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A peptide corresponding to a potential polyphosphoinositide binding site of phospholipase C-β_2 enhances its catalytic activity

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A peptide corresponding to a basic consensus amino acid motif present in both actin-binding proteins and phosphoinositide-specific phospholipases C was synthesized and its effect on the activity of a recombinant phospholipase C-β_2 (PLCβ_2) expressed in baculovirus-infected insect cells was studied. The peptide markedly and specifically stimulated the activity of the enzyme. This stimulatory effect required a particular primary and/or secondary structure of the peptide and occurred without lowering the affinity of the enzyme for Ca^{2+}. The function of the PLCβ_2 segment corresponding to the peptide might be to bind and offer the substrate to the catalytic domain of this enzyme in a more favorable configuration or, alternatively, to interact with a hypothetical inhibitory constraint.

Phospholipase C; Polyphosphoinositide; Actin-binding protein; Synthetic peptide; Signal transduction; Baculovirus

1. INTRODUCTION

The phospholipase C (PLC)-catalyzed hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdInsP_2) constitutes a fundamental part of a major transmembrane signal transduction pathway [1,2]. A still growing number of PLC isoforms has been identified in mammalian cells [3,4]. These isoforms present two homology domains, designated X and Y, which seem to be required for their catalytic activity. The mechanism of interaction with and cleavage of the substrate remains, however, essentially unknown.

In addition to its role as a precursor for second messengers, PtdInsP_2 appears to be involved in the regulation of several actin-binding proteins, namely gelsolin [5,6], villin [6], profilin [7,8], gCap39 [9], cofilin [10,11], destrin [10] and deoxyribonuclease I [10]. Potential high affinity polyphosphoinositide binding sites have been proposed in these proteins, based partially on mutagenesis experiments [5]. Furthermore, it has been reported that synthetic peptides whose sequences correspond to those binding sites can bind tightly to PtdInsP_2, compete with the proteins for the binding to this phospholipid, and dissociate protein/PtdInsP_2 complexes [5,6]. Both the basic character and the particular structure of these peptides seem to be required for their interaction with the acidic headgroup of PtdInsP_2 [5,6].

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Abbreviations: Ins$P$, inositol monophosphate; InsP_2, inositol 4,5-bisphosphate; InsP_3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; PtdInsP_2, phosphatidylinositol 4,5-bisphosphate; Sf9 cells, *Spodoptera frugiperda* cells.

It has been suggested that a similar potential PtdInsP_2 binding site exhibiting the amino acid motif KXXXKXKK and comprising the carboxyl-terminal-most region of the X domain exists in various intracellular PLCs [5]. We have studied the effect of a synthetic peptide corresponding to the proposed PtdInsP_2 binding site in PLCβ_2 on the activity of a recombinant PLCβ_2 expressed in *Spodoptera frugiperda* cells (Sf9 cells). Our findings show that this peptide markedly and specifically stimulates the catalytic activity of this enzyme.

2. MATERIALS AND METHODS

2.1. *Materials*

Lysine-phenylalanine (1:1), and lysine-alanine (1:1 and 2:1) random copolymers were obtained from Sigma (Deisenhofen, FRG). L-lysine trimer, tetramer and pentamer were purchased from Bachem (Heidelberg, FRG). All other materials were from standard vendors or from sources previously described [13,14].

2.2. *Peptide synthesis*

The peptide NH_2-CGGLPSPEDLRGKILIKNKK-$CONH_2$ was produced in a fully automated synthesizer employing the Fmoc strategy. The product was purified by preparative HPLC on a Parcosil ProRP C_{18} 300-7 reverse-phase column. The purified peptide was characterized by analytical HPLC and laser desorption mass spectrometry. The lyophilized peptide was dissolved in 50 mM Tris-HCl, pH 7.4, prior to the experiments. The peptide corresponds to amino acids 448–464 of PLCβ_2 [12]. The amino-terminal cysteine and two glycines were added to facilitate coupling to a carrier protein for eventual antibody production.

2.3. *Production of recombinant phospholipase C*

Spodoptera frugiperda cells (Sf9 cells, Invitrogen) were transfected with a 10:1 mixture of transfer vector and a modified baculovirus DNA (Baculogold, Pharmingen) [14]. The transfer vector (pVL1393) encoded either β-galactosidase (Invitrogen) or a deletion mutant of

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PLCβ_2 (PLC$\beta_2\Delta$). 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 [15]. Second, it carries a serine-to-alanine replacement in position two due to the introduction of an *NcoI* 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 (P. Schnabel et al., in preparation). For production of the recombinant proteins, Sf9 cells were maintained and infected exactly as previously described [14]. The cells were washed [14] and homogenized by two freeze-thaw cycles in hypotonic buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 μg/ml soybean trypsin inhibitor, 3 mM benzamidine, 1 μM pepstatin, 1 μM leupeptin, 100 μM phenylmethylsulfonyl fluoride. After centrifugation at 50,000 × *g* for 20 min at 4°C, the soluble fraction was collected. The expression of PLCβ_2 was verified by SDS-PAGE and immunoblotting [16].

2.4. *Phospholipase C assay*

Phospholipase C activity was assayed using exogenous radiolabelled substrate essentially as previously described [17]. 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, 50 mM Tris-maleate, pH 7.4, 3 mM EGTA, 80 mM KCl, 10 mM LiCl, 10 mM 2,3-bisphosphoglycerate, 1 mM ATP, 1.2 mM sodium deoxycholate, and 1 mM free Ca^{2+}, unless otherwise specified. The reaction was terminated by adding 350 μl of $CHCl_3/CH_3OH$/concentrated HCl (500:500:3, by vol.), vortexing and adding 100 μl of 1 M HCl containing 5 mM EGTA. Radioactivity released into the upper aqueous phase was quantitated by liquid, scintillation counting. To adjust the concentration of free Ca^{2+} to the desired value, the amount of $CaCl_2$ to be added to the assay was calculated as previously described [17]. In some cases, the relative amounts of inositol, glycerophosphoinositol, Ins*P*, InsP_2 and InsP_3 present in the aqueous phase were quantitated following a previously described method [18].

2.5. *Miscellaneous*

Protein concentrations were determined according to Bradford [19] using bovine IgG as standard. All experiments were repeated at least twice. Data from representative experiments are shown as means of triplicate determinations ± SD.

3. RESULTS

We initiated our work by studying the effect of a peptide corresponding to a previously proposed PtdInsP_2 binding site in PLCβ_2 on PtdInsP_2 hydrolysis by soluble fractions of Sf9 cells expressing PLC$\beta_2\Delta$, a deletion mutant of PLCβ_2. Much to our surprise, the peptide markedly stimulated the activity of PLC$\beta_2\Delta$ (Fig. 1). Half-maximal stimulation was observed at approximately 15 μM peptide, corresponding to a PtdInsP_2/peptide molar ratio of approximately 2. The stimulatory effect of the peptide, which by itself did not exhibit PLC activity, ensued without delay and was clearly noticed even at incubation periods as short as 5 min (data not shown). Additional experiments revealed that InsP_3 was the predominant (87%) product of peptide-stimulated PtdInsP_2 hydrolysis and that only a minor fraction (8%) appeared as InsP_2. Values for Ins*P*, inositol and glycerophosphoinositol were not significantly different from zero (data not shown).

Fig. 2 shows that the peptide specifically stimulates PLC$\beta_2\Delta$. Thus, 28 μM of the peptide led to an approximately 5-fold stimulation of the soluble PLC activity present in cells infected with baculovirus encoding PLC$\beta_2\Delta$. In contrast, no PLC activity could be detected in soluble preparations of β-galatosidase-baculovirus-infected cells, regardless of whether the peptide was absent or present in the incubation medium (Fig. 2A). PLC activity increased linearly with the amount of soluble protein present in the incubation (0–0.1 μg/tube), both in the absence or the presence of the peptide. Thus, the relative stimulation by the peptide remained constant within this range of protein concentration (Fig. 2b). Incubating the soluble fraction of cells expressing PLC$\beta_2\Delta$ for 10 min at 95°C completely abolished PLC activity, either in the absence or the presence of the peptide (data not shown).

Next we considered the possibility that the stimulatory effect of the peptide was confined to the mutant PLCβ_2 isozyme. We therefore performed a series of experiments with the full-length PLCβ_2 protein expressed under similar conditions in Sf9 cells. Our data showed that the peptide (28 μM) stimulated this enzyme as well (not shown).

Fig. 3 illustrates that both basal and peptide-stimulated PLC activity were absolutely dependent on and markedly stimulated by free Ca^{2+}. Furthermore, it is also apparent that the concentrations of free Ca^{2+} required to half-maximally and maximally stimulate the inositol phosphate formation were similar in the absence and in the presence of the peptide (approximately 0.15 μM and 1 μM, respectively).

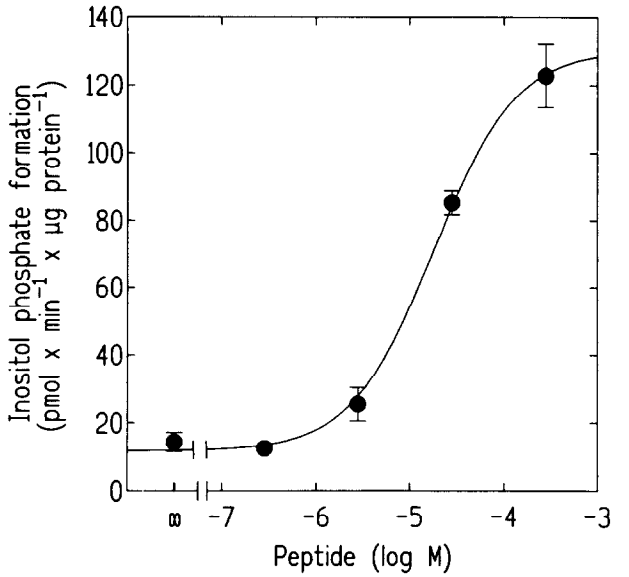

Fig. 1. Stimulation of inositol phosphate formation by the peptide CGGLPSPEDLRGKILIKNKK. Soluble proteins (0.1 μg per tube) of Sf9 cells infected with recombinant baculovirus encoding PLC$\beta_2\Delta$ were incubated at increasing concentrations of the peptide with a phospholipid suspension containing PtdInsP_2. See section 2 for experimental details. The incubation was performed in the presence of 1 mM free Ca^{2+}. Each value represents the mean ± S.D. of triplicate determinations.

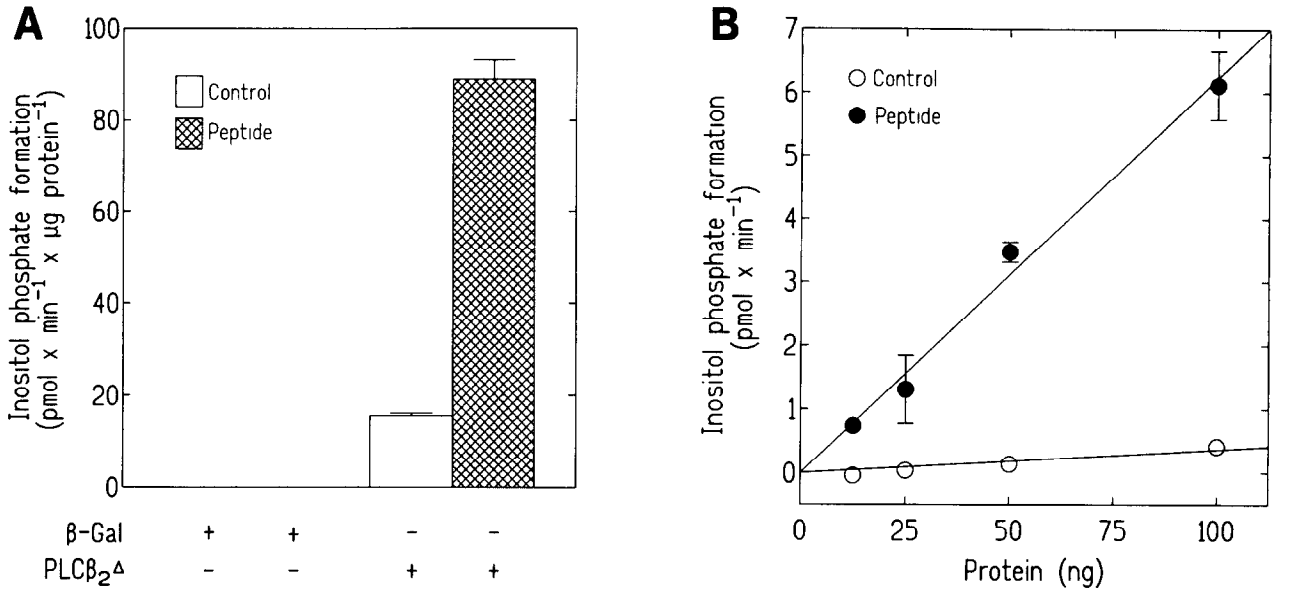

Fig. 2. PLCβ_2-specific stimulation of insositol phosphate formation by the peptide. (A) Sf9 cells were infected as indicated with recombinant baculovirus encoding either β-galactosidase or PLC$\beta_2\Delta$. Soluble proteins (0.1 μg per tube) were incubated as indicated in the absence (open bars) or the presence (hatched bars) of the peptide (28 μM) with a phospholipid suspension containing PtdInsP_2 (B). Soluble proteins of Sf9 cells infected with PLC$\beta_2\Delta$-baculovirus were incubated at increasing protein concentrations in the absence (open symbols) or the presence (closed symbols) of the peptide (28 μM) with the phospholipid substrate.

Finally, we examined the specificity of the stimulatory effect of the peptide CGGLPSPEDLRGKILIKNKK on PLC$\beta_2\Delta$ activity by comparing its effect to the effects of the following peptides: EQGVLPDDLSG (residues 116–126 of $G_{\alpha i2}$), VLAGSAEEGVMTPEL (residues 109–123 of $G_{\alpha i3}$), KENLKDCGLF (residues 341–350 of $G_{t\alpha 1}$), KNNLKECGLY (residues 345–354 of $G_{\alpha i3}$) and CGGASENPFREKK (residues 56–65 of $G_{\gamma 2}$, amino-terminal cysteine and two glycines added). None of these peptides (in concentrations of either 28 or 280 μM) affected the activity of PLC$\beta_2\Delta$, under experimental conditions identical to those described above. Note that the latter peptide is similar to the one under study, both at its amino- and carboxyl-terminus. Lysine trimer, tetramer and pentamer (28 μM) did not affect enzyme activity. Similarly, lysine-alanine (1:1 and 2:1) and lysine-phenylalanine (1:1) random copolymers (28 μM) did not enhance the inositol phosphate formation by PLC$\beta_2\Delta$.

4. DISCUSSION

It has been shown by others that a peptide corresponding to a potential PtdInsP_2 binding site in cofilin binds to this phospholipid and inhibits its hydrolysis by various PLCs [11]. Hence, if the sequence LPSPEDLRGKILIKNKK of PLCβ_2 constitutes a PtdInsP_2 binding site, then a peptide comprising the same sequence would be expected to compete with the enzyme for PtdInsP_2 binding, resulting in an apparent inhibition of its activity. Our results show, however, that such a peptide strongly stimulates the catalytic activity of the enzyme. In addition, they indicate that this stimulation is dependent on the presence of an active PLCβ_2, is not associated with an alteration of the Ca^{2+} affinity of this enzyme, and requires a defined primary and/or secondary structure of the peptide (inasmuch as it could not be mimicked by other peptides or by amino acid oligo- or copolymers of basic character).

The observed stimulation of PLCβ_2 may be taken to suggest that the substrate is presented to the enzyme in a more favorable way in the presence of the peptide. One possibility would be that the energy threshold for the hydrolysis of PtdInsP_2 bound to the peptide is lower than that of free PtdInsP_2. This could actually be the function of the basic amino acid motif in the PLC. An alternative explanation is that the peptide might aggregate the phospholipid substrate via its lysine residues, thereby facilitating the processive hydrolysis PtdInsP_2. This possibility appears unlikely, however, due to the lack of effect of lysine oligo- or copolymers.

Regarding the binding of the peptide to PtdInsP_2, others have recently observed that the binding of a synthetic peptide corresponding to the carboxyl-terminus of the X domain of PLCδ, and therefore rather similar to ours, to PtdInsP_2 is weak in comparison to the binding of the intact enzyme [20]. However, in the case of actin-binding proteins, the corresponding peptides bind to PtdInsP_2 as strongly as the native proteins [6]. The reason for this discrepancy is probably that in PLCδ there is an additional PtdInsP_2 binding site located at the amino-terminal domain, which might be responsible

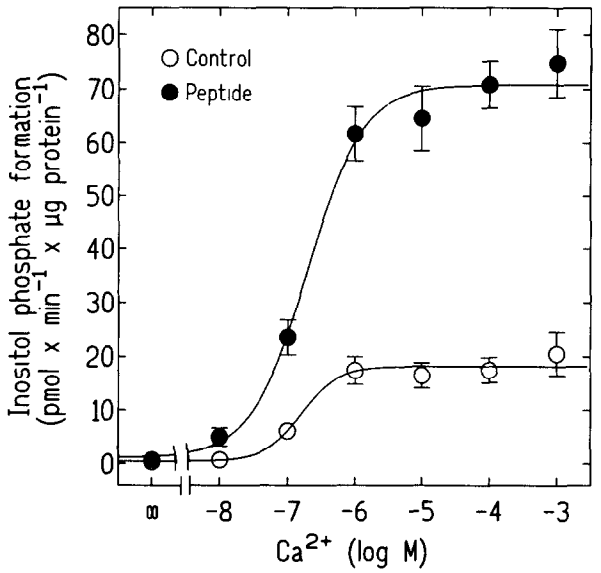

Fig. 3. Concentration-dependence on Ca^{2+} of the stimulation of inositol phosphate formation by the peptide. Soluble proteins (0.1 µg per tube) of Sf9 cells infected with recombinant baculovirus encoding PLC$\beta_2\Delta$ were incubated at increasing Ca^{2+} concentrations in the absence (open symbols) or the presence (closed symbols) of the peptide (28 µM) with a phospholipid suspension containing PtdInsP_2.

for the high-affinity binding of the enzyme to its substrate [21]. The existence of such a domain in PLCβ_2 deserves further investigation.

It is also possible to envisage the stimulatory effect of the peptide on PLCβ_2 as a result of a direct peptide/enzyme interaction. There are many known examples of this type of interaction [22]. It was recently proposed, based on experiments with synthetic peptides, that a site exists on PLC$_\gamma$ which inhibits the catalytic activity of this enzyme [23]. In order to explain our observations in a similar way we would have to postulate that PLCβ_2 is under an intra- or extramolecular inhibitory constraint, which can be released by the peptide. Thus, the peptide might exert its stimulatory effect by competing for binding to an inhibitory domain with a site required for catalytic activity. Considering the location of the possible inhibitory domain, we can already exclude the carboxyl-terminus of the enzyme, i.e. the domain which is necessary for stimulation by α_q subunits [15], since the peptide stimulates a PLCβ_2 mutant carrying a deletion in this region.

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