, it was not known whether the elements required for phorbol ester/DAG binding are contained in a sequence short enough for NMR study, and if so whether this peptide would assume the proper conformation for phorbol ester/DAG binding. However, analysis of the sequence homology of the regulatory domains of the α, βI, βII, and γ PKC isozymes reveals that the

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Abbreviations: DAG, 1,2-diacyl-*sn*-glycerol; PDBu, phorbol 12,13-dibutyrate; PKC, protein kinase C; PC, phosphatidylcholine; PS, phosphatidylserine.

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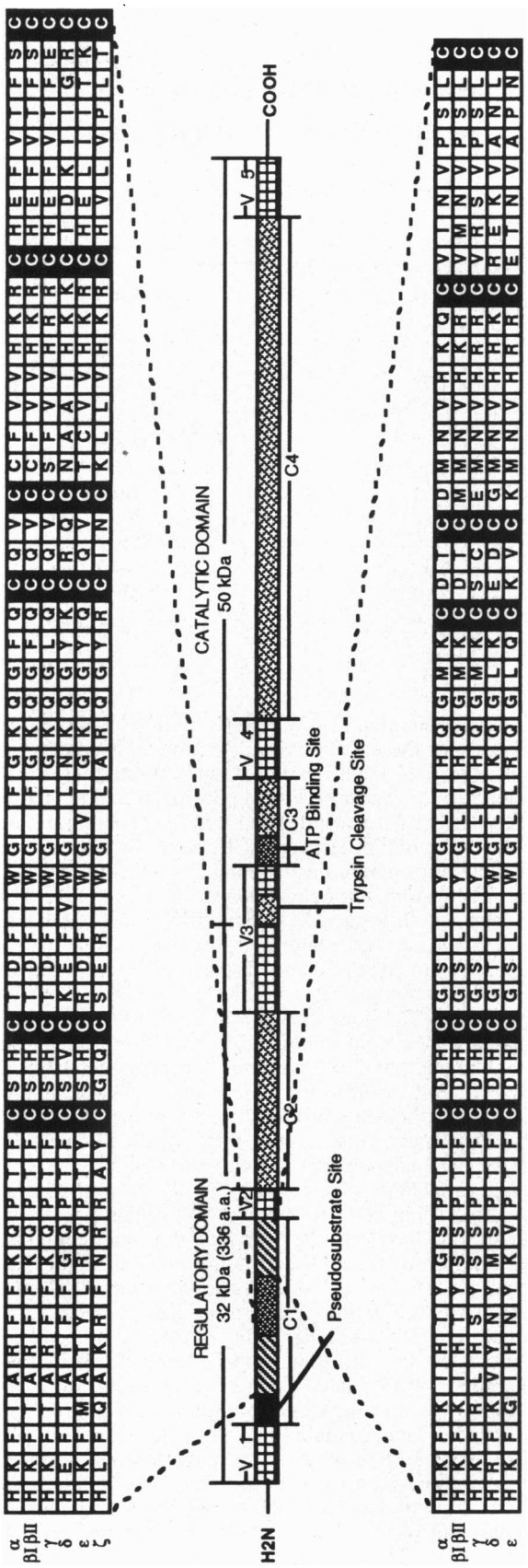

FIG. 1. Conserved structural features of PKC.

first conserved region (C_1) contains a sequence of amino acids which has been shown to be a pseudosubstrate for the phosphorylation site and consequently might not be involved in phorbol ester/DAG binding (29). Following this sequence are two ≈51-aa sequences, each of which contains a cysteine-rich tandem repeat (30, 31). The conservation of these sequences in phorbol ester-activatable PKC suggests that they could be directly involved in phorbol ester binding. In accord with this analysis, Ono *et al.* (32) demonstrated that mutations of either of these regions in fragments of rat brain PKC-γ with as few as 162 aa affected phorbol ester binding. A subsequent analysis by Burns and Bell (33) expanded on these findings, showing that a sequence incorporating aa 91–174 of rat brain PKC-γ could bind C-20-tritiated phorbol 12,13-dibutyrate ([^{3}H]P-DBu) (33). After completion of the present study, the Bell group (34) reported that fusion proteins incorporating as few as 43 aa of the second cysteine-rich region of PKC attached to glutathione *S*-transferase bind [^{3}H]PDBu.

To elucidate the structural factors involved in normal and abnormal PKC activation, we initiated a study of the synthesis, NMR analysis, and photoaffinity labeling of PKC subdomains associated with phorbol ester/DAG binding (35). While efforts to obtain subdomains of suitable size for NMR solution structure determination through cloning have been complicated by difficulties encountered in proteolysis of fusion proteins, we have found that the desired peptides can be prepared in quantity and high purity by solid-phase synthesis. Our studies focused on the regions of greatest homology, starting with the two histidine- and cysteine-rich portions of the C_1 region of rat brain PKC-γ. To prevent racemization and oxidation during synthesis, the carboxyl-terminus was extended in each case from the final cysteine to a glycine. Fig. 2 shows the final sequences which were chosen for synthesis: peptide B, incorporating aa 36–87 of the first cysteine-rich repeat, and peptide C, incorporating aa 101–151 of the second cysteine-rich repeat. This paper describes the synthesis and binding activity of these PKC subdomains and spectroscopic information on their solution behavior.

MATERIALS AND METHODS

Materials. Unless otherwise noted, reagents were obtained from Sigma and used without further purification. Ecolite-(+) scintillation fluid was from ICN; [^{3}H]PDBu, from DuPont/New England Nuclear; and teleocidin B-4, from the Department of Food Science and Technology, Kyoto University, Japan (36). Purified rat brain PKC (mixture of isozymes) was a gift of Professor Wray H. Huestis and Mr. Mike Moxness, Stanford University.

Synthesis of Peptides. Peptides B and C were synthesized by the Protein and Nucleic Acid Facility, Stanford University, with a Milligen/Biosearch 9050 automated peptide synthesizer using the fluorenylmethoxycarbonyl–polyamide strategy (37). Crude material was purified by HPLC using a C_{18} Vydac column with elution by a 40-min linear gradient of 10–100% acetonitrile in 0.1% trifluoroacetic acid. Samples of purified peptides exhibited satisfactory amino acid and mass spectral analyses.

[^{3}H]PDBu Binding Assay. The binding of [^{3}H]PDBu to peptides B and C was evaluated by a slight modification of the procedure of Tanaka *et al.* (38). The standard mixture (300 μl) contained 50 mM Tris/HCl buffer (pH 7.5 at 25°C), phospholipid at 0–200 μg/ml, 0.17% dimethyl sulfoxide, 0.2% ethanol, 2.5–50 nM [^{3}H]PDBu, bovine γ-globulin at 4 mg/ml, and peptide at 0.8–15 μg/ml in a 1.5-ml Eppendorf tube. Small unilamellar phospholipid vesicles were prepared in 50 mM Tris/HCl buffer (pH 7.5) by sonication at 0°C for 1 min before

PEPTIDE B:

H_2N-HKFTARFFKQPTFCSHCTDFIWGIGKQGLQCQVCSFVVHRRCHEFVTFECPG-CO_2H

PEPTIDE C:

H_2N-HKFRLHSYSSPTFCDHCGSLLYGLVHQGMKCSCCEMNVHRRCVRSVPSLCG-CO_2H

FIG. 2. Primary sequences of peptides B and C.

addition to the above mixture. Nonspecific binding was measured in the presence of 10 μM nonradioactive PDBu. Each sample mixture was prepared in triplicate. Samples were incubated for 30 min at 30°C in a shaker bath, removed, and cooled at 0°C for at least 5 min. The bound [^{3}H]PDBu–peptide complex was separated from free ligand by rapid filtration through a 2.1-cm-diameter Whatman GF/B filter, pretreated with freshly prepared 0.3% polyethylenimine for a minimum of 1 hr. The sample tube was rinsed with 1 ml of ice-cold 20 mM Tris/HCl (pH 7.5), and the rinse solution was then passed through the filter. The filter was further washed with four 1-ml portions of ice-cold 20 mM Tris/HCl (pH 7.5). Each filter was then placed in a scintillation vial, 10 ml of Ecolite-(+) scintillation fluid was added, and the solution was made homogeneous by vortexing. Radioactivity (dpm) was determined with a Beckman scintillation counter. After determination of the initial radioactivity of the samples, they were stored for 48–72 hr to allow for complete extraction of bound [^{3}H]PDBu from the filter. Samples were then shaken, and radioactivity was again determined.

NMR Spectroscopy. Proton NMR spectra were recorded at 400 MHz with either a Varian XL-400 or General Electric GN-400 Omega instrument operating at 20°C. Following conversion from their native formats, free inductive decays (FIDs) were processed and analyzed with FELIX 2.10 (Biosym Technologies, San Diego) on a Silicon Graphics R3000 Indigo/Elan workstation. Each FID was apodized with a 90° phase-shifted sinebell squared function before Fourier transformation.

Atomic Absorption Spectroscopy. Atomic absorption spectroscopy was carried out on a Perkin–Elmer 2380 spectrophotometer. Seven zinc standards ranging from 0–25 ng of Zn^{2+} per ml were prepared by diluting a commercial (Aldrich) standard solution of $ZnCl_2$ in doubly distilled water. The response of these samples was measured to establish a calibration curve for the instrument. Protein samples in a concentration range of 10–200 μg of protein per ml in doubly distilled water were analyzed and compared with the calibration curve to determine zinc content.

RESULTS AND DISCUSSION

Peptides B and C bound significant [^{3}H]PDBu in the presence of phosphatidylserine without added Ca^{2+} at pH 7.5. Scatchard analysis gave binding constants (K_d) of 6.4 μM and 414 nM, respectively (Fig. 3). For comparison, the K_d of [^{3}H]PDBu binding to rat brain PKC (mixture of isozymes) in this assay was determined to be 2.6 nM, similar to previously reported values (38, 39). These results localize the features for phorbol ester recognition to relatively short subsequences of PKC and are in accord with results of Ono *et al.* (32), who proposed that one of the two sequences of the cysteine-rich region of rat brain PKC-γ was sufficient for phorbol ester binding, although quantitative differences between the sequences in binding to PDBu were not discussed. Similarly, Bell and colleagues (33) have reported that a mutant (86 aa) containing only the second cysteine-rich region bound [^{3}H]PDBu to a greater degree than a mutant (146 aa) containing only the first cysteine-rich region (33). While the binding of [^{3}H]PDBu to peptides B and C is weaker than to PKC itself, it is sufficiently strong for their use in NMR studies and for photoaffinity labeling of the PDBu binding site (35).

Addition of 2 mM $CaCl_2$ to the assay mixture produced no significant change in the binding of [^{3}H]PDBu to either peptide B or C, unlike PKC itself, which requires Ca^{2+} for maximal binding (39). This result is consistent with the finding (32) that only polypeptides incorporating the second conserved region (C_2) exhibit Ca^{2+}-dependent binding. Curiously, addition of 2 mM disodium EDTA to the assay reduced binding of [^{3}H]PDBu to peptide B by ≈80%. Whether this reduction is the result of residual ion chelation by EDTA, a Na^+-dependent salt effect, or an interaction between EDTA and [^{3}H]PDBu or peptide B itself is unknown. Enhancement of phorbol ester binding to PKC in the presence of Zn^{2+} has been demonstrated by cellular homogenate and whole-cell assays (40), and x-ray fluorescence (41) and atomic absorption (42) measurements have demonstrated that PKC itself binds at least four zinc atoms. Likewise, neuronal chimaerin, a protein with substantial homology to the PKC regulatory domain, has been found to bind zinc (43). However, EDTA-dependent reduction of [^{3}H]PDBu binding to peptide B was not reversed by addition of $ZnCl_2$ to the assay mixture.

Both peptides B and C exhibited the strong requirement for phosphatidylserine (PS) characteristic of PKC (20–22). Replacement of PS in the standard assay mixture with phosphatidylinositol, sphingomyelin, phosphatidylcholine (PC), or phosphatidylethanolamine drastically reduced or completely eliminated the ability of peptides B and C to bind [^{3}H]PDBu. No specific binding was observed in the absence of phospholipid (Fig. 4). Binding was maximal for both peptides at a PS concentration of 50 μg/ml and either decreased (peptide B) or remained constant (peptide C) at higher PS concentrations.

As a further test of the structural correspondence between PKC and peptide C, the ability of PS to support PDBu binding to peptide C in mixed PS/PC vesicles was also investigated. In a 1992 study, Lee and Bell (44) demonstrated that the addition of phosphatidyl-(3-hydroxypropionate), phosphatidate, phosphatidylethanol, phosphatidylglycerol, and phosphatidylinositol could cause a 2- to 5-fold reduction in the amount of PS needed to activate PKC in Triton X-100 mixed micelles (44). This behavior was also exhibited by peptide C (Fig. 5): with mixed PS/PC vesicles prepared by resonication of phospholipid mixtures, as little as 16% PS could support nearly 50% maximal [^{3}H]PDBu binding. As a control, phospholipid mixtures prepared by simple mixing (without resonication) yielded binding results directly analogous to those obtained with varying concentration of PS alone.

The ability of other compounds known to interact with the phorbol ester binding site on PKC to competitively inhibit the binding of [^{3}H]PDBu to peptides B and C was determined by varying the concentration of the compound in question in the presence of a constant concentration of [^{3}H]PDBu. Dose–

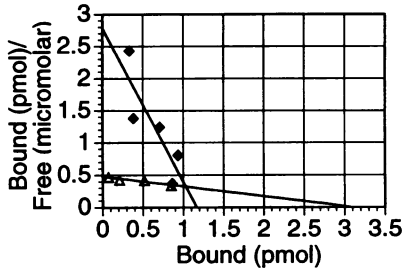

FIG. 3. Scatchard analysis of [^{3}H]PDBu binding to peptides B (△) and C (◆).

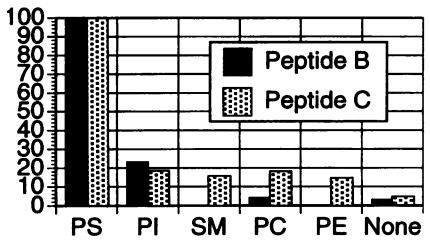

FIG. 4. Dependence of specific binding on phospholipid type. PI, phosphatidylinositol; SM, sphingomyelin; PE, phosphatidylethanolamine.

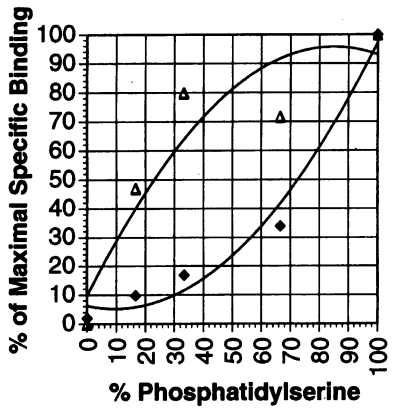

FIG. 5. Binding of [^{3}H]PDBu to peptide C in mixed PS/PC vesicles. △, Lipids sonicated following mixing to promote reorganization; ◆, lipids mixed without further sonication.

response curves were plotted for each compound, and the concentration at which 50% of [^{3}H]PDBu binding was inhibited (IC_{50}) determined. Table 1 gives the results of this analysis for teleocidin B-4, 1,2-dioctanoyl-*sn*-glycerol, and PDBu. As expected, teleocidin B-4 and 1,2-dioctanoylglycerol significantly inhibited the binding of [^{3}H]PDBu to peptides B and C. The observed IC_{50} values are close to those observed for PKC itself. However, only teleocidin B-4 showed differential inhibition of [^{3}H]PDBu binding, with K_i (peptide C) a factor of 10 stronger than K_i (peptide B). Detailed structure–activity studies on the binding of the teleocidins to these two peptides could give new insights into molecular recognition by PKC and isozyme selective activation, information of considerable medicinal consequence.

To verify that the observed results were indeed due to the specific binding of [^{3}H]PDBu to the peptides and not an artifact of the assay, several control experiments were conducted. No specific binding was observed in the absence of peptide, indicating that the bovine γ-globulin present as a carrier protein for [^{3}H]PDBu does not interfere in the assay. No PDBu binding was detected when peptides B and C were replaced with insulin, a protein of similar molecular weight but unrelated sequence. Likewise, metallothionein, a metal-chelating protein with a zinc finger-like substructure (45), did not bind [^{3}H]PDBu even at high (10 μg/ml) protein concentration. The similarity of the binding requirements of these model peptides to PKC itself was further explored with 4α-phorbol 12,13-didecanoate, a stereoisomer which is incapable of activating PKC (46). No inhibition of [^{3}H]PDBu binding to peptides B or C was observed with 4α-phorbol 12,13-didecanoate up to 100 μM, indicating that these peptides not only recognize activators but, like PKC itself, discriminate small changes in structure.

NMR experiments indicate that both peptides B and C are "well behaved" in solution. No concentration-dependent changes in multiplet structure, linewidth, or chemical shift are observed for aqueous solutions of peptide B, demonstrating that the peptide can be examined at relatively high (5 mM) concentrations without concern for potential aggregation. Importantly, substantial increases in chemical shift dispersion are visible on addition of PS, providing evidence that association with phospholipid causes an increase in the order and stability of the solution structure of the peptide. This is particularly notable in the region of the spectrum corresponding to the aromatic residues (Fig. 6) and is consistent with the observed phospholipid requirement for binding of [^{3}H]PDBu to peptides B and C, as well as to PKC itself. A recent report (34) has shown that fusion proteins incorporating sequences corresponding to our peptide C bound one to two atoms of zinc, and it was suggested that this zinc binding was required for phorbol ester binding. However, no change in the ^{1}H NMR spectrum of peptide C was observed in our studies when one or two molar equivalents of $ZnCl_2$ were added. In contrast, differences in the linewidth and chemical shift dispersion are generally observed in the NMR spectra of zinc-binding proteins between samples on the addition of stoichiometric quantities of Zn^{2+} (47, 48). We have also performed atomic absorption experiments on HPLC-purified samples of peptide C and have found no bound zinc present.

Table 1. Inhibition of [^{3}H]PDBu binding to peptides B and C

Compound	IC_{50}, nM	
	Peptide B	Peptide C
PDBu	36	50
1,2-Dioctanoyl-*sn*-glycerol	3,160	10,000
Teleocidin B-4	344	32
4α-Phorbol 12,13-didecanoate	>100,000	>100,000

In summary, we have identified two peptides corresponding to the activator region (phorbol ester/DAG-binding domain) of several PKC isozymes that exhibit binding behavior analogous to that of PKC itself. These peptides specifically bind phorbol esters and exhibit a phospholipid dependency similar to that found for the entire protein. However, a requirement for zinc was not found, suggesting that the relationship of zinc with PKC might extend beyond the minimum phorbol ester/DAG recognition domain. Other activators of PKC such as teleocidin B-4 and DAG were also found to bind to these peptides. Similarly, 4α-phorbol didecanoate, which differs from PE at only one stereogenic center and does not activate PKC, did not exhibit binding to these peptides. Finally, NMR analysis indicates that these peptides do not self-aggregate in solution but undergo conformational changes upon association with specific phospholipids in a fashion similar to that proposed for PKC on the basis of circular dichroism studies (49). These observations indicate that PKC might not be preorga-

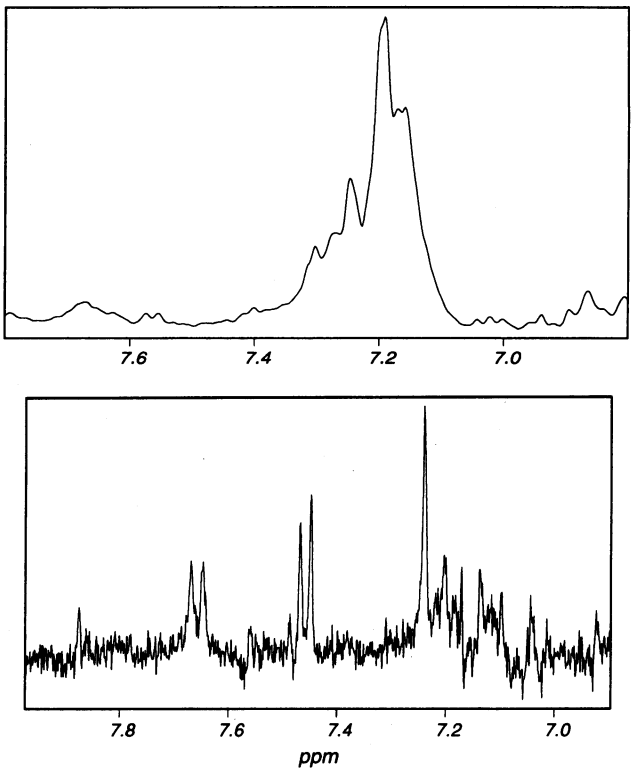

FIG. 6. ^{1}H NMR spectra (400 MHz) of peptide B in 99% ^2H_2O without (*Upper*) and with (*Lower*) PS.

nized for ligand binding (activation) except when it is in the region of the cell where its endogenous activator is generated. The similar solution and binding behavior of these peptides and PKC, coupled with their size and preliminary NMR spectra, suggests that these peptides could serve as molecular probes for the structural requirements for normal and abnormal PKC activation and for the development of new medicinal leads based on this receptor.

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