Influence of lipid on the structure and phosphorylation of protein kinase C α substrate peptides

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The structure and phosphorylation of two protein kinase C (PKC) α substrate peptides were investigated in varying lipid systems using enzyme activity assays and circular dichroism (CD) spectroscopy. The α -peptide, which exhibits the typical PKC α substrate motif and is based on the pseudosubstrate region of PKC α , was phosphorylated to a similar extent in bovine brain phosphatidylserine vesicles or diheptanoylphosphatidylcholine (PC₇) micelles (both with 5 mol % 1,2-dioleoyl-*sn*-glycerol), whereas neuromodulin (NM)-peptide, which does not exhibit this motif by virtue of its primary structure, was phosphorylated to a much lesser extent in the PC₇ micellar system. CD spectra of the peptides indicated that NM-peptide underwent a dramatic structural change in the presence of dimyristoylphosphatidylserine (DMPS) vesicles, whereas spectra acquired in PC₇ micelles were similar to those acquired in buffer

alone. No significant structural change was observed in the α -peptide in the presence of either lipid. PKC activity assays conducted with a series of NM-peptides successively substituted with nitroxide spin labels at each residue position suggested that several residues distal to the phosphorylation site are necessary for substrate recognition. The effect of these substitutions is not consistent with the binding of the NM-peptide to PKC in an extended structure, but is consistent with the binding of this peptide in a helical conformation. Furthermore, the docking of a helical NM-peptide to the substrate binding site of PKC suggests that the interaction is energetically feasible. These results suggest that PKC may recognize some non-linear substrate motifs and that lipid binding may convert a protein into a better PKC substrate.

INTRODUCTION

Protein kinase C (PKC), a family of phospholipid-dependent serine/threonine protein kinases, is an essential component of various signalling pathways leading to growth, differentiation and malignant transformation. All PKC enzymes are activated by interaction with membrane lipids, especially phosphatidylserine (PS). The classic PKC isoforms, α , β and γ , require Ca²⁺ and diacylglycerol (DAG) in addition to PS. PKCs δ , ϵ , η , and θ are independent of Ca²⁺, and PKCs ζ and λ require only the phospholipid (reviewed in [1,2]). PKC is regulated intrasterically via the interaction of a portion of its amino terminus, the pseudosubstrate sequence, with the substrate binding site on the catalytic domain (for review see [3]). The pseudosubstrate sequence resembles the phosphorylation site in a PKC substrate, with many basic residues surrounding an Ala instead of a phosphorylatable residue [4]. When the appropriate cofactors are present, the pseudosubstrate is released from the active site and the enzyme attains its active conformation. The sequence of the α -peptide, a PKC substrate, is based on the PKC pseudosubstrate region with Ser replacing the nonphosphorylatable Ala residue.

Numerous PKC substrates have been identified *in vitro* and

many have been placed on important signalling pathways. However, isozyme-specific differences in substrate phosphorylation have not been well characterized and not all substrates exhibit a pattern of basic residues that matches those in the pseudosubstrate motifs. Neuromodulin (NM), a 24.7 kDa PKC substrate in nerve growth cones, has one basic residue at i+2 relative to the phosphorylated Ser and one at i-3 [5]. The nearest additional basic residues are at i+7 from the PKC phosphorylation site and closer to the C-terminus. The unphosphorylated form of NM binds calmodulin (CaM) at a site near the PKC phosphorylation site and may play a role in sequestering CaM. When phosphorylated by PKC, NM may release CaM, thereby linking PKC activation to the regulation of free CaM in these cells [6].

An improved understanding of PKC substrate motifs and structures would facilitate the identification of additional substrates and would aid in the design of specific inhibitors directed at altering signal transduction pathways. In an attempt to characterize these recognition motifs better, we have examined structural features of PKC substrate peptides derived from the NM phosphorylation site (NM-peptide) and from the more classical pseudosubstrate motif of PKC α . Our results suggest that, unlike the pseudosubstrate, a helical NM-peptide is required

Abbreviations used: PKC, protein kinase C; CD, circular dichroism; α -peptide, a peptide based on the pseudosubstrate region of PKC α ; PC₇, diheptanoylphosphatidylcholine; NM, neuromodulin; NM-peptide, a peptide based on the PKC phosphorylation site of NM; DMPS, dimyristoylphosphatidylserine; PS, phosphatidylserine; DAG, diacylglycerol; CaM, calmodulin; DMPA, dimyristoylphosphatidic acid; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; bbPS, bovine brain phosphatidylserine; DO, 1,2-dioleoyl-*sn*-glycerol; PKM, the cofactor-independent catalytic fragment of PKC; TFE, trifluoroethanol; MeOH, methanol; PROSEC, an algorithm to estimate protein secondary structure from CD spectra; EPR, electron paramagnetic resonance; MTSSL, methanethiosulphonate spin label.

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for recognition by PKC and that lipids play a role in inducing or stabilizing the appropriate secondary structure.

EXPERIMENTAL

Materials

1,2-dimyristoyl-*sn*-glycero-3-phosphate (DMPA), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DMPG), 1,2-dimyristoyl-*sn*-glycero-3-[phospho-L-serine] (DMPS), bovine brain L- α -phosphatidylserine (bbPS), 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (PC₇), and 1,2-dioleoyl-*sn*-glycerol (DO) were purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.).

Peptide synthesis

The α -peptide (RFARKGSLRQKNV), substituted α -peptides (Arg-1 $\rightarrow$ Leu, Arg-4 $\rightarrow$ Leu, Lys-5 $\rightarrow$ Leu, Ser-7 $\rightarrow$ Ala, Arg-9 $\rightarrow$ Leu, and Lys-11 $\rightarrow$ Leu), NM (neuromodolin)-peptide (KIQASFRGHITRKKLKG) and the His-9 $\rightarrow$ Pro substituted NM-peptide (KIQASFRGPITRKKLKG) were synthesized at the Biomolecular Research Facility, University of Virginia, Charlottesville, VA, U.S.A. The series of spin-labelled NM-peptides and spin-labelled α -peptides were synthesized by solid-phase methods and purified as described previously [7]. These peptides contain a single cysteine substitution that is derivatized with a methanethiosulphonate spin label (MTSSL) (Figure 1). This side chain, which we will refer to as R1, has a hydrophobicity between that of tryptophan and methionine [8]. Stock solutions of peptides were made by weighing the appropriate amount of peptide and dissolving it in distilled water.

PKC purification

PKC α was purified from Sf-9 insect cells transfected with PKC α -containing baculovirus generously provided by Drs. Peter Parker, Silvia Stabel and Dorian Fabbro. Enzyme was purified by sequential chromatography on Q-Sepharose and phenyl-Superose columns as described [9,10]. PKC was quantitated by binding of [³H]phorbol-12,13-dibutyrate [11] and purity was assessed by gel electrophoresis followed by silver staining and subsequent Western-blot analysis using isozyme-specific antibodies.

Figure 1 Structure of a peptide derivatized with a methanethiosulphonate spin label

The cofactor-independent catalytic fragment of PKC (PKM) was produced by treating PKC α with trypsin (1:1 w/w) for 5 min. The digestion was halted by the addition of soybean trypsin inhibitor (five times the amount of trypsin by weight). PKC and PKM were separated by FPLC on a Q-Sepharose column. The separation was checked by Western-blot analysis which revealed a single band at 50 kDa. The concentration of PKM produced was determined by densitometry analysis and by comparing the activity of a known concentration of PKC with varying volumes of PKM. It was determined to be approx. 1.6 nM by both methods.

Preparation of lipid solutions

All phospholipids were stored in chloroform, under Ar, at -70°C prior to use. For the kinase assays, stock solutions of bbPS with 5 mol % DO, or PC₇ with 5 mol % DO, were prepared by aliquoting stock solutions of lipid in chloroform into borosilicate culture tubes using gas-tight syringes and drying down the lipid mixture under a stream of N₂ gas. The mixtures were lyophilized from benzene/methanol (19:1, v/v) for at least 12 h in the dark, hydrated and allowed to stand for approx. 1 h and then vortexed and aliquoted. DMPA, DMPC, DMPS and PC₇ used in CD spectroscopy experiments were similarly prepared. All of the lipids, with the exception of PC₇, were sonicated for 10 min. For electron paramagnetic resonance (EPR) spectroscopy, extruded lipid vesicles containing bbPS:PC (40:60) were prepared as described [7].

Assay of PKC activity

Kinase activity was assessed by measuring the ability of the enzyme to phosphorylate a peptide substrate in a reaction mixture (100 μl) containing substrate peptide (30 μM), 300 μM CaCl₂, 40 μM [γ -³²P]ATP (10–20 mCi/mmol), 5 mM MgCl₂, 20 mM Mops (pH 7.4 at 30 $^{\circ}\text{C}$), 0.8 nM PKC α or PKM, and either 200 μM bbPS with 5 mol % DO or a range of PC₇ concentrations (100–500 μM with 5 mol % DO) to ensure that peak activity was observed. The reaction was terminated after 3 min at 30 $^{\circ}\text{C}$ by spotting 50 μl onto P-81 ion exchange paper (Whatman). Papers were washed three times in 50 mM NaCl to remove unreacted ATP and dried. Bound radioactivity was determined by scintillation counting. Activity in the absence of lipid was subtracted from the activity observed in the presence of the lipid to give lipid-dependent PKC α activity.

CD measurements on substrate peptides

CD spectra were acquired in 10 mM Tris, trifluoroethanol (TFE) or methanol (MeOH) (30 μM peptide in aqueous or organic solvents) or in 1 mM DMPA, DMPC, DMPG, DMPS (bbPS was not used because it exhibited an interfering absorbance) or in 5 mM PC₇ (18 μM peptide in the presence of phospholipids) with a Jasco J-720 spectropolarimeter using a 1 mm cell at room temperature with the following conditions: band width 2.0 nm, sensitivity 100 mdeg, response 0.25 s, starting wavelength 250 nm, ending wavelength 196 nm, scan speed 50 nm/min, step resolution 0.5 nm. Each spectrum shown represents an average of 10 scans after subtraction of an averaged background scan. The CD spectra were subsequently deconvoluted by the PROSEC computer algorithm [12].

Substrate binding to lipids determined by EPR

Spin-labelled peptides, Cys-10-NM peptide or Cys-10- α -peptide were mixed to a final concentration of 50 μ M, with 50 mM bbPS:PC (40:60) vesicles in 100 mM KCl, 1 mM Mops, pH 7.0. EPR spectra were recorded on an X-band Varian Century Series spectrometer fitted with a loop gap resonator using a 100 gauss scan range, a microwave power of 2 mW and a modulation amplitude of 1.25 gauss (p-p). The EPR spectra of nitroxide labels such as R1 are sensitive to motion on the nanosecond time scale, and binding of the labelled peptides to membrane surfaces is easily quantitated from the amplitude of the high-field ($m_1 = -1$) resonance using procedures described previously [13,14]. Briefly, if the peptide is in equilibrium between membrane and aqueous phases, the EPR spectrum will be a composite representing the two populations. The quantities of peptide in each phase can be calculated by calibrating the high-field resonance amplitude in terms of the aqueous spin concentration.

RESULTS

Sequence requirements for phosphorylation of PKC substrate peptides in PS vesicles

Although PKC substrates typically exhibit a phosphorylatable Ser or Thr surrounded by basic amino acids, the sequence motif varies considerably among different substrates. To help define structural requirements for recognition by the enzyme, two different substrate peptides (α -peptide and NM-peptide) were compared and amino acid substitutions were made to determine which residues were essential. Peptides in which charged amino acids essential to substrate recognition have been replaced should be phosphorylated to a lesser extent than the native peptide or peptides with substitutions in nonessential residues. Substitution of individual basic residues in α -peptide (Arg-1 $\rightarrow$ Leu, Arg-4 $\rightarrow$ Leu, Lys-5 $\rightarrow$ Leu, Arg-9 $\rightarrow$ Leu, and Lys-11 $\rightarrow$ Leu) resulted in phosphorylation that was at least 35% of that of native α -peptide (Table 1), suggesting that none of these residues alone is necessary for substrate recognition by the enzyme. Substitution of the phosphorylatable serine (Ser-7 $\rightarrow$ Ala), of course, eliminated phosphorylation.

A series of substituted spin-labelled NM-peptides was synthesized for use in future EPR studies (see 'Experimental'). As with the α -peptide, native amino acids were replaced by a residue with a hydrophobic side-chain (Cys nitroxide-labelled). The Cys nitroxide spin-label has a side-chain surface area similar to that of phenylalanine. Each peptide was tested for its ability to act as a PKC substrate. In contrast to the substituted α -peptides, NM-peptides with the hydrophobic Cys-nitroxide (substitution R1) at Gln-3 or Ala-4, adjacent to the target Ser-5, were phosphorylated to only 10 or 15%, respectively, relative to the native peptide (Table 1), indicating that they are necessary for substrate recognition. This reduction in phosphorylation could be the result of steric hindrance or poor contact in the binding site resulting from the slightly larger side-chain volume of the spin-label. However, peptides with cysteine-labelled substitutions at Thr-11, Arg-12, Lys-13 or Lys-16 also exhibited significantly reduced phosphorylation despite the fact that these amino acids are at least six residues C-terminal to the phosphorylation site. If NM-peptide interacts with PKC in an extended conformation, in a manner similar to that previously modelled for the α -peptide with PKC [15], these residues should not interfere with the NM-peptide/PKC association. Hence, these results suggest that more distal residues also are necessary for enzyme recognition, possibly by contributing to an appropriate secondary structure.

Table 1 PKC α phosphorylation of substituted α -peptides and substituted NM-peptides

Kinase activity was measured as described in 'Experimental' in the presence of bbPS vesicles. The original residue and position of substitution are indicated. α -peptide residues were substituted with Leu with the exception of the Arg-1* peptide which was substituted with a Cys and subsequently spin-labelled (see 'Experimental') and the Ser-7 peptide which was substituted with Ala. NM-peptide residues were substituted with a Cys which was subsequently spin-labelled (see 'Experimental'). Results are shown as means $\pm$ S.E. of duplicate determinations from a representative of 2–3 experiments depending on the peptide.

Substituted residue	PKC α activity (c.p.m./80 fmol enzyme) $\times 10^{-3}$	Percent phosphorylation relative to native peptide
Substituted α -peptides		
None	543 $\pm$ 17	100
Arg-1*	236 $\pm$ 2	44
Arg-1	208 $\pm$ 66	38
Arg-4	352 $\pm$ 5	65
Lys-5	369 $\pm$ 16	68
Ser-7	0 $\pm$ 32	0
Arg-9	305 $\pm$ 14	56
Lys-11	283 $\pm$ 25	52
Substituted NM-peptides		
None	68.5 $\pm$ 8.0	100
Lys-1	41.1 $\pm$ 1.3	60
Ile-2	32.1 $\pm$ 1.6	47
Gln-3	6.6 $\pm$ 1.3	10
Ala-4	10.6 $\pm$ 0.5	15
Ser-5	2.7 $\pm$ 0.6	4
Phe-6	34.6 $\pm$ 1.2	50
Arg-7	15.1 $\pm$ 5.0	22
Gly-8	12.6 $\pm$ 0.8	18
His-9	49.7 $\pm$ 4.6	73
Ile-10	23.5 $\pm$ 9.8	34
Thr-11	5.2 $\pm$ 1.2	8
Arg-12	1.6 $\pm$ 1.6	2
Lys-13	8.0 $\pm$ 2.0	12
Lys-14	14.2 $\pm$ 0.1	21
Leu-15	31.5 $\pm$ 3.7	46
Lys-16	3.1 $\pm$ 1.0	5
Gly-17	11.3 $\pm$ 0.4	16

Since the aforementioned substituted residues of the α -peptide and NM-peptide were replaced with two different hydrophobic moieties, an α -peptide with a residue substitution identical to those incorporated into the series of NM-peptides was made. An α -peptide with Cys replacing Arg-1 was synthesized and was subsequently labelled with a methanethiosulphonate spin label. This spin-labelled α -peptide (Arg 1 $\rightarrow$ Cys nitroxide) was phosphorylated 44% relative to native α -peptide whereas the corresponding Leu-substituted α -peptide (Arg 1 $\rightarrow$ Leu) was phosphorylated 38% relative to the native peptide. These results suggest that the Leu side chain and spin-labelled moieties, while not identical, are comparable in the extent to which they affect PKC-mediated peptide phosphorylation.

In helix-inducing solvents, the NM-peptide exhibits helical character [7]. When the NM-peptide is viewed as an alpha helix (Figure 2B), the PKC phosphorylation site is surrounded by basic residues and more closely resembles the typical PKC substrate motif than does the NM-peptide in a linear structure. In contrast, the α -peptide does not reveal this concentration of basic residues when presented in helical format (Figure 2A).

Figure 2 PKC α phosphorylation of (A) substituted α -peptide and (B) substituted NM-peptide, presented in the context of an α -helix

The mean % phosphorylation of each substituted peptide with respect to the native is shown.

Furthermore, NM-peptide residues Arg-12 and Lys-16, which appear to be necessary for substrate recognition, flank the phosphorylated Ser-5 when presented in the helical format. Peptides with substituted residues on the opposite side of the helix from the phosphorylation site (Ser-5) also exhibited reduced phosphorylation. When residues Ala-4, Thr-11, Arg-7, Lys-14 and Gln-3 were replaced, peptide phosphorylation dropped to 25% or less of that of native NM-peptide, suggesting that these residues also play a role in substrate recognition, perhaps by interacting with lipid or other residues in the PKC binding pocket.

The observation that residue Thr-11 of the NM-peptide was required for substantial phosphorylation of the peptide by PKC

Figure 3 CD spectra of α -peptide and NM-peptide, mean residue ellipticity ($[\theta]_{\text{nmv}}$) as a function of wavelength, in the presence of (A) 10 mM Tris buffer and (B) 1 mM DMPS vesicles

Each spectrum represents an average of 10 scans.

raised the question of whether it also was recognized as a phosphorylation site. We therefore verified the site of phosphorylation under the conditions of the kinase assay via mass spectrometry. The peptide was found to be singly phosphorylated during the time course of the activity assays, with Ser-5 as the only phosphorylated residue detected (data not shown).

Structural analysis of NM-peptide and α -peptides in PS vesicles

The results of the kinase assays suggested that NM-peptide may be in a helical conformation when it interacts with PKC, not an extended conformation as is the α -peptide. To examine the possibility that the NM-peptide and the α -peptide adopt differing secondary structures at the membrane surface, the CD spectra of both peptides were acquired in aqueous solution and in the presence of lipid vesicles. The spectra were subsequently deconvoluted by the program PROSEC to obtain an estimate of secondary structure content. In Tris buffer, NM-peptide and α -peptide exhibited similar spectra with very little helical character (Figure 3A). This is consistent with previous results for the NM-peptide using NMR and EPR [7]. However, in the presence of DMPS vesicles at low ionic strength, the NM-peptide underwent a dramatic structural change (Figure 3B). Deconvolution of this spectrum and analysis via the PROSEC algorithm indicated

Table 2 Percent secondary structure of substrate peptides in various environments

Percent secondary structure was determined from CD spectra using the PROSEC computer program. Peptide concentrations were 30 μ M in TFE, MeOH and Tris and 18 μ M in the presence of phospholipid. Phospholipid concentrations were 1 mM with the exception of PC₇ where it was 5 mM and the NM-peptide (Pro substituted for His) where [DMPS] = 144 μ M.

Solvent	<i>n</i>	α -helix	β -sheet	Turn	Random coil
α-Peptide (13-mer)					
TFE	6	10 $\pm$ 5	43 $\pm$ 15	6 $\pm$ 10	41 $\pm$ 7
MeOH	4	1 $\pm$ 3	29 $\pm$ 20	24 $\pm$ 10	46 $\pm$ 9
DMPA	1	8	3	45	44
DMPG	3	8 $\pm$ 6	8 $\pm$ 14	28 $\pm$ 8	56 $\pm$ 8
DMPS	3	1 $\pm$ 2	56 $\pm$ 7	18 $\pm$ 5	25 $\pm$ 10
Tris	5	0 $\pm$ 0	36 $\pm$ 16	19 $\pm$ 9	45 $\pm$ 7
PC ₇	3	0 $\pm$ 0	25 $\pm$ 22	30 $\pm$ 15	45 $\pm$ 8
DMPC	1	0	25	34	41
NM-peptide					
TFE	3	60 $\pm$ 8	7 $\pm$ 10	0 $\pm$ 0	33 $\pm$ 2
MeOH	3	56 $\pm$ 12	19 $\pm$ 15	0 $\pm$ 0	25 $\pm$ 3
DMPA	2	25 $\pm$ 18	2 $\pm$ 3	53 $\pm$ 12	20 $\pm$ 28
DMPG	4	27 $\pm$ 9	0 $\pm$ 0	59 $\pm$ 1	14 $\pm$ 8
DMPS	9	26 $\pm$ 8	13 $\pm$ 15	52 $\pm$ 10	9 $\pm$ 7
Tris	3	0 $\pm$ 0	31 $\pm$ 18	19 $\pm$ 9	51 $\pm$ 10
PC ₇	3	0 $\pm$ 0	24 $\pm$ 21	24 $\pm$ 9	52 $\pm$ 14
α-Peptide (17-mer)					
TFE	1	37	19	0	44
DMPS	2	0 $\pm$ 0	54 $\pm$ 8	6 $\pm$ 2	40 $\pm$ 5
Tris	1	0	26	19	55
PC ₇	2	0 $\pm$ 0	40 $\pm$ 9	17 $\pm$ 7	43 $\pm$ 2
NM-peptide (Pro substituted for His)					
DMPS	1	17	46	26	11

26 $\pm$ 8 % helical character (Table 2). In contrast, the spectrum of α -peptide changed very little in the presence of DMPS and exhibited insignificant helical character (Figure 3B and Table 2). While algorithms for analysis of CD spectra may not provide accurate absolute percentages of secondary structure, the results of such analyses are useful for comparing relative structural changes induced by different environments or between different peptides. Examination of Table 2 reveals that the α -peptide does not exhibit a propensity toward helix formation in any of the solvent systems tested. This is in contrast to the NM-peptide, which showed a greater degree of helical character in classic helix-inducing solvents (TFE and MeOH) and in the presence of small unilamellar vesicles composed of acidic phospholipids.

To investigate the DMPS-dependence for these structural changes in NM-peptide, CD spectra were acquired in the presence of increasing concentrations of DMPS. These and similar spectra for α -peptide were deconvoluted to provide percent secondary structure as a function of PS concentration (Figures 4A and 4B). NM-peptide exhibited an increase in turn and α -helical content and a decrease in β -sheet and random coil as the DMPS concentration was increased to 150 μ M, whereas the α -peptide exhibited an increase in β -sheet content and a decrease in turn and random coil over the same range of DMPS concentrations. No significant helical conformation was detected for the α -peptide. Similar results were obtained in other acidic lipids, but not in the presence of zwitterionic PC₇ micelles (Table 2). Based on the charge of these two peptides, the binding constant of the NM-peptide is not expected to be substantially different from

Figure 4 Percent secondary structure of (A) α -peptide and (B) NM-peptide as a function of DMPS concentration

Structural content (Δ)- α -helix, ($\bullet$)- β -sheet, ($\blacktriangle$)-turn and ($\circ$) random coil was determined by deconvolution of the corresponding spectrum with the PROSEC algorithm.

that of the α -peptide to PS. Thus the differences in secondary structure are unlikely to be attributed to differences in the binding affinity of these two peptides to the DMPS vesicles.

Differences between the DMPS-induced structural changes in NM-peptide vs. α -peptide also could be a function of the difference in length (17-mer vs. 13-mer) of the peptides. To investigate this possibility, a longer α -peptide (17-mer) was examined via CD in the presence of DMPS vesicles. Although the 17-mer α -peptide exhibited helical character in TFE (37% compared with 60% for the NM-peptide), the spectra of the 17-mer α -peptide acquired in the presence of DMPS was similar to those of the 13-mer α -peptide acquired under the same conditions and also exhibited minimal helical character (Table 2). This result suggests that the observed structural differences between α -peptide and NM-peptide are not simply a function of their differing length.

Model for the binding of NM-peptide to PKC

These CD experiments suggest that NM-peptide exhibits a secondary structure with helical character when bound to the surface of vesicles composed of acidic phospholipids. To assess the possibility that a helical conformation might be maintained

Figure 5 Ribbon diagram of NM-peptide in a distorted helical conformation complexed with the catalytic region of PKC β II

PKC backbone (teal), NM-peptide (yellow), ATP (orange), Mn^{2+} (pink dots), basic residues of NM-peptide (light blue), acidic residue in the substrate binding pocket of PKC (red) (left). View of complex rotated 90° around the vertical axis (right).

when NM-peptide interacts with PKC, the NM-peptide, with an entirely α -helical secondary structure, was docked to the catalytic region of PKC β II using the program AFFINITY [16,17]. The coordinates for PKC β II, obtained from the Protein Data Bank, are based on the structure modelled by Orr and Newton [15]. The helical NM-peptide was placed in the PKC substrate-binding site by aligning the phosphorylation site, Ser-5, with that of a docked α -peptide. Energy minimization of the helical NM-peptide:PKC complex yielded a viable structure (Figure 5). Although the helical NM-peptide appears to be partly distorted, the docking and subsequent energy minimization indicate that the binding pocket in this proposed PKC structure can accommodate a substrate with substantial helical structure. It should also be noted that, while the AFFINITY program allows for dynamics of selected residues in the binding pocket, it does not carry out a global dynamics simulation. Thus, any hinge or global structural changes in PKC that might accompany binding of a helical peptide would not be allowed in this procedure.

Binding of NM-peptide and α -peptide to PS vesicles and PC_7 micelles

Since basic peptides, such as α -peptide [18] and NM-peptide [19], bind readily to negatively-charged PS vesicles, an EPR analysis of peptide-PKC interactions would be complicated by peptide-PS binding. To investigate differences in lipid binding of the two peptides, EPR spectra of NM-peptide spin-labelled at the tenth amino acid residue (Cys-10-NM peptide) and a similarly-labelled

α -peptide (Cys-10- α -peptide) were acquired in bbPS:PC (40:60) vesicles and in PC_7 micelles, a zwitterionic short-chained lipid system previously shown to activate PKC as determined by phosphorylation of histone [20]. The EPR spectra of both peptides in the presence of PS are highly anisotropic and characteristic of peptides bound to membranes [21] (Figures 6A and 6B). A minor component representing less than 2 or 3 % of the total peptide remains unbound in these samples. This is in contrast to the spectra of the peptides acquired in the presence of PC_7 micelles where the lineshapes are dominated by aqueous peptide signal (Figure 6C and 6D). Quantitation of the amplitudes of the high-field ($m_I = -1$) resonance indicated that 35 % of the α -peptide and 31 % of the NM-peptide are bound to the micelle under these conditions. Thus, PC_7 micelles may constitute a better lipid system to investigate peptide-PKC interactions since lipid-peptide interactions are reduced in the presence of the zwitterionic micelles relative to the negatively charged PS vesicles.

Structural analysis of NM-peptide and α -peptide in PC_7 micelles

To determine whether the peptides undergo structural changes in the presence of PC_7 , CD spectra were acquired with PC_7 micelles and deconvoluted with the PROSEC algorithm to yield estimates of secondary structure. The CD spectra of α -peptide and NM-peptide in PC_7 were remarkably similar, with neither exhibiting any helical character (Figure 7). These spectra were very similar to those of the peptides in buffer alone (Figure 3A).

Figure 6 EPR spectra of (A) Cys-10- α -peptide and (B) Cys-10-NM-peptide in the presence of bbPS:PC (40:60) vesicles and (C) Cys-10- α -peptide and (D) Cys-10-NM-peptide in the presence of 50 mM PC₇ micelles

Figure 7 CD spectra of α -peptide and NM-peptide, mean residue ellipticity ($[\theta]_{mwr}$) as a function of wavelength, in the presence of 5 mM PC₇ micelles

Each spectrum represents an average of 10 scans.

Phosphorylation of NM-peptide and α -peptide in PC₇ micelles

Since the NM-peptide did not undergo a structural change in the presence of PC₇ micelles similar to that which was observed in the PS lipid system, it is possible that the PKC phosphorylation of the NM-peptide may also be different in the two lipid systems. Phosphorylation of α -peptide and NM-peptide in the presence of PC₇ was compared to that in bbPS. Both peptides were phosphorylated to nearly the same extent in bbPS, but in PC₇ phosphorylation of α -peptide was 73 % of that observed in bbPS, whereas phosphorylation of NM-peptide in PC₇ was only 23 % relative to phosphorylation in the bbPS lipid system (Table 3). This result suggests that NM-peptide requires the presence of acidic phospholipids to be recognized as a substrate, whereas α -peptide has a less stringent lipid requirement. Combined with the CD data, which revealed a substantial change in secondary structure of NM-peptide versus α -peptide in DMPS, it raises the

Table 3 PKC α phosphorylation of α -peptide and NM-peptide in the presence of bbPS vesicles or PC₇ micelles

Kinase activity was measured as described in 'Experimental'. Data shown are means $\pm$ S.E. of maximal PKC activity for the lipid systems indicated from duplicate determinations from a single experiment. Results are representative of at least five experiments.

	PKC α activity (c.p.m./80 fmol enzyme) $\times 10^{-3}$	
	bbPS	PC ₇
α -Peptide	280 $\pm$ 45	205 $\pm$ 17
NM-peptide	284 $\pm$ 27	66 $\pm$ 27

question of whether the lipid requirement of the NM-peptide is due to a stabilization of the appropriate secondary structure.

Since the phosphorylation of the NM-peptide correlated with its tendency to exhibit α -helical character, we attempted to disrupt the helical structure to test the correlation. A Pro-replacement of the non-essential His in the middle of the peptide, however, did not eliminate PS-dependent helical character (Table 2), nor did it eliminate PKC-mediated phosphorylation (data not shown).

To determine whether the different lipid-dependence for recognition of α -peptide and NM-peptides by PKC extended to constitutively active PKC (PKM), the ability of PKM to phosphorylate the peptides was compared in the two lipid systems. No significant differences in peptide phosphorylation were observed in either lipid system or in the absence of lipid (data not shown). These results suggest that the presence of the regulatory portion of PKC contributes to recognition of certain substrate structures.

DISCUSSION

The substrate specificity of PKC has been investigated by many groups using synthetic peptides as substrates. The most common trait shared by these substrates is a primary sequence of Ser/Thr-Xaa-Lys/Arg where Xaa is usually an uncharged residue [22,23]. Although the sequences of PKC substrates exhibit great diversity apart from this consensus sequence, it has been observed that the presence of basic residues both C- and N-terminal to the target serine or threonine can enhance phosphorylation. However, kinetic studies suggested that regions more than six residues from the phosphorylation site have little influence on the kinetics of phosphorylation [22].

The commonly used α -peptide fits this motif, with several basic residues N- and C-terminal to the target serine. However, the PKC phosphorylation site in NM has an Arg residue two amino acids C-terminal to the phosphorylated serine but exhibits very little additional basic character proximal to the serine. The previously studied NM-peptide, which comprises the NM phosphorylation site, does have a cluster of basic residues near its C-terminus, but more distal to the site of phosphorylation. Examination of substituted NM-peptides revealed that several of these basic residues were necessary for substrate recognition despite the fact they are more than six residues from Ser-5, the phosphorylation site (Table 1). This contrasts with the α -peptide where kinase assays indicated that none of the basic residues

Table 4 Correlation of NM-peptide residue environment with PKC phosphorylation of substituted peptides

Residue environment was determined via visual inspection of Figure 5. Percent phosphorylation data are from Table 1. Lys-1, Lys-13 and Lys-16 are at the interface between PKC and solvent. Gly-8 and 17 positions are indeterminate.

NM-peptide residues	Percent phosphorylation of peptide with substituted residue relative to native peptide
Facing the PKC substrate-binding pocket	
Gln-3	10
Ala-4	15
Ser-5	4
Arg-7	22
Thr-11	8
Arg-12	2
Lys-14	21
Leu-15	46
Facing away from the PKC binding pocket	
Ile-2	47
Phe-6	50
His-9	73
Ile-10	34

alone could be considered essential for substrate recognition. This difference in basic residue requirement and location suggests that there may be a difference in the way a single PKC isozyme recognizes these two substrates.

Substrate recognition may be dependent not only on the sequence of a peptide, but also on the secondary structure. When viewed as an α -helix, the NM-peptide phosphorylation site is flanked by the more distal basic residues that were required for phosphorylation of the peptide by PKC (Figure 2B). The α -peptide phosphorylation site is surrounded by basic residues by virtue of its primary structure and presentation of that sequence on a helical wheel did not increase the basic character proximal to the phosphorylation site (Figure 2A).

Previous molecular modelling of a PKC pseudosubstrate inhibitor peptide complexed with the catalytic domain of PKC suggested that the peptide adopts an extended structure when associated with the enzyme [15,24]. However, the results shown here indicate that the NM-peptide can be reasonably docked to PKC with a structure that is largely helical (Figure 5). Comparison of the extended conformation of the pseudosubstrate peptide with the distorted helical conformation of NM-peptide suggests that several interactions between substrate and enzyme are conserved. Lys-1 of NM-peptide occupies a region of the PKC binding pocket similar to that of Arg-1 of the pseudosubstrate peptide (Arg-19 in the PKC sequence). This pocket was previously noted to contain a cluster of acidic residues [15]. Phe-6 and Ile-10 of NM-peptide appear to be interacting with hydrophobic residues Phe-501, Pro-505 and Ile-508 of PKC. These hydrophobic PKC residues were also shown to associate with Leu-8 of the pseudosubstrate peptide (Leu-26 in the PKC sequence) [15]. In this NM-peptide:PKC structure, residues of the NM-peptide appear to form ion pairs with residues in the PKC substrate binding pocket. These include (NM-peptide residue to PKC residue): Arg-7 to Asp-466, Arg-12 to Glu-390, Lys-13 to Asp-381, Lys-14 to Asp-492 and -382 and Lys-16 to Asp-382 (Figure 5).

Additionally, it is possible to correlate those amino acid residues of NM-peptide that interact with PKC in this model with the effect of substituting these residues on peptide phosphorylation (Table 4). Substitution of residues facing the PKC binding pocket, with the exception of Leu-15, causes a reduction in phosphorylation of greater than 75% relative to native NM-peptide. Conversely, substitution of residues facing away from PKC results in a less severe reduction in phosphorylation. Lys-13 and -16, located at the interface region, may be required to interact with acidic lipids or form ion pairs with PKC residues as mentioned above. Taken together, the molecular modelling of the NM-peptide docked to PKC and the results of the kinase assays using substituted peptide substrates suggest that this substrate interacts with PKC in a partly helical conformation.

CD spectroscopy was used to evaluate conformational changes which occurred due to changes in the peptide environment and revealed a predominantly random-coil structure for both peptides in aqueous solution. Spectra acquired in the presence of PS vesicles suggested that NM-peptide undergoes a major structural change upon binding to the vesicle surface, whereas α -peptide does not. Structural changes in peptides upon membrane binding are well documented and have been investigated by several groups. Peptides of mitochondrial presequences have been shown by CD to adopt an ordered secondary structure in the presence of small unilamellar vesicles composed of L- α -lecithin. The helical content of these peptides has been shown to increase upon the addition of the anionic phospholipid cardiolipin as well as in the presence of both anionic and zwitterionic micelles [25]. CD analysis of peptides derived from parathyroid hormone (PTH) and parathyroid-hormone-related protein (PTHrP) indicate that these peptides are helical in the presence of palmitoyl-oleoyl-phosphatidyl serine vesicles, monomyristoyllecithin or SDS micelles. However, regular secondary structure of PTH and PTHrP peptides was not stabilized in DMPC vesicles [26].

NM-peptide, too, appears to require acidic phospholipids for stabilization of its secondary structure. CD spectra of NM-peptide acquired in the presence of DMPG and DMPA (Table 2) are similar in appearance and secondary structural content to those acquired with DMPS vesicles (Figure 3B). This is in contrast to spectra of α -peptide and NM-peptide acquired in the presence of PC₇ micelles. This short-chain zwitterionic lipid did not induce a structural change in either substrate. Similarly, CD spectra of both peptides acquired in the presence of DMPC vesicles were comparable in appearance to those acquired with PC₇ micelles and indicated that the peptides were not helical. This difference in the propensity of the peptides to form helices was further underscored by the observation that α -peptide exhibited very little helical character even in the presence of helix-inducing solvents such as TFE and MeOH, whereas NM-peptide exhibited at least 55% helical content in these solvents (Table 2). Structural differences between these two peptides cannot be attributed simply to differences in length of the two peptides. The CD spectrum of a 17-mer α -peptide with a serine four residues from its N-terminus, as in NM-peptide, suggests that its structure is similar to the 13-mer α -peptide in both the PS and PC₇ lipid systems (Table 2).

In addition to influencing substrate-membrane binding, phospholipids have been shown to affect phosphorylation by PKC in a substrate-dependent manner. Bazzi and Nelsestuen [27] first observed that some substrates like histone required PS, Ca²⁺ and DAG, whereas poly-Lys-Ser required only PS. Phosphorylation of protamine sulphate was cofactor-independent [27,28]. Nakadate et al. [29] observed that phosphorylation of histone was enhanced in the presence of PS even with the cofactor-independent catalytic fragment of PKC (PKM), consistent with

a substrate requirement for lipid. Bruins and Epand [30] observed that the cationic amphiphile cholesteryl-3 β -carboxyamido-ethylene-trimethylammonium iodide modulates the PKC phosphorylation of histone or protamine sulphate. More recently, Seki et al. [31] demonstrated that preincubation of myristoylated alanine-rich C kinase substrate (MARCKS) with the phosphoinositides phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP) or phosphatidylinositol-4,5-bisphosphate (PIP₂) inhibited PKC phosphorylation. Preincubation of NM or neurogranin (NG) with PI had little effect on phosphorylation, whereas preincubation with PIP stimulated the phosphorylation of both NM and NG. Preincubation with PIP₂ stimulated phosphorylation of NG, but inhibited NM phosphorylation. These results suggest that a specific interaction between substrate and lipid may be required for PKC recognition and subsequent phosphorylation.

In the present work, we present evidence for a link between lipid-induced substrate structure and phosphorylation by PKC. Activity assays reveal that both the α -peptide and NM-peptide are adequate substrates in the presence of the acidic phospholipid PS. In contrast, the α -peptide is readily phosphorylated in the PC₇ micellar lipid system, whereas NM-peptide is phosphorylated to a much lesser extent. These results can be correlated with the presence or absence of structural changes induced in the peptides as the result of the presence of phospholipid as discussed above. Thus, α -peptide exhibits a largely random structure in DMPS and in PC₇ and PKC phosphorylation of α -peptide is supported in either lipid system. In contrast, CD spectra of the NM-peptide revealed significant secondary structure in PS but not in PC₇ and NM-peptide was phosphorylated to a much greater extent in the PS lipid system than in the PC₇ lipid system (Table 3). Differences in phospholipid binding of the substrate peptides also failed to account for differential phosphorylation by PKC. EPR spectra of spin-labelled peptides indicate that α -peptide and NM-peptide both bind similarly to PS and less effectively to PC₇ (Figure 6). These results suggest that NM-peptide requires the presence of acidic phospholipids to be recognized by PKC.

The secondary structural changes in NM-peptide induced by addition of DMPS vesicles were an increase in α -helix and turn and a decrease in β -sheet and random coil (Figure 4B). Zhang and Vogel [32] demonstrated via NMR that B50/neuromodulin peptide binds to apo-CaM in an α -helical conformation and that a helical structure is induced by binding. Qin et al. further demonstrated that NM-peptide interacts as an α -helix and in a calcium-independent manner with the C-terminal lobe of CaM [33]. These studies as well as those described herein suggest that NM-peptide has the propensity to form a helical structure in numerous environments. To assess the importance of helical character of the NM-peptide in substrate recognition, a Pro for His-9 substitution was made. This substitution in NM-peptide did not disrupt its helical character significantly (Table 2). However, the molecular model of NM-peptide docked to PKC suggests that His-9 may lie within the central loop of the peptide (Figure 5) where a Pro would be expected to cause minimal disruption of the structure. Retention of α -helical character in the Pro-substituted peptide was consistent with its PS-dependent phosphorylation by PKC.

Previous NMR, EPR and CD experiments indicated that NM-peptide assumes an extended structure upon binding to the membrane surface [7]. However, differences in the lipid systems and ionic-strength conditions used in the prior experiments versus those described herein are significant and appear to account for the differences in NM-peptide structure. It is evident from all of the experiments that NM-peptide has the propensity to form an α -helix in certain solvents and in the presence of acidic

phospholipids and this structure may be necessary for substrate recognition.

PKM phosphorylation of both of these peptide substrates appears to occur in a lipid-independent manner. Nakabayashi et al. demonstrated that PKM exhibited an altered substrate specificity relative to PKC in the phosphorylation of myosin light-chain kinase [34]. A role for the regulatory domain in substrate selection was shown for PKC- ϵ [35] and PKC- η [36] phosphorylation of histone. Deletion analysis implicated the pseudosubstrate region of PKC- η in contributing to the substrate specificity [36]. Dekker et al. suggested a model in which the lipid cofactors alter the conformation of PKC such that the interaction between the active site and the pseudosubstrate is weakened but not abolished as it is in PKM or in PKC bearing mutations in the pseudosubstrate region. They propose that the pseudosubstrate still competes with exogenous substrate in the lipid-activated PKC and that the extent to which mutants mimic native PKC will depend, in part, on the lipid context [36]. Our results are consistent with this model; however, the evidence for lipid-dependent changes in substrate structure (Figures 3 and 4) suggests that PKC may not be the only molecule undergoing structural changes required for substrate phosphorylation.

Although we do not yet have direct structural data on the association of the NM-peptide with PKC, taken together the above data argue that a helical motif is important in PKC recognition and phosphorylation of this peptide. The binding of peptides containing basic residues to acidic phospholipids has been shown to be an important event in the eventual phosphorylation of these substrates by intact PKC and we hypothesize that this binding event may serve not only to co-localize substrate and enzyme, but may also induce or stabilize a substrate conformation that can be recognized by PKC. This induced conformation appears to be substrate-specific and it cannot be generalized that a single secondary structure is required for recognition of all substrates. These results suggest that proteins may be recognized as PKC substrates by virtue of their secondary and not exclusively their primary structures.

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