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Isolation and Characterization of Fatty Acid Derivatives from an Actinomycetes and Examination of the Effects on Activities of Phospholipase C and Protein Kinase C

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In our screening to search inhibitors of phosphoinositide(PI)-specific phospholipase C (PI-PLC), two inhibitors, MT965-A and -B were isolated from a culture broth of an actinomycetes. MT965-A and -B were identified as fatty acid derivatives, 14-methylpentadecanoic acid and 16-methyllinoleic acid methyl ester, respectively, based on the spectral data including NMR and MS. Both inhibitors directly inhibited not only *in vitro* PLCγ1 activity but also the platelet-derived growth factor (PDGF)-induced inositol phosphates (IPt) formation in NIH 3T3γ1 cells overexpressing PLCγ1. However, the inhibitors enhanced *in vitro* protein kinase C (PKC) activity. On examination of the effects of various fatty acids (FAs) on activities of PLC, PKC, and PDGF-induced IPt formation, the unsaturated FAs (UFAs) showed the same activities like the inhibitors, but the saturated FAs (SFAs) did not show similar activities. It was inferred that the chain length, degree of unsaturation, methyl esterification, branching with a methyl group, and *cis*-configuration were important for their activity.

Key words: Phospholipase C, actinomycetes, fatty acids, inositol phosphates, protein kinase C

Phospholipases play the key role in generating the lipid-derived second messengers implicated in signal transduction processes, and a PI-specific PLC-involved signaling is a major pathway in the receptor tyrosine kinase signalings (4). The hydrolysis of a minor membrane phospholipid, phosphatidylinositol 4, 5-bisphosphate [PtdIns(4, 5)P_2](PIP$_2$), by the PI-PLC is one of the earliest key events. The hydrolysis produces two intracellular messengers, inositol triphosphates [Ins(1, 4, 5)P3] (IP$_3$) and diacylglycerol (DAG). IP$_3$ induces the release of Ca^{2+} from internal stores and DAG activates PKC (3, 18). The increase in intracelullar Ca^{2+} concentration and activation of PKC induce a series of events that ultimately lead to DNA synthesis and cell proliferation (15). Polypeptide growth factors, such as PDGF, epidermal growth factor (EGF), fibroblast growth factor (FGF), and nerve growth factor (NGF) are known to stimulate turnover of PIP$_2$ by activating PLCγ1 in a wide variety of cells (20). There is much evidence available regarding the involvement of PLC-mediated PI-turnover in the regulation of cell proliferation (16, 17). Microinjection of PLCγ1 antibody or PIP$_2$ antibody decreased PI-turnover activity and inhibited DNA synthesis (5, 21). Moreover, PI-turnover activity is increased in a number of human tumors such as breast cancer (1), non-small cell lung and renal cell carcinomas (20), and glial tumors (6). Thus, an inhibitor of PLC would be a possible candidate for cancer chemotherapy and it is also a useful reagent for exploring the mechanism of the signal transduction.

In our screening to search inhibitors of PLC, two inhibitors MT965-A and -B were isolated from a culture broth of an actinomycetes and identified as fatty acids. Therefore, the present paper describes the isolation, structure determination of the inhibitors, and examinations for the effects of other authentic fatty acids as well as the inhibitors on the activities of PLC, IPt formation and PKC.

Materials and Methods

Materials

PDGF, fetal bovine serum (FBS), bovine serum al-

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bumin (BSA) and Dulbecco's modified Eagle's medium (DMEM) were obtained from GIBCO/BRL (Grand Island, NY). Histone H1 type IIIS was purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]PI, myo-[2-^{3}H] inositol, and [^{32}P-γ]ATP were obtained from Amersham (Arlinghton Heights, IL). Dowex AG 1-X8 anion exchange resin was purchased from Bio-Rad Laboratories (Hercules, CA). HPLC grade organic solvents were obtained from Burdick & Jackson (Meskegon, MI). All FAs were obtained from Sigma Co.. For experiments, the FAs were dissolved in 99.8% methanol and stored as stock solution (10 mg/ml) under a nitrogen atmosphere at -75°C.

Strain

A unidentified actinomycetes SL50965 was isolated from a soil sample collected in Yusong, Korea. The strain was grown at 28°C and maintained on the Bennett's agar medium (glucose 1%, yeast extract 0.1%, beef extract 0.1%, Bacto peptone 0.1%, and agar 2%, pH 7.2).

Cell line

NIH 3T3γ1 cells overexpressing PLCγ1 were constructed by transfection of PLCγ1 cDNA into NIH 3T3 fibroblasts (10). The cells were cultured in DMEM supplemented with 10% of FBS at 37°C in a CO_2 incubator.

Fermentation

An actinomycetes SL50965 was cultured in 1 L Erlenmeyer flask containing 200 ml of the fermentation medium (glucose 1%, soluble starch 2%, soybean meal 2.5%, beef extract 0.1%, yeast extract 0.4%, NaCl 0.2%, K_2HPO_4 0.005%, and $CaCO_3$ 0.2%, not adjusted pH). The fermentation was carried out on a rotary shaker (150 rpm) at 28°C for 7 days.

In vitro assay of PLCγ1

PLCγ1 was purified (over 95% purity) from bovine cerebellum through DE-52, matrix green gel affinity, phenyl 5-PW and Mono Q column chromatography. The enzyme activity was assayed using [^{3}H]PI as substrate according to the method of Rhee *et al.* (19).

In vitro assay of PKC

The assay was performed as previously described (7). PKC partially purified from bovine cerebellum through DE-52 and phenyl 5-PW column chromatography was assayed for its phosphorylating activity on histone H1 type IIIS.

Assay of IPt formation

The assay was carried out by the method of Lee *et al.* (10). NIH 3T3γ1 cells (6×10^6 cells/ml) were fasted in inositol-free DMEM containing 1 μCi/ml of myo-[2-^{3}H]inositol for 24 hrs. The cells were rinsed twice with phosphate buffered saline (PBS) and preincubated in inositol-free DMEM containing 20 mM HEPES, pH 7.5, 20 mM LiCl and 1 mg/ml BSA at 37°C for 15 min. The reaction mixture was incubated with PDGF for 30 min and the incubation was stopped by adding ice-cold 5% $HClO_4$. IPt were extracted for 30 min on ice and the acid soluble fraction was applied to a Dowex AG 1-X8 anion exchange column. The radioactivity of the IPt eluted with varying concentration of ammonium formate was measured by using the liquid scintillation counter.

Spectral analysis

Electron ionized mass (EI-MS) spectrum was measured on a Hewlett-Packard MS-Engine-5989A mass spectrometer. Nuclear magnetic resonance (NMR) spectra were aquired on a Varian UNITY 300 at 300 MHz.

Results and Discussion

Isolation and structure determination of MT 965-A and -B

After the fermentation, an equivalent volume of acetone was added to 5 L of the culture broth obtained, and the extraction was continued for 5 hrs at room temperature. The mixture was filtered through a filter paper (Whatman No. 2) under reduced pressure. The filterate was concentrated and suspended in 500 ml of water. The suspended mixture was extracted twice with equal volumn of ethylacetate and the organic layer was concentrated under the reduced pressure to dryness. The residue was applied to a silica gel column, and the column was developed with a mixture of chloroform-methanol (100:1). The eluated fractions were assayed for the inhibitory activity against PLCγ1 and then the active fractions were pooled and concentrated. The residue was applied to a Sephadex LH-20 column and eluted with a mixture of chloroform-methanol (2:1). MT965-A and -B were finally purified with preparative HPLC using a ODS column (YMC-Pack A-323-10, 250×10 mm) and eluted with a mixture of methanol-water (9 :1) at a flow rate of 2 ml/min. The inhibitors were collected and concentrated under reduced pressure to give a white powdery substance (for MT965-A) and a colorless waxy (for MT965-B) substance.

The ^{1}H NMR spectra of both MT965-A and -B were classically similar to that of the fatty acids (Fig. 1). It was found that MT965-A contained 16

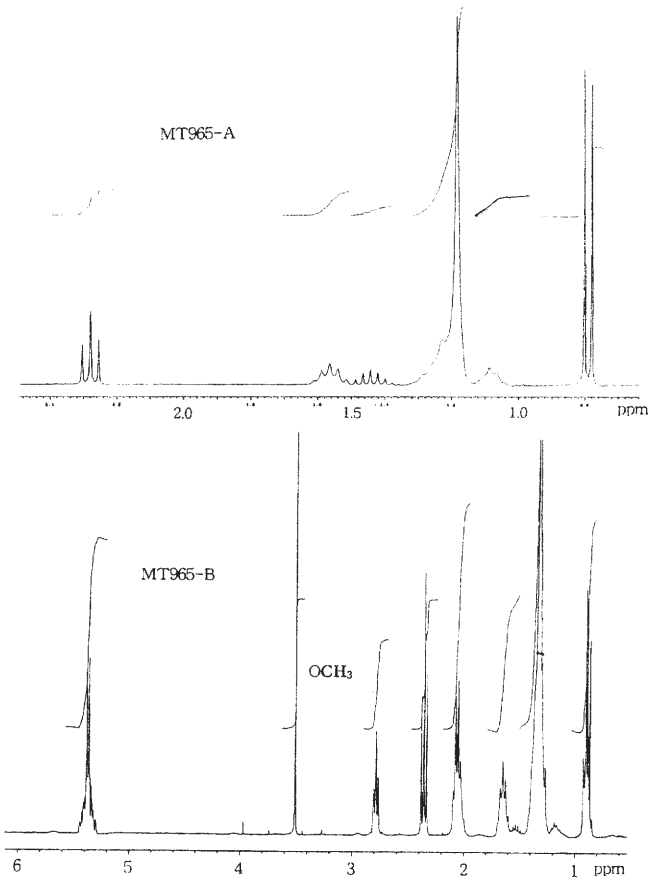

Fig. 1. ^{1}H NMR spectra of MT965-A and -B (in $CDCl_3$).

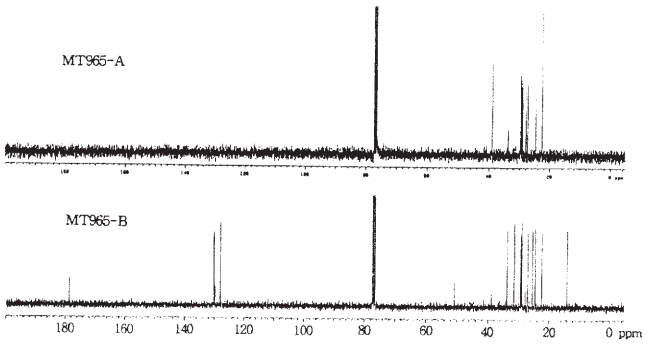

Fig. 2. ^{13}C NMR spectra of MT965-A and -B(in $CDCl_3$).

carbons consisting of 2 methyl carbons, 12 methylene carbons, 1 methine carbon and 1 carbonyl carbon, and that MT965-B contained 20 carbons consisting of 2 methyl carbons, 1 methoxy carbon, 12 methylene carbons and 5 methine carbons (4H, 2× HC=CH), respectively by the ^{13}C (Fig. 2) and distortionless enhancement polarization transfer (DEPT) NMR spectra (data not shown). In EI-MS spectrum (Fig. 3), MT965-A showed the molecular ion peaks at m/z 256. Based on the above spectral analysis, MT965-A was found to be 14-methylpentadecanoic acid (isopalmitic acid) with a methyl group branching at the 14-C position of pentadecanoic acid (Fig. 4). The structure was reconfirmed by comparing the ^{1}H and ^{13}C NMR spectra of the corresponding authentic fatty acid (data not shown). For MT965-B, when the ^{13}C NMR spectrum (Fig. 2) was compared with that of the authentic linoleic acid methyl ester (LAME) (data not shown), an additional two carbon signals (one methyl carbon at δ_C 22.6 and one methine carbon at δ_C 28.0) were found. It clearly indicated the presence of an extra methyl group in the aliphatic chain of LAME. Although homo (^{1}H-^{1}H) and hetero (^{1}H-^{13}C) correlated spectroscopys (COSY) NMR were carried out to elucidate the precise position of an additional methyl group, we could not do so, due to the fact that the corresponding signals were relatively very weak compared to other signals, and they also overlapped with aliphatic methylene signals. However, based on the chemical shifts and mutiplicities of the methyl carbon signal and proton signal (δ_H 0.9), and on their possible connectivities with the others in the proton signals, it could be indicated that the

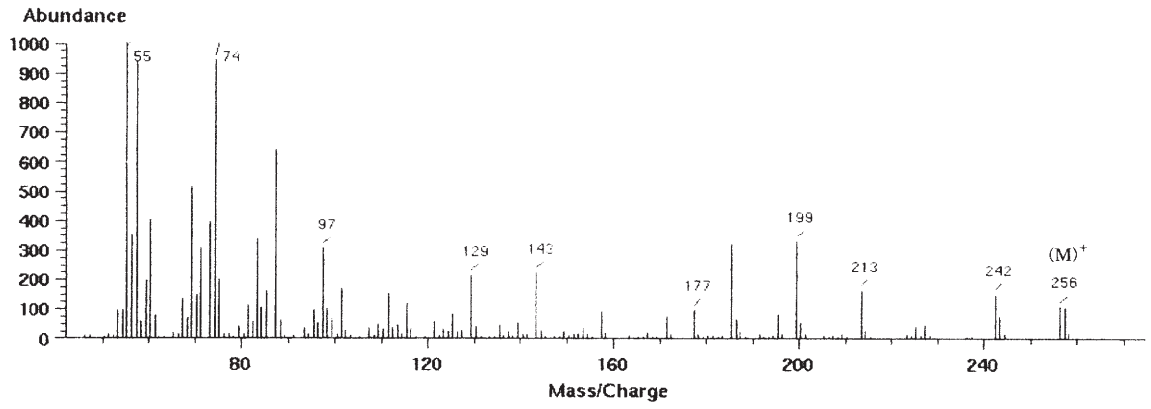

Fig. 3. EI-MS spectrum of MT965-A.

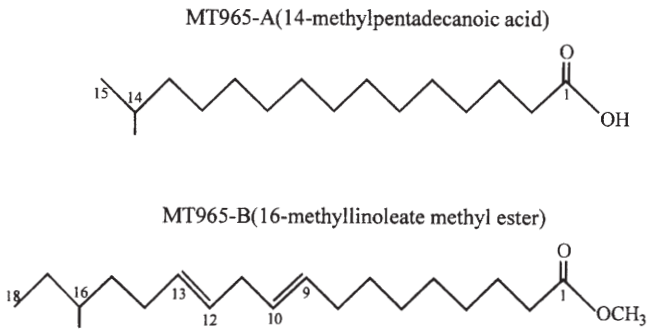

Fig. 4. The proposed structures of MT965-A and -B.

methyl group must be located in a position far distant from a carbonyl ester terminal, and that it did not present in isoform along with another methyl group. These results gave a position of 16-C as the most possible one, as shown in Fig. 4. Therefore, MT965-B was determined to be 16-methyl LAME with molecular weight of 308.

Effects of FAs on the activities of PLCγ1, IPt formation and PKC

MT965-A and -B directly inhibited the activity of PLCγ1 *in vitro* showing the IC_{50} values of 80 and 30 μM, respectively (Table 1). As expected, both compounds suppresed the formation of IPt in NIH 3T3γ1 cells stimulated by PDGF. The inhibition of IPt formation could be due to the inhibition of the PLCγ1-mediated hydrolysis of PI. In contrast to the inhibitory activities on PLCγ1 and suppression of IPt formation, both compounds enhanced the activity of PKC *in vitro*. MT965-B showed approximately 3-fold higher activity than that of MT965-A. The potency of the activation was similarly parallel with that of the inhibition on PLCγ1. Since, MT965-A and -B were isolated as inhibitors of PLCγ1 and identified as FAs, we examined the effects of the various authentic FAs on the activities of PLCγ1, IPt formation and PKC. As shown in Table 1, the UFAs especially oleic acid (OA), linoleic acid (LA), linolenic acid (LNA) and palmitoleic acid (PLA), showed more potent inhibitory activities on PLCγ1 than those of other FAs tested, whereas the SFAs such as pentadecanoic acid (PDA), palmitic acid (PA) and stearic acid (SA) were ineffective. Increasing the concentration of SFAs up to 800 μM failed to inhibit the activity. The ineffective activity of SFAs on the activities of PLC and PKC have previously been reported (8, 11, 14). The UFAs inhibiting PLCγ1 also enhanced the PKC activity as in the case of MT965-A and -B. The long chain monounsaturated FAs (MUFAs) such as nervonic acid (24:1) and erucic acid (22:1) appeared to be somewhat less effective. But the highly polyunsaturated FAs (PUFAs) including arachidonic acid (AA, 20:4), docosahexaenoic acid (DHA, 22:6) and eicosapentanoic acid (EPA, 20:5) were relatively more effective than MUFAs. Interestingly, the inability of SFAs was restored by the methyl esterification of a free carboxyl group (e. g. PA and SA) or by branching with a methyl group (e. g. MT965-A). It was also interesting that the methyl esters of SFAs that showed the inhibitory activities on PLCγ1 and activating activities on PKC did not inhibit the IPt formation in NIH 3T3γ1 cells. Thus it was inferred that the methyl esters could be modified during their incorporation into NIH 3T3γ1 cells. We also found that the methyl esters (e. g. LAME and OAME) were more effective than the corresponding UFAs, LA and OA. This result, as well as the inability of SFAs, is in conflict with the previous report (12) that the negative charge of FAs is necessary for PKC activation. Although OA and elaidic acid (EA) had the same number of carbons and double bond, the former, *cis*-configuration was more active than EA, its *trans*-isomer. It has been previously described (11, 12) that most of the biologically active FAs that showed activities of PLC and PKC had almost all *cis*-configuration. There fore, from the above results it could be concluded that; 1) only the UFAs and not the SFAs had the inhibitory activity on PLCγ1 and IPt formation, and the stimulatory activity on PKC, 2) the chain length, degree of unsaturation, methyl esterification, branching with a methyl group, and *cis*-configuration structure of

Table 1. Effects of the fatty acids and their derivatives on the activities of PLC, IPt formation and PKC

Fatty acids	PLC (IC_{50}, μM)	PKC[a] (activating fold)	IPt formation (IC_{50}, μM)
MT965-A	80	2.0	100
MT965-B	30	3.0	40
Arachidonic acid (20:4)	60	2.5	65
Docosahexaenoic acid (22:6)	75	2.5	120
Elaidic acid (18:1)	210	1.2	280
Erucic acid (22:1)	240	1.2	300
Linoleic acid (18:2)	50	2.8	70
Linolenic acid (18:3)	35	3.0	55
Nervonic acid (24:1)	270	1.1	330
Oleic acid (18:1)	40	3.0	50
Palmitoleic acid (16:1)	60	2.8	80
Petroselinic acid (18:1)	90	2.2	120
Eicosapentanoic acid (20:5)	80	2.2	130
Pentadecanoic acid (15:0)	>800	NE[b]	NE
Palmitic acid (16:0)	>800	NE	NE
Palmitic acid methyl ester	90	1.8	NE
Stearic acid (18:0)	>700	NE	NE
Stearic acid methyl ester	100	1.8	NE
Linoleic acid methyl ester	40	2.8	NE

[a] Assayed at a concentration of 200 μM, [b] no effect.

UFAs were important for their activities. It was also confirmed that UFAs might be closely associated with a PLC-mediated signaling cascade.

In the present study, the UFAs showed the inhibitory action on the activities of PLCγ1 and PI-turnover, in contrast to the previous reports in which UFAs & activated PLC (8, 9, 13). Irvine *et al.* (9) indicated that UFAs such as OA and AA stimulated PLC activity in rat brain cytosol. Negishi *et al.* (13) have also described that AA stimulated Ca^{2+}-sensitive PLC in bovine adrenal chromaffin cells, and the effect was specific for AA. Futhermore, Hwang *et al.* (8) reported that UFAs did not directly affect the PLCγ1 activity, but enhanced the activation of PLCγ1 activity by a tau protein. In addition, the incubation of human trophoblasts with AA also stimulated PLC activity (22). Recently, in consistent with the present result, Awad *et al.* (2) have concluded that the decrease in proliferation of HT-29 colon cancer cells with supplementation with LNA complexed to BSA may be mediated through its inhibitory effect on PI-PLC. Since, the actual mechanism by which FAs directly modulate the activity of PLC is still unknown, it is difficult to precisely explain our conflicting result. Hwang *et al.* (8) suggested the possibility that AA interacts with one of the two pleckstrin homology (PH) domains of PLCγ and cooperates with tau bound to the Src homology 3 (SH3) domain to enhance enzyme activity. Unfortunately, no significant activation of UFAs on PLCγ1 was measured at lower concentrations in our experiments, it was conceivable that the activity of UFAs may be sensitive to or dependent on their local concentration. Although other studies have shown a stimulatory effect of UFAs on PLC activity, the present result strongly suggested the inhibitory activity of UFAs including MT 965-A and -B on the enzyme activity, because they inhibited not only *in vitro* PLCγ1 activity but also IPt formation in the cells. However, since little is known about the mechanism for the modulating activity, especially for the inhibiting activity of UFAs on PLC, further investigations are needed to elucidate the present result.

On the other hand, UFAs directly activate PKC and a potential role of free FAs as second messengers has been extensively suggested (11, 12, 14, 15). It is now accepted that UFAs are necessary for the full or sustained activation of PKC, and thereby, like Ca^{2+}, DAG, or IP_3, they play a role as regulatory molecules in signaling through PKC pathway. These studies were consistent with the result obtained in our experiment. However, the mechanism by which FAs modulate the activity of PKC also remains to be poorly understood.

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