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Structural Requirements of Lyngbyatoxin A for Activation and Downregulation of Protein Kinase C†

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ABSTRACT: Structure–activity studies of novel synthetic analogues of lyngbyatoxin A reveal that the lactam ring but not the 7-linalyl moiety of lyngbyatoxin A is essential for the in vitro stimulation of protein kinase C (PKC). (−)-Indolactam V (ILV), which contains no hydrophobic substituent at C-7, or analogues containing either a linalyl or *n*-hexyl group at C-7 were equally efficacious in stimulating HeLa cell PKC in vitro and in competing with phorbol 12,13-dibutyrate for binding to PKC in intact cells. The hydrophobicity of alkyl groups at C-7, however, influenced the potency of these compounds to bind to and activate PKC. In addition, these compounds exhibited differences in their ability to translocate PKC. Lyngbyatoxin A (0.1 μM) like TPA induced a rapid translocation of PKC from the cytosol to the membrane and subsequently led to a sustained decrease in both cytosolic and membrane PKC activity. In contrast, (−)-*n*-hexylILV (0.1 μM) and (−)-ILV (1 μM) produced a transient and attenuated decrease in cytosolic PKC activity. At concentrations that produced half-maximal PKC stimulation, (−)-ILV did not cause any downregulation of PKC whereas lyngbyatoxin A and (−)-*n*-hexylILV led to 60% and 40% PKC downregulation, respectively. Western blot analyses with monoclonal antibodies to PKC isoforms indicated that reduction in PKC activity by chronic exposure to TPA or lyngbyatoxin A analogues could be explained by downregulation of PKCα. Constitutive expression of PKCβ and PKCγ isoforms was low in HeLa cells and was not affected significantly by TPA or lyngbyatoxin A analogues. These results indicate that the structural requirements for PKC activation may be separated from those required for PKC downregulation.

Protein kinase C (PKC),[1] a calcium-activated, phospholipid-dependent enzyme, is an important element in signal transduction (Nishizuka, 1986). It phosphorylates a variety of proteins and regulates many cellular processes including cell proliferation, differentiation, and gene expression (Nishizuka, 1986). PKC is now recognized as a family of closely related polypeptides with at least eight members including α, βI, βII, and γ (Nishizuka, 1988; Huang et al., 1986; Jaken & Kiley, 1987).

The activity of PKC is highly regulated. Both calcium and acidic phospholipids like phosphatidylserine are required for its activity (Kishimoto et al., 1980). Diacylglycerol (DAG),

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[1] Abbreviations: PKC, protein kinase C; DAG, diacylglycerol; ILV, indolactam V; PDBu, phorbol 12,13-dibutyrate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; DMSO, dimethyl sulfoxide; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid.

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transiently generated by the hydrolysis of inositol phospholipids, permits activation of PKC by physiologic concentrations of calcium (Kishimoto et al., 1980). PKC is the primary receptor for tumor-promoting phorbol esters (Neidel et al., 1983; Sando et al., 1983), which can substitute for the endogenous stimulator DAG and activate the enzyme by a similar mechanism (Castagna et al., 1982). PKC usually resides in the cytoplasm in an inactive form. Activators of PKC induce a rapid translocation of the enzyme from the cytosol to the plasma membrane (Kraft & Anderson, 1983). Prolonged cellular exposure to phorbol esters leads to proteolytic degradation or downregulation of PKC (Rodriguez-Pena & Rozengurt, 1984). A structurally diverse group of tumor promoters including indole alkaloids, ingenols, and aplysiatoxins also activate PKC and compete for phorbol ester binding (Jeffrey & Liskamp, 1986; Wender et al., 1986; Irie et al., 1987).

To understand the mechanism of activation of PKC, several groups have examined the structural requirements of various activators (Jeffrey & Liskamp, 1986; Wender et al., 1986; Irie et al., 1987; Itai et al., 1988; Nakamura et al., 1989; Kozikowski et al., 1989). Such structure–activity studies together with computer graphic analyses have revealed commonalities among structurally distinct classes of PKC activators. It has been proposed that these activators possess functional groups that occupy similar spatial positions: a hydrophobic domain necessary for intercalation into membranes and hydrophilic residues critical for interaction with PKC (Blumberg, 1988; Rando, 1988).

While there has been considerable attention directed toward the structural requirements for PKC activation by various agonists, no systematic study has been conducted to identify the structural features necessary for PKC downregulation. An important distinction between the tumor-promoting phorbol esters and the physiologic activator DAG is that the latter does not appear to cause appreciable downregulation of PKC (Issandou et al., 1988; Issandou & Rozengurt, 1989). In addition, phorbol esters are not rapidly metabolized like DAG and are known to induce cellular responses that are not shared by the physiologic stimulator (Issandou et al., 1988; Issandou & Rozengurt, 1989; Strulovici et al., 1989). Development of synthetic PKC modulators should be extremely useful in understanding PKC-mediated processes, but systematic structural modification of phorbol esters has been difficult due to complexity and instability of these synthetic compounds (Wender et al., 1986).

We have synthesized a series of simplified analogues of a natural tumor promotor, lyngbyatoxin A, and have demonstrated that the lactam ring structure is essential for activation of PKC but that the complex linalyl group at the 7-position of the indole ring is dispensable (Kozikowski et al., 1989). In the present study, we show that the hydrophobic group at the 7-position of (−)-ILV is important in determining the ability of lyngbyatoxin A to downregulate PKC. In addition, we demonstrate that activators of PKC cause selective downregulation of the PKCα isoform in HeLa cells.

Experimental Procedures

Materials

Calf thymus histone (III-S) and PDBu were purchased from Sigma Chemical Co. Phosphatidylserine was from Supelco, and 1,2-diolein was from Avanti Polar Lipids, Inc. [γ-^{32}P]-ATP was purchased from Amersham, and [^{3}H]PDBu was from Du Pont. DE-52 was obtained from Whatman Lab Sales Inc. PKC substrate peptide [Ser^{25}]PKC(19–31) was purchased from Bachem. Acrylamide, bisacrylamide, ammonium persulfate, SDS, gelatin, molecular weight markers, and Triton X-100 were from Bio-Rad. Monoclonal antibodies to specific PKC isoforms (MC-1a, MC-2a, and MC-3a) were obtained from Seikagaku America Inc. Polyclonal antisera specific to PKCα was kindly provided by Dr. William Hait, Yale University, New Haven, CT. Polyvinylidene difluoride membrane was from Millipore. Rabbit anti-mouse IgG, goat anti-rabbit IgG, rabbit peroxidase anti-peroxidase, and diaminobenzide tetrahydrochloride were purchased from Cappel Research Reagents. TPA and (−)-ILV were obtained from LC Services Corp. Lyngbyatoxin A was a generous gift from Dr. S. Sakai of Chiba University. Analogues of lyngbyatoxin A were synthesized and chemically characterized as described previously (Kozikowski et al., 1989).

Methods

Cell Culture. Human cervical carcinoma (HeLa) cells were maintained as exponentially growing monolayer cultures in Dulbecco's-modified minimal essential medium (DMEM) supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 25 mM HEPES buffer (pH 7.4), penicillin (100 units/mL), and streptomycin (100 μg/mL) and kept in a humidified incubator at 37 °C with 95% air and 5% CO_2.

Isolation of PKC. PKC was partially purified from HeLa cells using DE-52–cellulose chromatography (Basu et al., 1990). Briefly, cells were harvested and cell pellets were washed with ice-cold phosphate-buffered saline without any divalent cations. All subsequent procedures were carried out at 4 °C. Cells were homogenized in a solution containing 20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 2 mM EDTA, 5 mM EGTA, 10 mM β-mercaptoethanol, 1 mM phenylmethanesulfonyl fluoride, and 0.01% leupeptin (buffer A). The homogenate was centrifuged at 100000g for 1 h. The cytosolic fraction was subjected to DE-52 chromatography and eluted with a solution of 0.1 M NaCl in 20 mM Tris-HCl (pH 7.5), 0.25 M sucrose, 0.5 mM EDTA, 0.5 mM EGTA, and 10 mM β-mercaptoethanol. The particulate PKC was resuspended in buffer A, extracted by stirring for 30 min with 0.5% Triton X-100, and centrifuged at 100000g for 1 h. The resulting supernatant was subjected to DE-52 chromatography as mentioned above. To isolate total PKC, cell homogenate was treated with 0.5% Triton X-100 before centrifugation.

PKC Assay. The enzyme activity was determined by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into histone III-S. The reaction mixture (100 μL) contained the enzyme preparation (1–3 μg of protein), 20 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 400 μg histone, 20 μM [γ-^{32}P]ATP, 5 mM magnesium acetate, and 0.5 mM $CaCl_2$ with or without 25 μg/mL phosphatidylserine and 2.5 μg/mL DAG. Calcium- and phospholipid-dependent PKC activity was determined by subtracting the activity determined in the absence of phosphatidylserine and DAG from that in the presence of phosphatidylserine and DAG. In the presence of either calcium or phospholipid alone, the enzyme activity was less than 5% of the activity when both were present. To determine the activation of PKC by TPA or lyngbyatoxin A analogues in the in vitro assay, we omitted DAG from the assay and used 0.1 mM $CaCl_2$ instead of 0.5 mM $CaCl_2$. All reactions were incubated at 30 °C for 5 min and terminated by spotting onto Whatman P-81 filter papers. The filters were washed four times in 75 mM phosphoric acid, dried, and counted in a liquid scintillation counter. The assay was linear with regard to both time and amount of enzyme. Protein was determined by the method of Bradford (1976) using bovine serum albumin as a standard. One unit of protein kinase C activity is defined

as that amount of enzyme which catalyzes the transfer of 1 pmol/min of phosphate from ATP to histone at 30 °C.

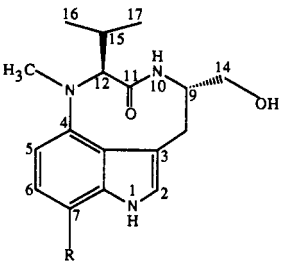
For downregulation studies, HeLa cells were plated in 96-well microtiter plates (Costar) and treated for 17–19 h with indicated concentrations of TPA or lyngbyatoxin A analogues at 37 °C and 5% CO_2. Cell numbers as determined by a colorimetric assay (Carmichael et al., 1987) remained unaltered following such incubation. In addition, viability of cells was not affected on the basis of Trypan blue dye exclusion assay. At the end of the incubation, the residual PKC activity was determined as described before (Heasley & Johnson, 1989). Briefly, cells were washed with 20 mM HEPES-buffered DMEM (pH 7.4) and incubated with or without 1 μM TPA for 10 min at 37 °C. The medium was then aspirated and replaced with 40 μL of a salt solution (Heasley & Johnson, 1989) supplemented with 30 μg/mL digitonin, 10 mM magnesium chloride, 25 mM β-glycerophosphate, 5 mM EGTA, 2.5 mM $CaCl_2$, 0.1 mM [γ-^{32}P]ATP, and 100 μM substrate peptide [Ser^{25}]PKC(19–31). PKC activity determined both in the presence and in the absence of TPA was maximum at 50 μg/mL digitonin and remained unaltered up to 100 μg/mL digitonin. After a 10-min incubation at 37 °C, the reaction was terminated with 5% TCA. Aliquots of reaction mixtures were spotted on Whatman P-81 filter papers and processed as described above. The assay was linear up to 15 min.

[^{3}H]PDBu Binding. HeLa cells (10^6 cells/mL) were incubated with 10 nM [^{3}H]PDBu (20 Ci/mmol) and various concentrations of TPA or lyngbyatoxin A analogues in HEPES-buffered DMEM containing 2 mg/mL fatty acid-poor bovine serum albumin for 30 min at 25 °C. At the end of the incubation, the reaction mixture was cooled in ice, filtered through Whatman GF/C filter paper, and washed twice with ice-cold phosphate-buffered saline (pH 7.4) containing 2 mg/mL bovine serum albumin. The nonspecific binding was determined in the presence of 50 μM unlabeled PDBu.

Western Blot Analyses. HeLa cell extracts, partially purified by DE-52 chromatography, were subjected to sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis (Laemmli, 1970) and then transferred electrophoretically to polyvinylidene difluoride membrane (Burnette, 1981). Immunoblotting was performed with isoform-specific monoclonal antibodies to PKCα, -β, and -γ or polyclonal antisera to PKCα using the peroxidase–antiperoxidase reaction as described previously (Huang et al., 1986). For studies with polyclonal rabbit antisera to PKCα, however, the membrane was incubated with goat anti-rabbit IgG but not rabbit anti-mouse IgG. Immunoreactive bands were visualized with diaminobenzide tetrahydrochloride and H_2O_2. The molecular weights of the immunostained proteins were estimated by comparing them either with Coomassie blue stained gels containing protein markers that were run in parallel or with the position of prestained molecular weight markers. The relative intensity of the immunoreactive bands was quantified by densitometric scanning of the immunoblots using an Ultroscan XL laser densitometer (LKB).

Results

Effects of Lyngbyatoxin A Analogues on PKC Activation. We compared the effects of lyngbyatoxin A analogues (Figure 1) that contained either no hydrophobic group (ILV) or six-carbon-containing (*n*-hexylILV) or ten-carbon-containing (linalylILV or lyngbyatoxin A) alkyl substituents at the 7-position of the indole ring on PKC activation. As shown in Figure 2A, all these compounds activated HeLa cell PKC in a concentration-dependent manner. Both lyngbyatoxin A and



(-)-Indolactam V R = H

(-)-*n*-Hexylindolactam V R = $(CH_2)_5CH_3$

(-)-Lyngbyatoxin A R = $-C(CH_3)(CH=CH_2)-(CH_2)_2CH=C(CH_3)_2$

FIGURE 1: Structure of lyngbyatoxin A analogues.

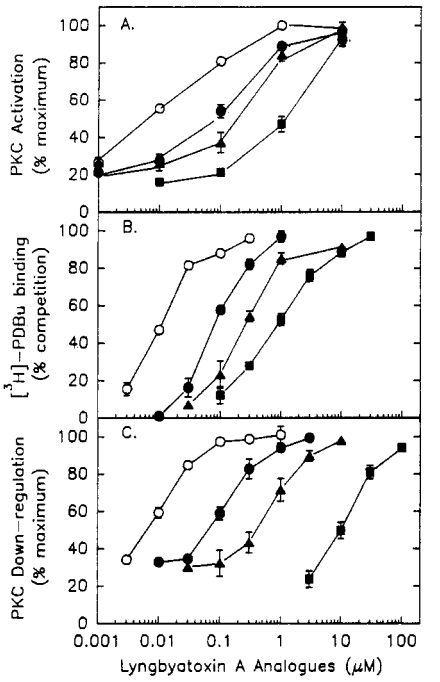


FIGURE 2: Effects of varying concentrations of lyngbyatoxin A analogues on PKC activation, downregulation, and PDBu binding to PKC. (A) PKC activity was determined in vitro in the presence of TPA (○), lyngbyatoxin A (●), (-)-*n*-hexylILV (▲), or (-)-ILV (■) as described under Experimental Procedures. The results are representative of 2–5 experiments. PKC activity in the presence of 1 μM TPA was considered as 100%. PKC activity in the absence or presence of 1 μM TPA was 98.3 ± 4.1 and 879 ± 39 pmol min^{-1} mg^{-1}, respectively. (B) HeLa cells were treated with 10 nM [^{3}H]PDBu and indicated concentrations of TPA (○), lyngbyatoxin A (●), (-)-*n*-hexylILV (▲), or (-)-ILV (■) as described under Experimental Procedures. The results are an average of 2–4 experiments done in duplicate. (C) HeLa cells were treated without or with various concentrations of TPA (○), lyngbyatoxin A (●), (-)-*n*-hexylILV (▲), or (-)-ILV (■) for 17 h in microtiter plates. At the end of the incubation, PKC activity was determined in intact cells using the [Ser^{25}]PKC(19–31) peptide as described under Experimental Procedures. All results were normalized to the downregulation of PKC seen with 1 μM TPA, which was defined as 100%.

the shorter alkyl chain containing (-)-*n*-hexylILV caused almost equivalent stimulation of PKC over the entire concentration range tested. Although (-)-ILV, which is devoid of any hydrophobic substituent at C-7, was 10-fold less potent than (-)-*n*-hexylILV or lyngbyatoxin A, it was equally efficacious in stimulating PKC at 10 μM.

Table I: Comparison of PKC Activation, Downregulation, and PDBu Binding by TPA and Lyngbyatoxin A Analogues[a]

compounds	activation	down-regulation	PDBu binding	downregulation/ activation	downregulation/ PDBu binding
		EC_{50} (μM)			
TPA	0.007	0.007	0.01	1.0	0.7
lyngbyatoxin A	0.08	0.065	0.08	0.8	0.81
(−)-*n*-hexylILV	0.19	0.40	0.25	2.1	1.6
(−)-ILV	1.2	10	0.91	6.3	11
		EC_{80} (μM)			
TPA	0.09	0.025	0.03	0.28	0.83
lyngbyatoxin A	0.60	0.27	0.27	0.45	1.0
(−)-*n*-hexylILV	0.82	1.6	0.80	1.95	2.0
(−)-ILV	5.8	30	4.0	5.2	8.0

[a] The concentrations required for 50% (EC_{50}) or 80% (EC_{80}) effect on PKC activation, downregulation, and PDBu binding were determined from Figure 2.

The ability of lyngbyatoxin A analogues to activate PKC in vitro correlated approximately with their ability to bind to PKC in intact cells (Figure 2B). Lyngbyatoxin A was less potent than TPA in activating PKC (Figure 2A) as well as in competing with [^{3}H]PDBu for binding to PKC (Figure 2B). The concentrations of lyngbyatoxin A, (−)-*n*-hexylILV, and (−)-ILV required for 50% inhibition of [^{3}H]PDBu binding were 0.08 μM, 0.25 μM, and 0.91 μM, respectively (Table I). These results indicate that lyngbyatoxin A analogues can interact with PKC in intact cells.

Effects of Lyngbyatoxin A Analogues on Subcellular Distribution of PKC. Translocation of PKC from cytosol to the plasma membrane is believed to be a function of PKC activation. We compared the ability of lyngbyatoxin A analogues to promote the translocation of PKC. In the absence of any exogenous activator, 85% of the total activity in HeLa cells was associated with cytosol. As shown in Figure 3, exposure of cells to 0.1 μM TPA (Figure 3A) or lyngbyatoxin A (Figure 3B) for 10 min caused a rapid decline in cytosolic PKC activity with a concomitant increase in particulate PKC activity. The continued presence of these compounds resulted in nearly a complete loss of both cytosolic and particulate PKC activity. In contrast, 0.1 μM (−)-*n*-hexylILV elicited only a partial translocation of PKC; cytosolic PKC activity decreased 30% in 10 min and was accompanied by a parallel increase in membrane PKC activity (220% of control) (Figure 3C). Incubation of cells for longer than 10 min with (−)-*n*-hexylILV caused a gradual disappearance of cytosolic PKC activity down to 50% of the initial value at 4 h. Membrane PKC activity also decreased slightly from 220% at 10 min to 150% of the control in 4 h and remained essentially unaltered for the next 20 h. When the incubation was extended beyond 4 h, cytosolic PKC activity progressively reappeared and reached 75% of the control value by 24 h. A 1.0 μM concentration of (−)-ILV, which caused stimulation of PKC equivalent to 0.1 μM lyngbyatoxin A or (−)-*n*-hexylILV, induced a transient and partial translocation of cytosolic PKC to the membrane (Figure 3D). The cytosolic PKC activity decreased 15% following a 10-min incubation with a concomitant increase in membrane PKC activity; the maximum decrease (25%) in cytosolic activity was observed during a 1–4-h incubation with (−)-ILV.

Effects of Lyngbyatoxin A Analogues on PKC Downregulation. Because sustained activation of PKC by TPA ultimately leads to its downregulation, we examined the effects of varying concentrations of lyngbyatoxin A analogues on PKC downregulation (Figure 2C). We have used a PKC-specific substrate peptide [Ser^{25}]PKC(19–31), which contains a serine instead of an alanine in the pseudosubstrate domain of PKC, to monitor downregulation. PKC activity was determined directly in intact cells after permeabilizing cells with low

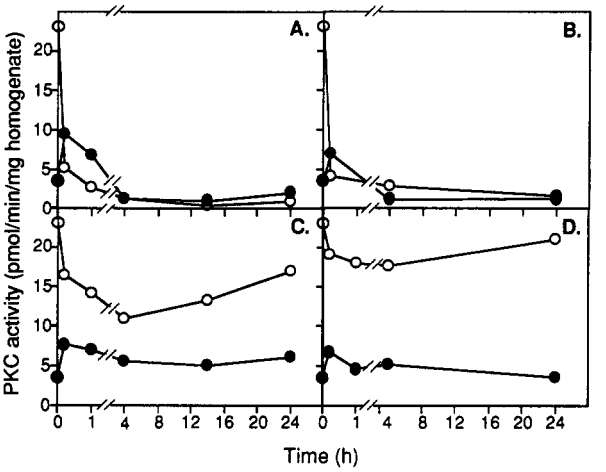


FIGURE 3: Effects of TPA and lyngbyatoxin A analogues on the subcellular distribution of PKC. HeLa cells were treated without or with 0.1 μM TPA (A), 0.1 μM lyngbyatoxin A (B), 0.1 μM (−)-*n*-hexylILV (C), or 1 μM (−)-ILV (D) for indicated periods of time at 37 °C. At the end of the incubation, PKC was isolated and the enzyme activity was determined in the cytosolic (○) and particulate fractions (●) as described under Experimental Procedures.

concentrations of digitonin. The maximum decrease in PKC activity was noted with 0.1 μM TPA; in these cells, the residual PKC activity was 20–30% of that seen in untreated control cells. Although lyngbyatoxin A was less potent than TPA in activating as well as downregulating PKC, both compounds were equally effective in inducing PKC downregulation at concentrations that caused equivalent PKC stimulation. No PKC downregulation was observed with a concentration of ILV (1.2 μM) that resulted in 50% PKC stimulation; only 50% of maximum PKC downregulation was achieved with 10 μM (−)-ILV, which produced full PKC activation (panel A versus panel C in Figure 2). A 0.19 μM concentration of (−)-*n*-hexylILV, which caused half-maximal PKC stimulation, led to a 38% downregulation of PKC (Figure 2C). Table I shows that the ratios of both EC_{50} and EC_{80} for PKC downregulation and activation or PDBu binding were similar for TPA and lyngbyatoxin A and increased with decreasing hydrophobicity of alkyl substituents at C-7.

Effects of TPA and Lyngbyatoxin A Analogues on the Downregulation of PKC Isoforms. To investigate whether TPA and lyngbyatoxin A analogues cause differential downregulation of PKC isoforms, we performed Western blot analyses using monoclonal antibodies against PKCα, -β, and -γ isoforms. As shown in Figure 4A, untreated HeLa cells contained significant amounts of immunoreactive PKCα. Treatment of HeLa cells with 0.1 μM TPA for 24 h reduced the amount of PKCα to 15% of the control (based upon laser densitometry). Lyngbyatoxin A (0.1 μM) also caused sub-

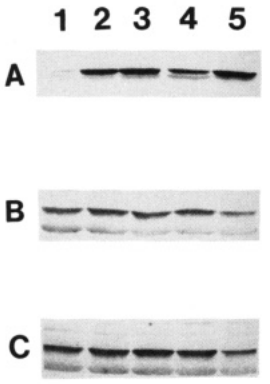


FIGURE 4: Effects of TPA and lyngbyatoxin A analogues on PKC isoforms. HeLa cells were treated for 24 h at 37 °C with either 0.1 μM TPA (lane 1), (−)-*n*-hexylILV (lane 2), (−)-*tert*-butylILV (lane 3), lyngbyatoxin A (lane 4), or 0.05% DMSO (lane 5). At the end of the incubation, PKC was isolated by DE-52 chromatography and Western blot analyses were performed using monoclonal antibody against PKCα (A), PKCβ (B), or PKCγ (C) as described under Experimental Procedures. The intensity of the immunoreactive bands in panels B and C has been photographically enhanced compared to panel A to improve presentation.

stantial downregulation (30% of control) of PKCα. In contrast, after a 24-h treatment with 0.1 μM (−)-*n*-hexyl or (−)-*tert*-butylILV, there was little or no decrease in amount of PKCα, respectively. Because the decrease in PKC activity by (−)-*n*-hexylILV or (−)-ILV was maximum when the cells were exposed for 4 h instead of 24 h to the compounds, we compared the fate of immunoreactive PKCα following incubation with various agents for 4 and 24 h. A polyclonal antisera specific to PKCα developed by the method of Markowske et al. (1988) was used in this study (Figure 5). The change in immunoreactive PKCα induced by a 24-h exposure to various compounds was similar when detected by a PKCα-specific monoclonal or polyclonal antibody. Both TPA and lyngbyatoxin A caused substantial downregulation of PKCα by 4 h, reducing the amount of PKCα to 35% of the control (based on laser densitometry). Further incubation with TPA but not lyngbyatoxin A caused complete disappearance of PKCα. A 4-h exposure to (−)-*n*-hexylILV caused a 12% decrease in PKCα with little change in the PKCα level by 24 h. (−)-ILV (1 μM) had no effect on the levels of immunoreactive PKCα.

The intensities of immunoreactive bands corresponding to PKCβ and -γ isoforms were low in DMSO-treated HeLa cells (Figure 4B,C) and did not decrease during a 24-h treatment with TPA. Similar results were obtained when cells were exposed for 24 h to lyngbyatoxin A or its analogues.

Discussion

Protein kinase C can be activated by a structurally diverse group of compounds including DAGs, phorbol esters, and indole alkaloids. The structural features necessary for PKC activation have been proposed, but the structural requirements for PKC downregulation, which can occur after PKC activation, have not been determined. Recently, we reported on a series of PKC activators, which, unlike phorbol esters, are easily synthesized and modified (Kozikowski et al., 1989). We have now used several of these synthetic analogues of lyngbyatoxin A to study the relationship between PKC activation and downregulation.

Computer-assisted molecular modeling of PKC activators suggests strong similarities in two portions of the molecules: A hydrophilic region consisting of the C-14 hydroxyl group, the C-11 carbonyl group, and the N-10 amide group of lyngbyatoxin A, which correspond to the C-20 hydroxyl group,

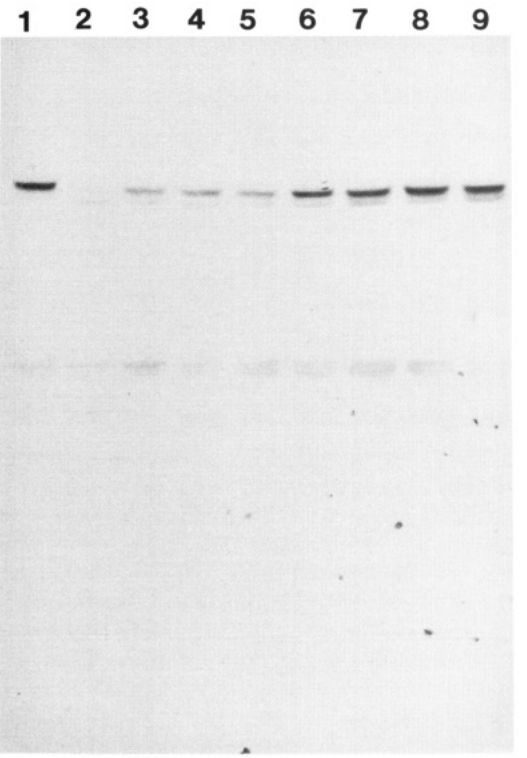


FIGURE 5: Effects of TPA and lyngbyatoxin A analogues on the fate of PKCα. HeLa cells were treated without (lane 1) or with 0.1 μM TPA (lanes 2 and 3), lyngbyatoxin A (lanes 4 and 5), (−)-*n*-hexylILV (lanes 6 and 7), or 1 μM (−)-ILV (lanes 8 and 9) for 24 h (lanes 2, 4, 6, and 8) or 4 h (lanes 3, 5, 7, and 9). At the end of the incubation, PKC was isolated and Western blot analysis was performed using polyclonal antisera specific to PKCα as described under Experimental Procedures.

the C-3 carbonyl group, and the C-4 hydroxyl group of the phorbol esters; and a hydrophobic domain at C-12 of TPA, which is equivalent to C-7 substitution of lyngbyatoxin A (Itai et al., 1988). Structure–activity studies with phorbol esters demonstrate the importance of hydrophilic residues for biological activity (Wender et al., 1986; Jeffrey & Liskamp, 1986; Rando et al., 1988). This appears to be true also with lyngbyatoxin A analogues. We have demonstrated that the lactam ring structure of lyngbyatoxin A containing C-14, N-10, and C-11 atoms is critical for PKC activation (Basu et al., 1991). Although the maximum stimulation of PKC was achieved with lyngbyatoxin A analogues that retained both the lactam ring structure and a hydrophobic group at the C-7 position, modification of the latter had no major effect on the ability of these compounds to activate PKC in vitro. This is consistent with the observations that biological activity is retained by pendolmycin, which contains an isopentenyl structure at the 7-position, and (−)-ILV, which is devoid of any hydrophobic tail at the 7-position (Umezawa et al., 1989; Heikkila & Akerman, 1989). Irie et al. (1987) have shown that hydrophobicity at position 7 has little effect on tumor-promoting activity but influences receptor binding presumably by allowing for more efficient interaction with the lipid bilayer.

It is generally accepted that hydrophobic moieties can function to anchor a molecule into membranes. Since prolonged association of PKC with membranes is accompanied by subsequent degradation of the enzyme, the ability of a compound to form a stable association with the membrane may be essential for inducing downregulation of PKC. We provide biochemical evidence that the hydrophobicity at the C-7 position of lyngbyatoxin A can influence the ability of the compound to downregulate PKC. Three independent methods were used to assess PKC downregulation. In the first method,

PKC was extracted from cells treated with or without TPA and lyngbyatoxin A analogues and the enzyme activity was determined in vitro using histone as the substrate (Figure 3; Basu et al., 1991). In the second method, we have employed a PKC-specific substrate peptide to monitor PKC downregulation in intact cells (Figure 2C). With the third approach, we have assessed PKC downregulation by Western blot analyses (Figures 4 and 5).

We have attempted to measure PKC activation with the [Ser^{25}]PKC(19–31) substrate in intact HeLa cells but have found high basal phosphorylation of the peptide after permeabilization of cells obscured the PKC stimulation by low concentrations of TPA and lyngbyatoxin A analogues. It is conceivable that the small peptide could reach the active site of the enzyme in a cofactor-independent way. Nevertheless, prolonged cellular exposure to 0.1 μM TPA markedly decreased PKC activity below the basal value of untreated cells, and reconstitution of PKC activity with TPA provided a rapid and convenient way to assess PKC downregulation in cells chronically treated with TPA or lyngbyatoxin A analogues. The downregulation results with the PKC substrate peptide (Figure 2C) agreed with those obtained by measuring histone phosphotransferase activity of the enzyme isolated from cells treated with TPA or lyngbyatoxin A analogues (Figure 3) (Basu et al., 1991). Although lyngbyatoxin A was less potent than TPA in both activating and downregulating PKC, the relationship between PKC activation and downregulation by these compounds was similar (Figure 2C and Table I). A similar result was obtained with (−)-*n*-octylILV (Basu et al., 1991). These data suggest that the hydrophobicity of an 8–10-carbon-containing alkyl group is sufficient to allow tight association of the enzyme to the membrane. In contrast, (−)-*n*-hexyl- and (−)-*tert*-butylILV at concentrations that caused an in vitro stimulation of PKC similar to that seen with lyngbyatoxin A induced only partial downregulation of PKC (Table I) (Basu et al., 1991). Interestingly, PDBu, which contains a 4-carbon alkyl group at the 12-position of the phorbol ring produced only a 25% PKC downregulation in HeLa cells (Basu et al., 1991). Furthermore, 10 μM (−)-ILV, which activated PKC maximally, induced only a 50% downregulation of PKC. The lack of PKC downregulation by (−)-ILV could not be explained by its poor entry into cells or its subsequent hydrolysis to inactive compounds; these compounds could displace [^{3}H]PDBu in intact cells, and unlike cell-permeable DAG, they were capable of exerting long-term effects in cells. For example, a 24-h pretreatment with either (−)-*n*-hexylILV or (−)-ILV caused a significant sensitization of HeLa cells to the anticancer agent cisplatin, a phenomenon that is thought to be mediated by activation of PKC (Basu et al., 1991). In addition, lack of downregulation by (−)-ILV could not be explained by direct inhibition of proteolytic degradation of PKC because a 24-h coincubation with TPA and (−)-ILV did not block TPA-induced downregulation of PKC.

Another distinction between lyngbyatoxin A and the simplified analogue (−)-*n*-hexylILV was the reversibility of PKC downregulation. PKC contains two cysteine-rich regions that can bind phorbol esters with different affinity (Bell & Burns, 1991). Recently, it has been demonstrated that binding of PKC to the membrane as well as its activation is a two stage process (Bazzi & Nelsestuen, 1989). A reversible association of PKC to the membrane is followed by the formation of an "irreversible" PKC–membrane complex. If this "irreversible" association of PKC with the membrane is necessary for inducing downregulation, then compounds that lack significant membrane-interactive properties may not cause PKC downregulation. We hypothesize the lyngbyatoxin A might resemble TPA in forming a tight ternary complex with PKC and membrane components, whereas the *n*-hexyl or *tert*-butyl derivatives could resemble the cell-permeable DAGs in that they can form only reversible complexes and therefore fail to promote substantial downregulation of PKC. High concentrations of these analogues may, however, allow the tight ternary complex to form and ultimately lead to PKC downregulation.

Our results with Western blots support the notion that there is differential downregulation of PKC isoforms by PKC activators. TPA or lyngbyatoxin A analogues caused selective reduction of PKCα. The depletion of PKC activity by TPA or lyngbyatoxin A analogues correlated well with the loss of the immunoreactive band corresponding to PKCα, the major immunoreactive species detected in HeLa cells. The constitutive expression of PKCβ or -γ isoforms as detected by immunoreactivity was extremely low in HeLa cells. Although PKCγ is known to be expressed in brain tissue and spinal cord, recent evidences indicate that it may also be expressed in certain leukemic cells (Isakov et al., 1990; Komada et al., 1991). The monoclonal antibody used in this study was shown not to cross-react with PKCα or PKCβ. We cannot, however, rule out the possibility that it may recognize some other isoforms of PKC, such as PKCϵ, that may be expressed in HeLa cells (Pfeffer et al., 1990). PKCβ and -γ isoforms appear to be induced moderately by TPA and lyngbyatoxin A analogues during prolonged incubation. The residual 20–30% PKC activity detected after prolonged treatment with TPA or lyngbyatoxin A may be contributed by PKC isoforms, such as PKCβ that resist PKC downregulation. In addition, the selective induction and/or activation of PKC isoforms by lyngbyatoxin A analogues may account for the reappearance of PKC activity after a 4-h incubation with (−)-*n*-hexylILV (Figure 3C). Lack of downregulation of PKCβ or PKCγ as well as induction of these isoforms following prolonged exposure to phorbol esters has also been noted by other investigators (Strulovici et al., 1989; Fournier et al., 1989; Isakov et al., 1990).

Because phorbol esters are structurally rigid, stable, and potent activators of PKC, they are often used as model compounds to study the mechanism of activation of PKC. The persistent action of phorbol esters as opposed to the transient activation of PKC by physiologic stimuli like growth factors, hormones, or DAG raises questions regarding the physiological significance of phorbol ester-mediated cellular processes. Differences in DAG- and TPA-mediated effects have been reported by many investigators (Kreutter et al., 1985; Issandou et al., 1988; Issandou & Rozengurt, 1989; Strulovici et al., 1989). Some of these differences may be attributed to the inability of DAG to promote substantial downregulation of PKC; multiple additions of DAG are required for sustained activation of PKC as well as induction of some cellular responses. Our novel analogues of lyngbyatoxin A offer promise for future studies of PKC function for several reasons: (1) They are effective activators of PKC. (2) They can be synthesized relatively easily allowing implementation of additional structural modifications. (3) Unlike the DAGs, they are not expected to be rapidly metabolized in intact cells by kinases or lipases. (4) Their structure–function relationships suggest that the structural requirements for PKC activation can be separated from those required for PKC downregulation.

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