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Inhibition of Phosphoinositide Hydrolysis and Cell Growth of Swiss 3T3 Cells by Myristoylated Phospholipase C Inhibitor Peptides[1]

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It has been demonstrated that the phospholipase C-γ (PLC-γ) molecule contains within it a phospholipase C inhibitor (PCI) region and that synthetic peptides based on the sequence of this region (PCI peptides) suppress the enzymatic activity of PLC isoforms [Y. Homma and T. Takenawa (1992) *J. Biol. Chem.* 267, 21884-21889]. In order to improve the permeability of the plasma membrane to PCI peptides, we synthesized myristoylated PCI peptides, myr-GLYRKAMRLRYPV [myr-PCI(Y)] and myr-GLFRKAMRLRFPV [myr-PCI(F)], which are identical except for the replacement of the two tyrosine residues in myr-PCI(Y) by phenylalanines in myr-PCI(F), and examined their inhibitory activity on PLC enzymes *in vitro* and *in vivo*. This fatty acid modification potentiated the inhibitory activity of the original PCI peptides and both myr-PCI(Y) and myr-PCI(F) suppressed the PIP_2-hydrolyzing activity of purified PLC isoforms *in vitro*. The K_i values of myr-PCI(Y) and myr-PCI(F) for purified PLC-γ1 were 3.5 and 55 μM, respectively. Myr-PCI(Y) at concentrations in the sub-micromolar range significantly suppressed IP_3 formation induced by EGF, PDGF, bombesin, or serum in Swiss 3T3 cells. Furthermore, myr-PCI(Y) also strongly inhibited cell proliferation induced by these stimuli. The inhibitory effect on IP_3 formation and proliferation of myr-PCI(F) was much less potent than that of myr-PCI(Y). These results suggest that myristoylated PCI peptides could be applied to living cells as specific inhibitors of PLC signaling pathways and that PLC pathways are at least in part required for growth in Swiss 3T3 cells.

Key words: cell growth, inhibitor, receptor, second messenger, synthetic peptide.

A wide variety of external stimuli act through a receptor-mediated mechanism to induce the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), resulting in the generation of intracellular second messengers, inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 initiates the release of Ca^{2+} from internal stores and activates Ca^{2+}-dependent systems such as calmodulin-dependent protein kinases, while DAG binds to and activates protein kinase C (*1, 2*). Since the production of these second messengers is catalyzed by a phosphoinositide-specific phospholipase C (PLC), this PLC is a critical enzyme in transmembrane signaling processes (*3, 4*).

Multiple members of the PLC superfamily have been identified in mammalian cells and can be divided into three main subfamilies (PLC-β, -γ, and -δ) (*3, 4*). These isoforms possess in their structures two conserved X and Y regions required for catalytic activity. In addition, PLC-γ contains a unique Z region consisting of two *src* homology 2 (SH2) and one SH3 motifs. This Z region is not essential for PIP_2-hydrolyzing activity (*5*), but participates in the regulation of PLC-γ itself by interacting with cellular components (*6-9*). Recent findings have demonstrated that PLC-γ possesses adjacent to its SH2 and SH3 motifs a phospholipase C inhibitor (PCI) region that strongly suppresses PIP_2-hydrolyzing activity. The primary structure of the PCI region, including a minimum octamer, YRKMRLRY, is important for enzyme inhibition and synthetic peptides identical to the PCI region inhibit the enzyme activity induced not only by PLC-γ, but also by PLC-β and PLC-δ (*10*). Furthermore, synthetic PCI peptides inhibit Ca^{2+}-inducible PLC activation in digitonin-permeabilized cells. Similar results are obtained in both agonist- and GTPγS-inducible PLC activation systems using purified plasma membranes (*11*). These observations suggest the possibility of developing a novel, specific inhibitor for PLC activity. In the present study, we modified the PCI peptides by conjugating myristic acid at the N-terminal end and examined their effect on cellular responses in Swiss 3T3 cells induced by external stimuli. The myristoylated PCI (myr-PCI) peptides suppressed IP_3 formation and proliferation induced by growth factors, indicating their applicability to intact cells.

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Abbreviations: PIP_2, phosphatidylinositol 4,5-bisphosphate; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; BrdU, 5-bromo-2'-deoxyuridine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline.

EXPERIMENTAL PROCEDURES

Materials—PIP_2 (bovine brain), phosphatidylethanolamine (soybean), and bombesin were purchased from Sigma. PDGF, EGF, and insulin were from Collaborative Research. [^{3}H]PIP_2, [^{3}H]thymidine, and the IP_3 assay kit were from Du Pont-New England Nuclear. The cell proliferation (BrdU assay) kit was from Amersham. The MTT-cell growth assay kit was from Chemicon International. Peptides were prepared on an automatic peptide synthesizer (Applied Biosystems, Perkin-Elmer) using *t*-butoxycarbonyl-protected amino acids including myristoyl glycine (Calbiochem-Novabiochem) and purified on a C18 reverse phase column by high-performance liquid chromatography. The synthetic peptides used in this study are as follows: PCI, GLYRKMRLRYPV; myr-PCI(Y), myristoylated GLYRKMRLRYPV; myr-PCI(F), myristoylated GLFRKMRLRFPV; myr-ψPKA, myr-GRRNAIHDI (*12*); myr-ψPKC, myr-FARKGALRQ (*12*).

Cell Culture—Swiss 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2 in a humidified incubator. Prior to stimulation, the cells were incubated for at least 24 h in serum-free, starvation medium consisting of insulin (5 μg/ml), transferrin (5 μg/ml), sodium selenite (5 ng/ml), and bovine serum albumin (1 mg/ml) in DMEM.

PLC Assay—A conventional *in vitro* PLC assay was carried out as described (*10*). Activation of endogenous PLC was assessed by measuring the intracellular IP_3 levels as follows: Swiss 3T3 cells were grown in 35-mm culture dishes, and the medium was replaced with the starvation medium after cells reached semi-confluency ($\sim 5\times10^5$ cells/dish). Cells starved for 24 h were pretreated with or without synthetic peptides for 30 min and then stimulated with growth factors for various periods. The reactions were stopped by adding ice-cold trichloroacetic acid (TCA), and IP_3 was extracted and assayed according to the manufacturer's recommended method.

Cell Growth Assay—Cells were grown in 96-well plates and the medium was replaced with the starvation medium after the cells reached semi-confluency ($\sim 1\times10^4$ cells/well). Cells starved for 24-48 h were pretreated with or without synthetic peptides for 1-24 h and then stimulated with growth factors. The following three methods were used to determine the effect of synthetic peptides on growth factor-dependent cell proliferation.

Method I ([^{3}H]thymidine assay): An aliquot (37 kBq/well) of [^{3}H]thymidine was added to each well 17 h after the start of the stimulation and the cultures were further incubated for 24 h. The cells were washed with PBS and the incorporated [^{3}H]thymidine was recovered on glass filters as TCA-insoluble material. Radioactivity was measured in a liquid scintillation counter.

Method II (BrdU assay): An aliquot of BrdU solution was added to each well 17 h after the start of stimulation and the cultures were further incubated for 1 h. The cells were washed with PBS and fixed with 50% (v/v) ethanol. Incorporated BrdU was detected according to the manufacturer's recommended method.

Method III (MTT assay): An aliquot of MTT solution was added to each well 48 h after the start of stimulation and the cultures were further incubated for 4 h. Cell viability was analyzed by MTT staining and determining the optical density at 590 nm according to the manufacturer's recommended method.

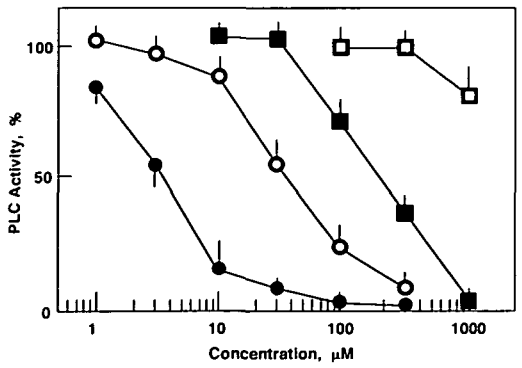

Fig. 1. **Inhibition of PLC isoforms *in vitro* by myristoylated PCI peptides.** PIP_2-hydrolyzing activity of purified PLC-γ1 was measured in the presence of various concentrations of PCI(Y) (■), PCI(F) (□), myr-PCI(Y) (●), and myr-PCI(F) (○). Results (means ± SE, $n=3$) are expressed as percentage of activity in the absence of myristoylated PCI peptides.

RESULTS

Effect of Myristoylation on PCI Peptide—It has been reported that the conjugation of myristic acid or palmitic acid to the N-terminal of synthetic peptides enhances their transmembrane potency and the modified peptides act as specific effectors or inhibitors for intact cells (*13-15*). Based on this information, we synthesized two PCI peptides, myr-GLYRKMRLRY and myr-GLFRKMRLRF, designated myr-PCI(Y) and myr-PCI(F), respectively, and compared their *in vitro* PLC-inhibitory activity with that of non-myristoylated PCI(Y) and PCI(F). As shown in Fig. 1, myr-PCI(Y) strongly suppressed PLCγ1-dependent PIP_2 hydrolysis in a concentration-dependent manner. Myr-PCI(F) also inhibited PIP_2 hydrolysis, but with about 15 times less potency than myr-PCI(Y). The K_i values of myr-PCI(Y) and myr-PCI(F) were about 3.5 and 55 μM, respectively. On the other hand, the K_i values of non-myristoylated PCI(Y) and PCI(F) were about 120 μM and >1,000 μM, respectively. These results are summarized in Table I, and suggest that the myristoylation of the N-terminal of PCI peptides potentiates their inhibition of PLC activity.

Myr-PCI(Y) inhibited not only the PIP_2 hydrolysis induced by PLCγ1, but also that induced by other PLC isoforms (Table I). Myr-PCI(Y) also inhibited the hydrolysis of PIP and PI induced by PLC isoforms (data not shown). These findings are consistent with previous observations on

TABLE I. **Summary of PLC-inhibitory effect of various PCI peptides.**

	K_i values (μM)		
	PLC-β1	PLC-γ1	PLC-δ1
PCI(Y)	140	120	200
PCI(F)	>1,000	>1,000	>1,000
myr-PCI(Y)	15	3.5	18
myr-PCI(F)	83	55	150

non-myristoylated PCI peptides (*10*). Other myristoylated peptides, such as myr-ψPKA and myr-ψPKC, and myristoyl glycine showed no inhibitory activity on PIP_2 hydrolysis.

Inhibition of IP_3 Formation by Myr-PCI—We next examined the effect of myr-PCI(Y) and myr-PCI(F) on stimulation-dependent PLC activation in Swiss 3T3 cells. Swiss 3T3 cells were preincubated with either 10 μM myr-PCI(Y) or myr-PCI(F) for 30 min and then stimulated with EGF (100 ng/ml) plus insulin (10 ng/ml) for various times. Intracellular IP_3 levels are shown in Fig. 2A. The IP_3 levels quickly increased to a maximum level within 30 s after stimulation and then decreased to the control level in a time-dependent manner. Myr-PCI(Y), and to a lesser extent, myr-PCI(F), inhibited the EGF-dependent IP_3 formation at all time points tested. At 30 s after stimulation, the IP_3 level of myristoyl glycine-treated control cells, myr-PCI(Y)-treated cells, and myr-PCI(F)-treated cells were 19, 3.2, and 17 pmol/10^6 cells, respectively. We further tested the appropriate interval for cell pretreatment with myr-PCI(Y) and similar results were obtained with cells pretreated for 5 to 60 min with myr-PCI(Y) (data not shown). On the other hand, myr-PCI(F) at 10 μM caused a moderate inhibition of IP_3 formation.

The concentration dependence of the effect of myristoylated PCI peptides on IP_3 formation was also examined. Swiss 3T3 cells pretreated with various concentrations of myr-PCI(Y) or myr-PCI(F) were stimulated with EGF plus insulin for 30 s. As shown in Fig. 2B, IP_3 formation was suppressed by myr-PCI(Y) in a concentration-dependent manner. The IC_{50} value was ~1 μM. At 30 s after stimulation, IP_3 formation in 10 μM myr-PCI(Y)-treated cells was less than 10% of the highest response observed in control cells. On the other hand, myr-PCI(F) at concentrations below 5 μM had no significant effect on IP_3 formation and the IC_{50} value was ~50 μM. Neither myr-ψPKA, myr-ψPKC, nor myr-glycine affected IP_3 formation.

Since myristoylated PCI peptides inhibited different PLC isoforms *in vitro*, we expected that myr-PCI(Y) would suppress the activation of different PLC pathways. In addition to EGF, various stimuli such as PDGF, bombesin, and serum are known to initiate PLC activation in Swiss 3T3 cells. We studied the effect of myr-PCI(Y) on PLC activation induced by these stimuli. As shown in Fig. 3A, myr-PCI(Y) suppressed the IP_3 formation induced by the different stimuli tested in a concentration-dependent manner. The IC_{50} values of myr-PCI(Y) for IP_3 formation induced by PDGF (100 ng/ml), bombesin (1 μM), and serum (5%; v/v) were ~1, ~1.5, and ~2.5 μM, respectively. On the other hand, when cells pretreated with 10 μM myr-PCI(Y) were stimulated with various concentrations of PDGF, bombesin, or serum, the inhibitory effect of

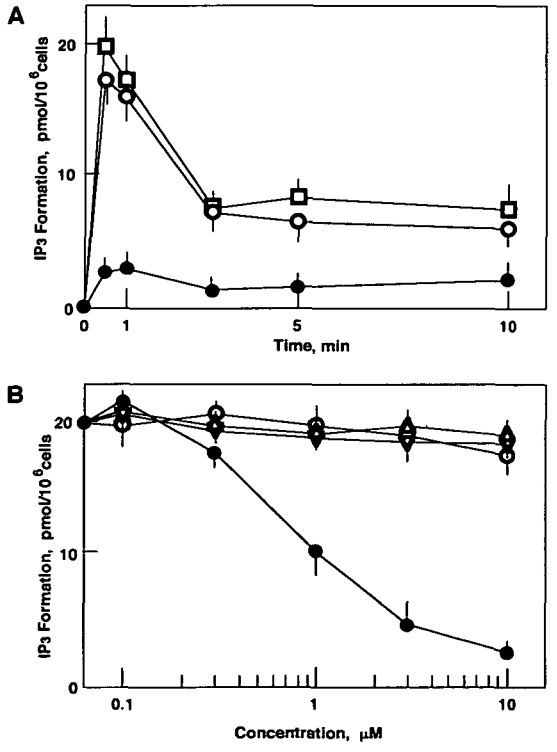

Fig. 2. **Inhibition of EGF-dependent IP_3 formation by myristoylated PCI peptides.** (A) Swiss 3T3 cells were pretreated for 30 min with either 10 μM myr-PCI(Y) (●), 10 μM myr-PCI(F) (○), or 10 μM myristoyl glycine (□) and stimulated with 100 ng/ml EGF plus 10 ng/ml insulin for the indicated periods. (B) Swiss 3T3 cells were pretreated for 30 min with various concentrations of myr-PCI(Y) (●), myr-PCI(F) (○), myr-ψPKA (△), or myr-ψPKC (▽) and then stimulated for 30 s with 100 ng/ml EGF plus 10 ng/ml insulin. Results are expressed as means±SE ($n=3$).

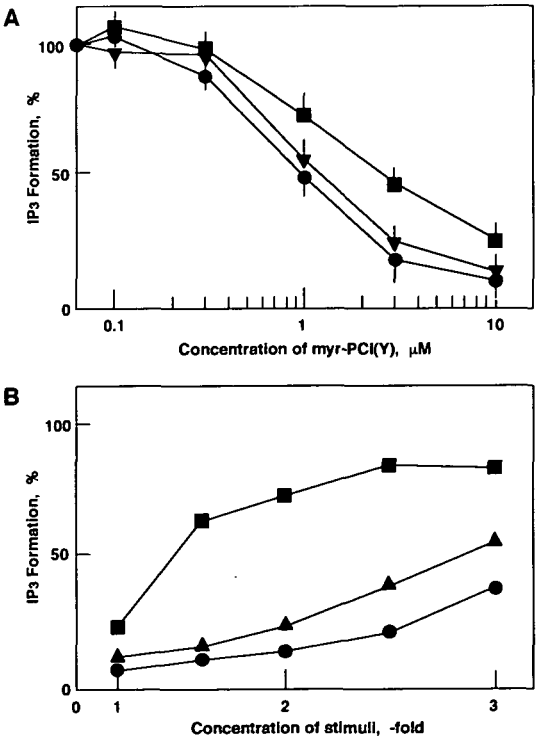

Fig. 3. **Inhibition of stimuli-induced IP_3 formation by myristoylated PCI peptides.** (A) Swiss 3T3 cells were pretreated with various concentrations of myr-PCI(Y) for 30 min and then stimulated for 30 s with 100 ng/ml PDGF (●), 1 μM bombesin (▼), or 5% (v/v) serum (■). (B) Swiss 3T3 cells were pretreated with 10 μM myr-PCI(Y) for 30 min and then stimulated for 30 s with various concentrations of EGF plus insulin (▲), PDGF (●), or serum (■); original (1-fold) solutions were 100 ng/ml EGF plus 10 ng/ml insulin, 100 ng/ml PDGF, or 5% (v/v) serum. Results (means±SE, $n=3$) are expressed as percentage of activity in the presence of myristoyl glycine.

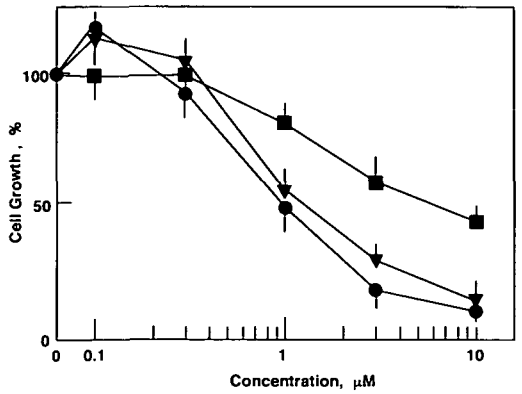

Fig. 4. **Inhibition of cell proliferation by myr-PCI(Y).** Swiss 3T3 cells were pretreated with various concentrations of myr-PCI(Y) for 30 min and then stimulated with 20 ng/ml EGF plus 10 ng/ml insulin (▼), 10 ng/ml PDGF (●), or 5% (v/v) serum (■). An aliquot of [^{3}H]thymidine was added to each culture 17 h after stimulation and the cultures were further incubated for 24 h. Results (means ± SE, $n=3$) are expressed as percentage of radioactivity detected in cells cultured in the presence of myristoyl glycine.

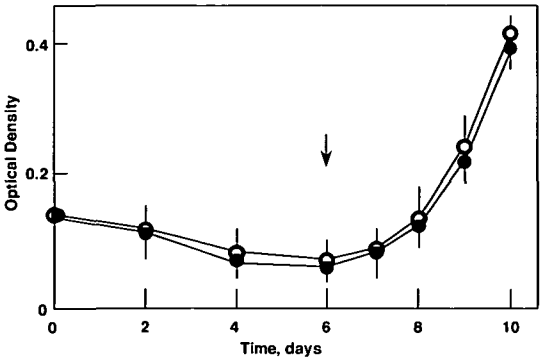

Fig. 5. **Effect of myristoylated PCI peptides on cell viability.** Swiss 3T3 cells were maintained for 6 days in serum-free, starvation medium (○) or starvation medium containing 10 μM myr-PCI(Y) (●) and then treated with growth medium (arrow) containing 5% (v/v) serum, but no myristoylated PCI peptides. Cell numbers were assessed by MTT assay. Results are expressed as means ± SE ($n=3$).

myr-PCI(Y) tended to be overcome by the potentiation of the stimulus (Fig. 3B). Among the four stimuli tested, myr-PCI(Y) was the most effective suppressor of the IP_3 formation induced by either EGF plus insulin or PDGF.

Inhibition of Cell Growth by Myr-PCI—Growth factors provoke cell proliferation through the activation of cellular signaling systems. Myr-PCI(Y) may provide a convenient tool with which to analyze the contributions of various PLC pathways to cell proliferation. Swiss 3T3 cells pretreated with various concentrations of myr-PCI(Y) for 30 min were stimulated with EGF (20 ng/ml) plus insulin (10 ng/ml), PDGF (10 ng/ml), or serum (5%), and the transition of the cell cycle into S phase was determined by [^{3}H]thymidine and BrdU assays. Similar results were obtained with each assay and those from the [^{3}H]thymidine assay are shown in Fig. 4. Myr-PCI(Y) suppressed cell growth induced by all stimuli tested in a concentration-dependent manner. The IC_{50} values for inhibition of cell growth induced by EGF, PDGF, and serum were ~1, ~1.5, and ~5 μM, respectively.

We confirmed that myr-PCI(Y) and myr-PCI(F) showed no cytotoxic effects on Swiss 3T3 cells. When these cells were maintained for 7 days or longer in serum-free starvation medium containing 10 μM myr-PCI(Y), we could detect no significant difference in cell numbers between myr-PCI(Y)-treated and non-treated control cells as assessed by MTT assay (Fig. 5). In addition, when cells pretreated with 10 μM myr-PCI(Y) for 6 days were stimulated with 5% serum on the 6th day, they started to proliferate (Fig. 5). Similar results were obtained using cells treated with 10 μM myr-PCI(F) or 10 μM myristoyl glycine (data not shown).

DISCUSSION

In order to study the contribution of PLC signaling pathways to the signal transduction of various external stimuli, inhibitors such as neomycin and U73122 that interfere with the interaction between PLC enzymes and the PIP_2 substrate (*16, 17*) have been used. However, these compounds are not fully specific in their action, partly because of their hydrophobicity. The identification of the PCI region in PLC-γ subspecies suggested the possibility of developing a novel, specific inhibitor of PLC activity. Since earlier studies had demonstrated that fatty acid modification of synthetic peptides, *e.g.*, myristoylation, improves their ability to permeate through the plasma membrane (*13-15*), we applied this modification method to produce myristoylated PCI peptides, myr-PCI(Y) and myr-PCI(F). Modification of the N-terminal of PCI peptides by myristic acid potentiates the inhibitory effect *in vitro*: the K_i values are 120 and 3.5 μM for PCI(Y) and myr-PCI(Y), respectively, and >1,000 and 55 μM for PCI(F) and myr-PCI(F), respectively. This indicates that myristoylation of PCI peptides induces structural alterations, resulting in their effective entry into substrate-containing vesicles and close association with PLC enzymes.

As we had expected, this modification produced a significant increase in the permeability of the plasma membrane to PCI peptides, leading to the inhibition of stimulation-dependent IP_3 formation, that is, the activation of endogenous PLC enzymes. Swiss 3T3 cells respond to various stimuli including EGF, PDGF, bombesin, and serum to produce IP_3 *via* different PLC activation systems. Myr-PCI(Y) strongly inhibited the IP_3 formation induced by all three stimuli tested, and the IC_{50} values were quite low, ~1 μM for IP_3 formation induced by either EGF or PDGF. Myr-PCI(F) at higher concentrations also inhibited IP_3 formation, while myristoyl glycine and non-myristoylated PCI(Y) and PCI(F) did not. These results are consistent with our previous observations (*10*) and imply the possible application of myr-PCI(Y) as a specific inhibitor of PLC signaling systems. The IC_{50} value (~1 μM) is significantly lower than its K_i values (*e.g.*, 4 μM for purified PLC-γ1) obtained from *in vitro* assay. This difference may indicate effective association of myr-PCI(Y) with endogenous PLC enzymes *via* unknown mechanisms.

Various signaling pathways are involved in cellular responses such as proliferation, differentiation, and secretion. Specific inhibitors of PLC activity are quite useful for understanding how PLC pathways contribute to signaling for the expression of cellular functions. In this study, we examined the effect of myr-PCI on proliferation induced by

EGF, PDGF, or serum in Swiss 3T3 cells. Cell stimulation by these ligands initiates the transition of the cell cycle to the S phase. Myr-PCI(Y) obviously suppressed the entry of the cell cycle into S phase induced by the three stimuli tested in a dose-dependent manner. Myr-PCI(F) at higher concentrations also inhibited proliferation. Although the effect of these peptides is influenced by the concentration of the stimulus used (*i.e.*, the intensity of the signals), the difference in the inhibitory effect of these two peptides on cell growth seems to reflect the difference in effect on PLC activity *in vitro*. These results suggest that PLC activation is, at least in part, required for the primary signals of Swiss 3T3 cell proliferation.

The inhibitory effects of myr-PCI(Y) on IP_3 formation and cell growth vary with the cells examined. Myr-PCI(Y) is effective in *erbB*-transformed NIH 3T3 cells, but is less effective in *v-raf*- or *B-raf*-transformed NIH 3T3 cells, neutrophils, and isolated pancreatic cells than in Swiss 3T3 cells (unpublished results). Therefore, some other modifications, including acylation by different fatty acids and substitution by D-type amino acids, would be required to produce PCI peptides effective in a wide variety of cells. In addition, the structural analysis of PCI peptides is also important to develop a novel inhibitor of PLC enzymes.

In summary, we synthesized myristoylated PCI peptides. Myristoylation of the peptides potentiated not only their inhibitory effect *in vitro* on PLC isoforms, but also their ability to permeate through the plasma membrane. Myr-PCI(Y) strongly suppressed IP_3 formation and cell proliferation induced by various external stimuli in Swiss 3T3 cells, while myr-PCI(F) was not very active. These results suggest that myr-PCI peptides will be useful as specific PLC inhibitors and as tools to analyze intracellular signal transduction systems.

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