Characterization of Putative Polyphosphoinositide Binding Motifs from Phospholipase Cβ_2[†]

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ABSTRACT: Several phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$)-regulated actin-binding proteins and most phosphoinositide-specific phospholipases C (PI-PLCs) comprise a basic amino acid motif (KxxxKxKK, where x denotes any amino acid), which was previously suggested to represent a $PtdInsP_2$-binding site commonly present in these proteins. We have shown earlier that a peptide corresponding to amino acids 448−464 of human PLCβ_2 (LPSPEDLRGKILIKNKK, peptide P1) markedly and specifically stimulated the activity of this enzyme [Simões et al. (1993) *FEBS Lett. 331*, 248]. Here, we present a detailed analysis of the effects of various peptides related to peptide P1 aimed at understanding the mechanisms of peptide-mediated PLCβ_2 stimulation. Peptide KILIKNKK (P2), which comprises only the basic amino acid consensus motif, also stimulated PLCβ_2, although higher concentrations were required to observe this stimulatory effect. The effects of P1 and P2 were not additive, indicating that the two peptides affect PLCβ_2 activity *via* the same mechanism. Peptide LPSPEDLRG (P3), composed of the amino-terminal half of P1, did not affect the activity of PLCβ_2. Peptide KILIKNKKQFSGPTSS (P4), which includes the nine amino acids flanking the carboxy-terminus of the KILIKNKK motif within the sequence of PLCβ_2, stimulated the enzyme but was indistinguishable in potency from P2. Circular dichroism analysis revealed that peptide P1 changes its conformation in the presence of $PtdInsP_2$ but not in the presence of other phospholipids including phosphatidylinositol 4-phosphate. The results suggest that the basic amino acid sequence physically interacts with $PtdInsP_2$. The role of the sequence corresponding to P1 within the native PLCβ_2 enzyme may be to offer the substrate to the actual catalytic residues in a configuration more favorable to hydrolysis.

Phosphatidylinositol 4,5-bisphosphate ($PtdInsP_2$)[1] hydrolysis catalyzed by intracellular phosphytidylinositol-specific phospholipases C (PI-PLCs) constitutes a key mechanism involved in several major signal transduction pathways, given that it can be triggered by activation of G-protein-coupled receptors or receptor-controlled tyrosine kinases (Nishizuka, 1992; Berridge, 1993). At least nine distinct PI-PLC isozymes have been identified in mammalian tissues. Their amino acid sequences share considerable similarity within two regions of about 170 and 260 amino acids, which are usually designated X and Y and appear to be essential for the catalytic activity of these enzymes (Meldrum et al., 1991a; Rhee & Choi, 1992). The similarities between the regions outside domains X and Y have been used to define the β, γ, and δ subfamilies of the PI-PLCs (Rhee et al., 1989). The number of members within each subfamily has been increasing rapidly, based on either protein purification or cDNA-cloning studies (Carozzi et al., 1992; Jhon et al., 1993; Kim et al., 1993; Lee et al., 1993a; Bahk et al., 1994; Ferreira et al., 1993; Ma et al., 1993; Su et al., 1994).

In addition to its role as a precursor for the second messengers $InsP_3$ and diacylglycerol, $PtdInsP_2$ seems to mediate a number of other cellular functions. Thus, $PtdInsP_2$ serves as substrate for 3-phosphorylation by phosphatidylinositol 3-kinase (Stephens et al., 1993). Very recently, $PtdInsP_2$ has been reported to promote the activation of phospholipase D by ADP-ribosylation factor (Brown et al., 1993) and support the interaction of the latter protein with its GTPase-activating protein (Randazzo & Kahn, 1994).

$PtdInsP_2$ has also been shown to interact with several actin-binding proteins, e.g., gelsolin (Yu et al., 1992; Janmey et al., 1992), villin (Janmey et al., 1992), profilin (Goldschmidt-Clermont et al., 1990, 1991), gCap39 (Yu et al., 1990), and cofilin (Yonezawa et al., 1990, 1991), as well as destrin and deoxyribonuclease I (Yonezawa et al., 1990). Potential high-affinity polyphosphoinositide-binding sites encoded by the amino acid consensus motifs KxxxKxKK and/or KxxxxKxKK (x denotes any amino acid; in some cases, lysine residues are replaced by arginine or histidine residues in the latter motif) have been proposed to exist in several of these proteins (Yu et al., 1992; Janmey et al., 1992). Synthetic peptides corresponding to those binding sites bind $PtdInsP_2$, compete with gelsolin for binding to $PtdInsP_2$, and dissociate gelsolin−$PtdInsP_2$ complexes (Yu et al., 1992; Janmey et al., 1992). Interestingly, phosphatidylinositol 3-kinase comprises

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[1] Abbreviations: PI-PLC, phosphoinositide-specific phospholipase C; $PtdInsP_2$, phosphatidylinositol 4,5-bisphosphate; PtdInsP, phosphatidylinositol 4-phosphate; PtdIns, phosphatidylinositol; PtdEtn, phosphatidylethanolamine; $InsP_3$, inositol 1,4,5-trisphosphate; Sf9 cells, *Spodoptera frugiperda* cells; CD, circular dichroism; peptide P1, LPSPEDLRGKILIKNKK; peptide P2, KILIKNKK; peptide P3, LPSPEDLRG; peptide P4, KILIKNKKQFSGPTSS.

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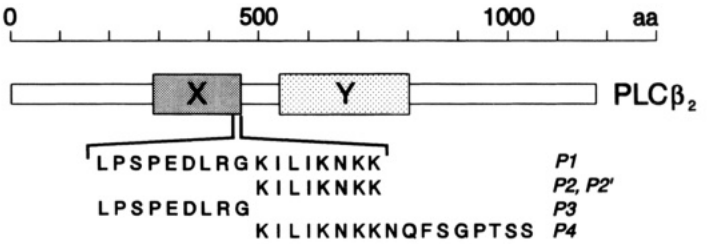

FIGURE 1: Linear representation of PLCβ_2. The two regions of high homology present in all phosphoinositide-specific phospholipases C and designated domains X and Y are shown as shaded boxes. The positions of the peptides used in this study within the native PLCβ_2 isozyme, their designation, and their primary structures are shown. The amino acid (aa) numbering is indicated.

the latter sequence motif in the inter-SH2 region of the p85 subunit (Dhand et al., 1994). Antibodies reactive with this region have been shown to inhibit the binding of PtdInsP_2 to the p85 protein (End et al., 1993).

The motif KxxxKxKK has also been identified at the carboxyl-terminus of domain X of various PI-PLCs (Yu et al., 1992), raising the possibility that PI-PLCs and certain actin-binding proteins may interact with PtdInsP_2 *via* structurally related domains. In fact, the actin- and PtdInsP_2-binding proteins profilin, cofilin, and gelsolin have been reported to inhibit various PI-PLCs (Goldschmidt-Clermont et al., 1991; Yonezawa et al., 1991; Banno et al., 1992).

To investigate whether the basic amino acid consensus motif KxxxKxKK represents a PtdInsP_2-binding site of PI-PLCs, we have synthesized a peptide corresponding to this motif within human PLCβ_2 (LPSPEDLRGKILIKNKK, residues 448−464; *cf.* Figure 1) and studied its effect on the activity of a recombinant PLCβ_2 (Simões et al., 1993). Much to our surprise, the peptide markedly and specifically stimulated the activity of the enzyme. The stimulatory effect of the peptide, which by itself did not exhibit PLC activity, ensued without delay and occurred without changing the affinity of the enzyme for Ca^{2+}. On the basis of these results, we speculated that the function of the PLCβ_2 segment corresponding to the peptide might be to bind PtdInsP_2 and facilitate its hydrolysis or, alternatively, to relieve the enzyme from a hypothetical inhibitory constraint. Here, we have determined which of the constituents of the stimulatory peptide are necessary for enzyme stimulation and have begun to dissect the molecular mechanisms of this stimulation. The results suggest that the sequence corresponding to the consensus motif does in fact constitute a PtdInsP_2-binding site in PLCβ_2.

MATERIALS AND METHODS

Materials. Inositol 1,4,5-trisphosphate, spermine, and spermidine were obtained from Sigma, Deisenhofen, FRG. L-Lysine tetramer was purchased from Bachem, Heidelberg, FRG. All other materials were from standard vendors or sources previously described (Camps et al., 1992a; Dietrich et al., 1992; Simões et al., 1993).

Peptide Synthesis. The peptides NH_2-LPSPEDLRGKILIKNKK-$CONH_2$ (peptide 1, residues 448−464 of PLCβ_2), NH_2-KILIKNKK-$CONH_2$ (peptide 2, residues 457−464), NH_2-KILIKNKK-COOH (peptide 2′, residues 457−464), NH_2-LPSPEDLRG-$CONH_2$ (peptide 3, residues 448−456), and NH_2-KILIKNKKNQFSGPTSS-$CONH_2$ (peptide 4, residues 457−473) were synthesized employing the Fmoc strategy and purified by preparative HPLC on a Parcosil ProRP C_{18} 300-7 reversed phase column. The purified peptides were further characterized by analytical HPLC and laser desorption mass spectrometry. The lyophilized peptides were dissolved in 50 mM Tris/HCl, pH 7.4, prior to the experiments. One amino-terminal cysteine and two glycines were added to peptides 1 and 3 to facilitate coupling to a carrier protein for eventual antibody production. For simplicity, these additions will not be specified throughout this communication.

Production of Recombinant Phospholipase C. PLC$\beta_2\Delta$, a deletion mutant of human PLCβ_2 (Park et al., 1992), was produced in baculovirus-infected *Spodoptera frugiperda* (Sf9) cells as previously described (Simões et al., 1993). Expression of the recombinant enzyme was monitored by SDS−PAGE and immunoblotting (Camps et al., 1992b). PLC$\beta_2\Delta$ differs from the wild-type PLCβ_2 isozyme in two respects. First, it lacks a carboxyl-terminal region (F819−E1166) necessary for stimulation by α_q subunits (Wu et al., 1993). Second, it carries a serine to alanine replacement in position 2 due to the introduction of a *Nco*I restriction enzyme site into its cDNA. PLC$\beta_2\Delta$ is indistinguishable from wild-type recombinant PLCβ_2 in terms of its interaction with PtdInsP_2, Ca^{2+}, and G-protein $\beta\gamma$ subunits (Schnabel et al., 1993), as well as its ability to be stimulated by peptide 1 (Simões et al., 1993).

Phospholipase C Assay. Phospholipase C activity was assayed using exogenous radiolabeled substrate essentially as described (Gierschik & Camps, 1994). In brief, 0.1 μg of soluble protein from Sf9 cells was incubated for 30 min at 25 °C in a volume of 70 μL containing 28 μM [^{3}H]PtdInsP_2 (5 Ci/mol), 280 μM phosphatidylethanolamine (PtdEtn), 50 mM Tris/maleate, pH 7.4, 80 mM KCl, 10 mM LiCl, 10 mM 2,3-bisphosphoglycerate, 1.2 mM sodium deoxycholate, 3 mM EGTA, and 1 mM free Ca^{2+}. To prepare the lipid substrate, a mixture of PtdEtn, PtdInsP_2 and [^{3}H]PtdInsP_2 in chloroform was evaporated to dryness under a stream of nitrogen. The lipids were then resuspended by continuous vortex mixing for 30 min in buffer (30 μL/sample) containing 116.5 mM Tris/maleate [taken from a 1 M stock adjusted to pH 7.5 at 20 °C with NaOH (Gomori, 1955)], 23 mM 2,3-bisphosphoglycerate, 23 mM LiCl, 7 mM EGTA, and 2.8 mM sodium deoxycholate. To determine the apparent K_M and V_{max} values for the enzyme, the lipid substrate was diluted keeping the mole ratio sodium deoxycholate/PtdEtn/PtdInsP_2 constant. After vortexing, the lipid suspension was sonicated for 15 min in a bath type sonicator (Sonorex RK 102; Bandelin, Berlin, Germany). The phospholipase C reaction was started by addition of the lipid substrate and terminated by adding 350 μL of chloroform/methanol/concentrated HCl (500:500:3, by volume) followed by vortex mixing and further addition of 100 μL of 1 M HCl containing 5 mM EGTA. Phase separation was accelerated by centrifugation for 2 min in an Eppendorf

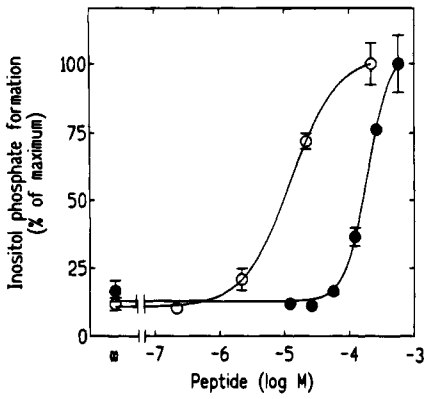

FIGURE 2: Stimulation of inositol phosphate formation by peptides KILIKNKK and LPSPEDLRGKILIKNKK. Soluble proteins (0.1 μg/tube) of Sf9 cells infected with recombinant baculovirus encoding PLC$\beta_2\Delta$ were incubated at increasing concentrations of either LPSPEDLRGKILIKNKK (open circles) or KILIKNKK (closed circles) with a phospholipid suspension containing PtdInsP$_2$. The reaction was terminated by the addition of chloroform/methanol/concentrated HCl and the mixture analyzed for inositol phosphates. See Materials and Methods for experimental details. The data were obtained in two independent experiments. Maximal inositol phosphate formation was 122.7 ± 9.3 (LPSPEDLRGKILIKNK) and 178.6 ± 18.4 (KILIKNKK) pmol min^{-1} μg of protein^{-1}. Each value represents the mean ± SD of triplicate determinations.

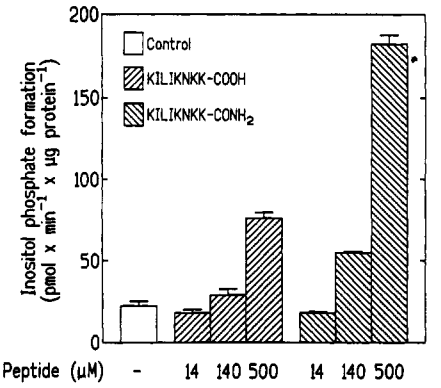

FIGURE 3: Comparison of the effects of peptides KILIKNKK-COOH and KILIKNKK-CONH$_2$ on inositol phosphate formation. Soluble proteins (0.1 μg/tube) of Sf9 cells infected with recombinant baculovirus encoding PLC$\beta_2\Delta$ were incubated in the absence (open bar) or presence (hatched bars) of the indicated concentrations of KILIKNKK-COOH or KILIKNKK-CONH$_2$ with a phospholipid suspension containing PtdInsP$_2$. Each value represents the mean ± SD of triplicate determinations.

microcentrifuge. The formation of water soluble inositol phosphates was determined by liquid scintillation counting.

Circular Dichroism Spectroscopy. Peptide secondary structure was monitored using circular dichroism (CD) spectroscopy. Spectra were taken on a Jasco J-500 automatic recording spectrapolarimeter coupled to a JP-Y data processor. Slit width was maintained at 1 nm throughout the spectral range. The instrument was calibrated using a 0.05% (w/v) solution of β-androsterone in dioxane. The circular dichroism of samples in a 1 mm quartz cuvette was measured from 190 to 240 nm at 5 nm/min scan speed, 2.0 s time constant, and 2.0 mdeg/cm sensitivity. Curves presented are the result of four signal-averaged scans with a similarly signal-averaged buffer base line subtracted. Curves were fitted using the CD spectra of model peptides in α-helix, β-sheet, extended coil, and reverse turn conformations, in a modification of the method described in Yang et al. (1986), and secondary structure content was estimated on this basis.

Miscellaneous. Protein concentrations were determined according to Bradford (1976) using bovine IgG as standard.

RESULTS

In Figure 1, we show the sequences of peptide P1, which has previously been shown to stimulate PLC$\beta_2\Delta$ (Simões et al., 1993), and the peptides introduced in this study (peptides P2−P4), as well as their positions within the native PLCβ_2 isozyme. Figure 2 shows a comparison of the effects of peptide P1 (LPSPEDLRGKILIKNKK) and peptide P2 (KILIKNKK) on inositol phosphate formation by PLC$\beta_2\Delta$. Note that the latter peptide corresponds exactly to the basic amino acid motif suggested to constitute a PtdInsP$_2$-binding site present in phospholipases C. Increasing concentrations of peptide P2 led to a marked stimulation of inositol phosphate formation, which was similar in maximal extent to the stimulation observed for peptide P1 (≈8.3-fold stimulation of basal activity in both cases). Interestingly, however, the potency of peptide P2 to stimulate PLC$\beta_2\Delta$ was lower than the potency of P1. Thus, half-maximal stimulation of enzyme activity was observed at approximately 12 μM P1 but required roughly 15 times higher concentrations (≈180 μM) of peptide P2. In additional experiments (results not shown), we found that stimulation of PLC$\beta_2\Delta$ by P1 was due to an increase in V_{max}, with only a minor, if any, change in K_M. Furthermore, we found that the stimulatory effects of peptides P1 and P2 were not additive, indicating that enzyme stimulation occurs *via* the same or similar molecular mechanisms.

To assess the importance of the carboxyl-terminal CONH$_2$ group present in both P1 and P2, we compared the effects of peptide P2 (NH$_2$-KILIKNKK-CONH$_2$) and peptide P2' (NH$_2$-KILIKNKK-COOH) on inositol phosphate formation by PLC$\beta_2\Delta$. Figure 3 shows that peptide P2' was capable of stimulating PLC$\beta_2\Delta$ activity when tested at 140 and 500 μM but that the degree of this stimulation was much lower than that observed for the same concentrations of peptide P2 (≈1.3- and 3.4-fold vs ≈2.5- and 8.2-fold stimulation of basal activity, respectively). These results demonstrate that the carboxyl-terminal CONH$_2$ group is not essential for but enhances peptide stimulation of phospholipase C.

The basic amino acid motif represented by peptide P2 comprises four lysine residues containing four positively charged ϵ-amino groups, which could conceivably interact with the negatively charged head group of PtdInsP$_2$. To examine the role of these four lysine residues in peptide-mediated phospholipase C stimulation, we studied the effect of lysine tetramer on the activity of PLC$\beta_2\Delta$. Lysine tetramer did in fact stimulate the formation of inositol phosphate formation by PLC$\beta_2\Delta$ (results not shown). Half-maximal stimulation and maximal (≈3.4-fold) stimulation were observed at approximately 400 μM and 3 mM, respectively. Next, we investigated whether other compounds comprising similar regularly spaced cationic centers were also capable of stimulating PLC$\beta_2\Delta$. We found that both spermine, $NH_2(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$, and spermidine, $NH_2(CH_2)_3NH(CH_2)_4NH_2$, markedly stimulated inositol phosphate formation by PLC$\beta_2\Delta$ (≈3.7- and 4.5-fold stimulation of basal activity, respectively) but that both compounds stimulated the enzyme only when present within a narrow range of concentrations (results not shown).

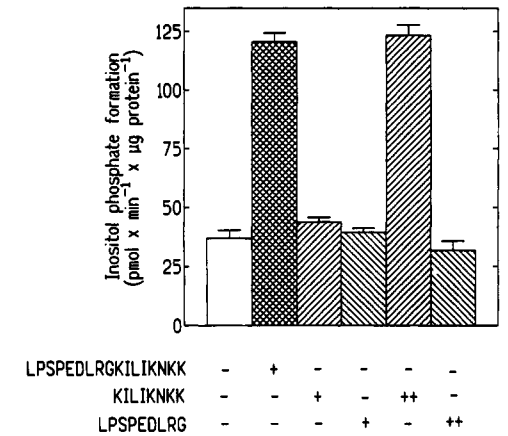

FIGURE 4: Comparison of the effects of peptides LPSPEDLRGKILIKNKK, KILIKNKK, and LPSPEDLRG on inositol phosphate formation. Soluble proteins (0.1 µg/tube) of Sf9 cells infected with recombinant baculovirus encoding PLC$\beta_2\Delta$ were incubated as indicated at the abscissa in the absence of peptide (−) or in the presence of either 28 (+) or 280 (++) µM LPSPEDLRGKILIKNKK, KILIKNKK, or LPSPEDLRG with a phospholipid suspension containing PtdInsP$_2$. Each value represents the mean ± SD of triplicate determinations.

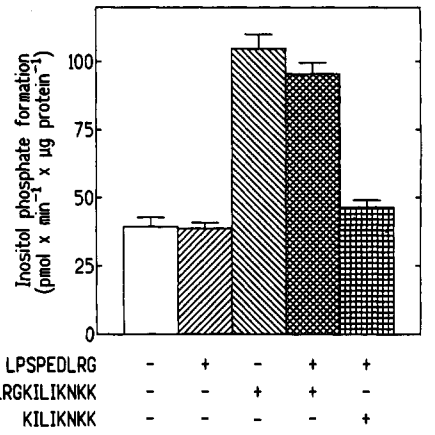

FIGURE 5: Effects of peptides LPSPEDLRGKILIKNKK, KILIKNKK, and LPSPEDLRG on inositol phosphate formation. Soluble proteins (0.1 µg/tube) of Sf9 cells infected with recombinant baculovirus encoding PLC$\beta_2\Delta$ were incubated as indicated at the abscissa in the absence (−) or presence (+) of 28 µM LPSPEDLRG, LPSPEDLRGKILIKNKK, and/or KILIKNKK with a phospholipid suspension containing PtdInsP$_2$. Each value represents the mean ± SD of triplicate determinations.

Specifically, maximal enzyme activity was evident at 100 µM spermine and 1 mM spermidine. The finding that the tetramine spermine was stimulatory at lower concentrations than the triamine spermidine is interesting, since it indicates that the number of positive charges affects the ability of this type of compounds to stimulate PLC$\beta_2\Delta$. In additional experiments (results not shown), we observed that the stimulatory effects of spermine and peptide P1 were not additive, suggesting that they are caused by the same or very similar mechanisms.

The finding that peptide P2 was by far less potent in stimulating PLC$\beta_2\Delta$ than peptide P1 prompted us to characterize the functional significance of the peptide sequence contained in the latter, but not in the former, peptide in more detail. Figure 4 shows that peptide P3 (LPSPEDLRG) had no effect on inositol phosphate formation by PLC$\beta_2\Delta$ when tested by itself at either low (28 µM) or high (280 µM) concentration. In contrast, peptides P1 and P2 caused marked (≈3.3-fold) increases in enzyme activity when examined at 28 and 280 µM, respectively. Figure 5 shows that peptide P3 (28 µM) did not interfere with stimulation of PLC$\beta_2\Delta$ by 28 µM P1. Furthermore, there was little, if any stimulation of inositol phosphate formation when peptides P2 and P3 were both present at 28 µM. Addition of 28 µM peptide P1 resulted in an approximately 2.7-fold increase in PLC$\beta_2\Delta$ activity in this experiment. On the basis of these results, it appears likely that the amino acids corresponding to the consensus amino acid motif for PtdInsP$_2$ binding are responsible for the PLC$\beta_2\Delta$ stimulation, whereas the residues present in P3 potentiate this stimulatory effect if covalently bound to the basic amino acid motif.

To determine whether the increased potency of peptide P1 to stimulate PLC$\beta_2\Delta$ was specifically caused by the amino-terminal elongation of the basic consensus motif, a carboxyl-terminally extended version of the motif was synthesized (KILIKNKKNQFSGPTSS, peptide P4) and its effect on inositol phosphate formation by PLC$\beta_2\Delta$ was compared to that of peptide P1 (LPSPEDLRGKILIKNKK). Note that the sequence NQFSGPTSS present in peptide P4 corresponds to the nine residues adjacent to the carboxyl-

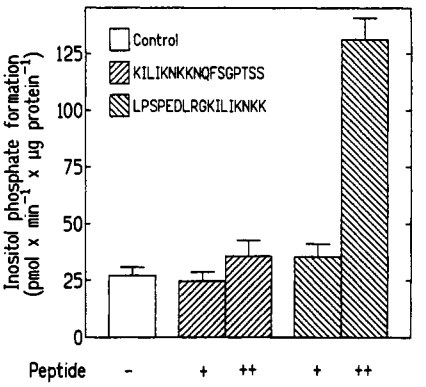

FIGURE 6: Comparison of the effects of peptides KILIKNKKQFSGPTSS and LPSPEDLRGKILIKNKK on inositol phosphate formation. Soluble proteins (0.1 µg/tube) of Sf9 cells infected with recombinant baculovirus encoding PLC$\beta_2\Delta$ were incubated as indicated in the absence of peptide (−) or in the presence of either 7 (+) or 70 (++) µM of KILIKNKKQFSGPTSS or LPSPEDLRGKILIKNKK with a phospholipid suspension containing PtdInsP$_2$. Each value represents the mean ± SD of triplicate determinations.

terminus of the basic motif within the sequence of PLCβ_2. Figure 6 shows that peptide P4 caused only a marginal stimulation of PLC$\beta_2\Delta$ when tested at 70 µM whereas peptide P1 led to an approximately 4.8-fold stimulation of enzyme activity when tested at the same concentration. In additional experiments (results not shown), we found that peptides P2 and P4 were capable of causing the same maximal stimulation of enzyme activity when present at high concentration (700 µM). Thus, the potentiating effect of the amino-terminal extension LPSPEDLRG cannot be mimicked by the similarly sized carboxyl-terminal extension NQFSGPTSS.

In the next experiments, CD spectroscopy was used to examine whether peptide P1 interacted physically with the phospholipase C substrate PtdInsP$_2$. The CD spectra of peptide P1 in the absence and presence of various phospholipids are shown in Figure 7. No changes of peptide conformation were observed when the peptide solution was supplemented with PtdEtn or combinations of this phospholipid with either phosphatidylserine or phosphatidylinositol 4-phosphate (PtdInsP). In marked contrast, peptide confor-

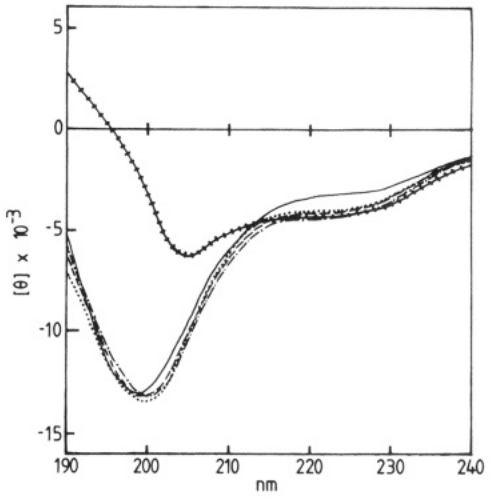

FIGURE 7: Far-ultraviolet circular dichroism spectra of the peptide LPSPEDLRGKILIKNKK. The peptide was dissolved at a concentration of 100 μg/mL in H_2O, and spectra were taken as described in Materials and Methods in H_2O (—) or in the presence of 280 μM PtdEtn and 1.2 mM sodium deoxycholate (— — —); 28 μM PtdInsP, 280 μM PtdEtn, and 1.2 mM sodium deoxycholate (— • — •—); 28 μM phosphatidylserine, 280 μM PtdEtn, and 1.2 mM sodium deoxycholate (• • •); or 28 μM $PtdInsP_2$, 280 μM PtdEtn, and 1.2 mM sodium deoxycholate (++++).

mation was significantly altered upon addition of PtdEtn and $PtdInsP_2$. Note that the phospholipids were used at concentrations identical to those used in the phospholipase C assay. Other components present in the assay were, however, omitted, in order to optimize the signal to noise ratio of the CD spectra. Interestingly, $CaCl_2$, present at 1 μM, a concentration at which phospholipase C stimulation by P1 is almost maximal (Simões et al., 1993), did not prevent the $PtdInsP_2$-induced conformational change of P1 (results not shown). In additional experiments (results not shown), we found that neither inositol 1,4,5-trisphosphate (28 μM) nor $CaCl_2$ (1 μM) affected peptide P1 conformation. The CD change of P1 observed upon addition of $PtdInsP_2$ is consistent with a transition from *ca.* 25% β-sheet, 47% coil, and 28% reverse turn in the absence of $PtdInsP_2$ to a much more compact conformation in its presence, with a large proportion of the coil converting to reverse turn. Taken together, these results not only suggest that peptide P1 interacts physically with the phospholipase C substrate $PtdInsP_2$ but that this interaction is highly specific for this phospholipid.

DISCUSSION

In this communication, we present a detailed structure–function analysis of peptide LPSPEDLRGKILIKNKK, which corresponds to the carboxyl-terminus of domain X in PLCβ_2 and has previously been found to activate this enzyme (Simões et al., 1993). The results show that the peptide can be divided into two functional constituents, a carboxyl-terminal portion, which is involved in enzyme stimulation, and an amino-terminal part, which increases the potency of the peptide to elicit this stimulatory effect. The results furthermore provide direct evidence for an immediate and specific peptide–$PtdInsP_2$ interaction, strongly suggesting that the peptide stimulates PLCβ_2 by binding to and offering the substrate to the catalytic domain of the enzyme in a more favorable configuration rather than by interacting with a hypothetical intrinsic inhibitory constraint.

LPSPMDLMYKILVKNKK	PLCβ_1
LPSPEDLRGKILIKNKK	PLCβ_2
LPSPQDLMGRILVKNKK	PLCβ_3
LPSPNDLKRKILIKNKR	PLCβ_4
LPSPNQLKRKILIKHKK	PLCγ_1
LPSPSQLREKIIIKHKK	PLCγ_2
LPSPEQLRGKILLKGKK	PLCδ_1 - b
LPSPEQLKGKILLKGKK	PLCδ_1 - r
LPSPEDLRGKILVKGKK	PLCδ_2
LPSPQELMGKILVKNKK	PLC*X*β
LPPPAMLRRKIIIKNKK	*plc-21*
LPPPCKLKRKILIKNKR	*norpA*
LPSPNDLKRKILIKKQT	Retinal PLCβ
LPTLDSLKYKILLKGHT	DdPLC
FRPQVSLKRKILIKNKR	PLCβ_x

FIGURE 8: Alignment of conserved residues present in phosphoinositide-specific phospholipases C in the proximity of the proposed phosphoinositide binding site. The amino acid sequence corresponding to peptide P1 [residues 448–464 of human PLCβ_2 (Park et al., 1992)] was aligned with the corresponding sequences of rat and bovine PLCβ_1 (Suh et al., 1988a; Katan et al., 1988), rat and human PLCβ_3 (Jhon et al., 1993; Carozzi et al., 1993), rat PLCβ_4 (Kim et al., 1993; Lee et al., 1993a), rat and bovine PLCγ_1 (Suh et al., 1988b; Stahl et al., 1988), rat and human PLCγ_2 (Emori et al., 1989; Ohta et al., 1988), rat and bovine PLCδ_1 [PLCδ_1-r and PLCδ_1-b, respectively (Suh et al., 1988a)], bovine PLCδ_2 (Meldrum et al., 1991b), *Xenopus laevis* PLCβ [PLC*X*β (Ma et al., 1993)], *Drosophila plc-21* (Shortridge et al., 1991), *Drosophila norpA* (Bloomquist et al., 1988), bovine retinal PLCβ (Ferreira et al., 1993), *Dictyostelium discoideum* PLC (Drayer et al., 1992), and *Artemia* PLCβ [PLCβ_x (Su et al., 1994)]. The sequences from different species are identical unless specified otherwise. Amino acid residues conserved in more than 70% of the 15 sequences shown are shaded.

The observations that peptide P2 alone was capable of stimulating PLCβ_2 activity, albeit at concentrations higher than those required for stimulation by the full length peptide, and that this stimulation could be mimicked by lysine tetramer and even by the polyamines spermine and spermidine suggest that the positive charges presented by the lysine residues present in the carboxyl-terminal portion of peptide P1 are important for enzyme stimulation. Importantly, the motif K/RxxxKxKK/R is present in almost all mammalian PI-PLCs currently known at the cDNA level, and in several nonmammalian PI-PLCs identified in *Xenopus* (Ma et al., 1993), *Drosophila* (Bloomquist et al., 1988; Shortridge et al., 1991), and in the brine shrimp *Artemia* (Su et al., 1994) (*cf.* Figure 8). The last two basic residues of the motif are missing in a PLCδ-related PLC found in *Dictyostelium discoideum* (Drayer & Van Haastert, 1992) and PLCβ-related PI-PLCs of bovine retina (Ferreira et al., 1993). It is worth noting, however, that most of the PI-PLCs, including the latter two variants, carry one or two basic residues immediately adjacent to the amino-terminus of the motif and that one of the two basic residues missing in the *Dictyostelium* PLC is replaced by a histidine. Furthermore, the spacing of the polyphosphoinositide-binding basic residues present in actin-binding proteins is also somewhat variable (Janmey et al., 1992; Yu et al., 1992), and replacing single lysine residues of one of the consensus motifs present in gelsolin by alanine does not affect polyphosphoinositide binding (Yu et al., 1992). Of interest, polyamines, basic proteins, and polymers of basic amino acids have previously been shown to stimulate phospholipase C (Haber et al., 1991). The fact that the stimulatory effects of peptide P1 and spermine on PLCβ_2 were not additive raises the possibility that polyamines elicit phospholipase C stimulation by mimicking an internal peptide sequence of these enzymes. It is worth noting in this context that polyamines are present

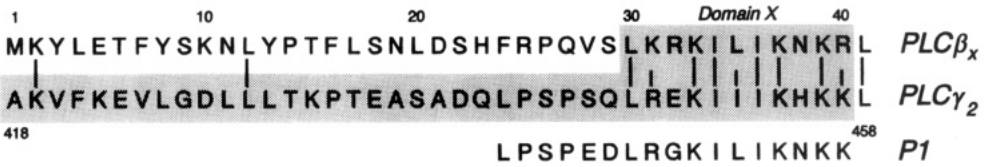

FIGURE 9: Alignment of the amino terminus of *Artemia* PLCβ_x with the corresponding carboxyl-terminal region of domain X of rat PLCγ_2. The alignment was generated using the PALIGN program (Myers & Miller, 1988) contained in the PC/GENE software package (IntelliGenetics, Mountain View CA). The structure−genetic matrix (Feng et al., 1985) was used, and both the open and unit gap costs were set to 10. A vertical bar designates that two aligned residues are identical. A half-vertical bar designates that two aligned residues are similar. Amino acids said to be similar are A, S, T; D, E; N, Q; R, K; I, L, M, V; and F, Y, W. Domain X of rat PLCγ_2 extends from residues 288 to 457 (Emori et al., 1989; Rhee & Choi, 1992). Note that residues 459−826 of PLCγ_2 are not essential for catalytic activity (Emori et al., 1989) and Leu-458 is poorly conserved among other PI-PLCs.

in eukaryotic cells at concentrations in the submillimolar to millimolar range and that polyamines are essential for cell growth and differentiation (Morgan, 1987, 1990). Whether polyamines interact with intracellular phospholipases C under physiological conditions and whether such an interaction plays a role in signal transduction deserve further studies.

The finding that peptide P1 specifically interacts with $PtdInsP_2$ is in agreement with previous reports on the preference of PLCβ isozymes for $PtdInsP_2$ in comparison to PtdInsP and/or PtdIns (Ryu et al., 1987; Harden et al., 1988; Park et al., 1992; James et al., 1994). The specific peptide−$PtdInsP_2$ interaction, together with the peptide-mediated stimulation of PLCβ_2 activity, raises the distinct possibility that the sequence corresponding to P1 corresponds to at least part of the catalytically relevant substrate-binding site of PLCβ_2. Other authors have previously shown the existence of a high-affinity $PtdInsP_2$-binding site located outside both the X and Y domains of PLCδ_1, most likely within its 60 amino-terminal residues (Cifuentes et al., 1993). However, this binding site appears to be required for membrane binding and processive $PtdInsP_2$ hydrolysis rather than for catalytic activity *per se* because proteolytic PLCδ_1 fragments lacking this binding site are still capable of hydrolyzing $PtdInsP_2$ (Cifuentes et al., 1993; Ellis et al., 1993). Several lines of evidence suggest that the catalytic core of PI-PLCs resides within the X and Y regions of these enzymes. Thus, deletion of sequences encompassing domains X or Y of PLCγ_1 or PLCγ_2, but not of the sequence between these domains, abolishes $PtdInsP_2$ hydrolysis (Bristol et al., 1988; Emori et al., 1989). Experiments using limited proteolysis of PLCδ_1 support the notion that both domains X and Y are required for catalysis and that an intact intervening peptide connecting these two domains is not required for $PtdInsP_2$ hydrolysis (Cifuentes et al., 1993; Ellis et al., 1993). Similar observations were made for PLCβ_2 (Lee et al., 1993b; Schnabel and Gierschik, unpublished results).

A cDNA encoding a highly truncated but nevertheless functional PLCβ-related PI-PLC, designated PLCβ_x, has recently been identified in the brine shrimp *Artemia* (Su et al., 1994). The enzyme lacks several hundred amino acids at the amino-terminus as compared to other PI-PLCs. When the amino-terminal residues of PLCβ_x encompassing the residual domain X residues are aligned with the corresponding region of rat PLCγ_2, the PI-PLC presently most thoroughly characterized by deletion mapping of its X and Y domains (Emori et al., 1988), it is evident that the homology between the two sequences is restricted to a very small region corresponding to residues 30−41 of PLCβ_x (Figure 9). With the exception of the carboxyl-terminal leucine, which is poorly conserved in several other PI-PLCs, this region is fully covered by the full length PLCβ_2 peptide P1 analyzed in this study. On the basis of these considerations, it appears likely that the residues represented by peptide P1, in particular the sequence LRGKILIKNKK, correspond to the catalytically relevant portion of domain X of PLCβ_2.

The fact that peptide P1 physically interacts with but does not hydrolyze $PtdInsP_2$ (Simões et al., 1993) strongly suggests that additional residue(s) are present in PI-PLCs to catalyze the actual hydrolysis of $PtdInsP_2$ to $InsP_3$ and diacylglycerol. We speculate that the role of peptide P1 within the native PLCβ_2 structure is to "offer" the phospholipid substrate to those catalytic residues in a conformation more favorable to hydrolysis. Our results are consistent with the notion that the sequences encoded by peptide P1 and the actual catalytic residues are isolated from each other within the nonactivated enzyme and that enzyme activation is caused by some structural change which brings these portions of the enzyme into close proximity. According to this model, addition of exogeneous peptide P1 would activate the enzyme by complementing the "catalytic core" and thereby mimicking the structural reorganization of the enzyme, which is normally necessary for enzyme activation.

Our work was initiated by the observation that a polyphosphoinositide-binding consensus motif identified in gelsolin and several other actin-binding proteins is also present in several PI-PLCs (Yu et al., 1992). It is tempting to speculate that the similarity between the polyphosphoinositide-binding domains of these two classes of proteins might be of some physiologic relevance. Of interest in this regard is a previous report showing that phosphorylated PLCγ_1 and PLCβ_1 were activated, albeit slightly, by physiological concentrations of profilin (Goldschmidt-Clermont et al., 1991). Also pertinent to this issue, Janmey and colleagues reported that preformed gelsolin−polyphosphoinositide complexes were specifically dissociated by gelsolin-derived peptides containing the basic consensus motif but not by several other agents known to inhibit the gelsolin−polyphosphoinositide interaction when added prior to complex formation (Janmey et al., 1992). If receptor-activated PI-PLCs were capable of dissociating preformed actin-binding protein−polyphosphoinositide complexes with similar specificity, this would provide a intriguing novel mechanism for cross-talk between transmembrane signaling and cytoskeletal organization.

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