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# Inhibition of Phosphoinositide-Specific Phospholipase C by Manoalide

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## SUMMARY

Manoalide is a novel sesterterpenoid which has previously been shown to be a potent inhibitor of venom phospholipases $A_2$. To determine whether manoalide inhibited other phospholipases, the sensitivity of phosphoinsitide-specific phospholipase C (PI-PLC) to inactivation by manoalide was examined using crude cytosolic PI-PLC and a PI-PLC purified to homogeneity from guinea pig uterus cytosol (PI-PLC I). Manoalide inhibited both cytosolic and purified PI-PLC I in a concentration-dependent fashion, exhibiting an $IC_{50}$ of 3–6 $\mu$M. Inactivation of PI-PLC I was calcium- and pH-dependent, with greater inactivation occurring at alkaline pH. Manoalide inhibited hydrolysis of all three phosphoinositides by purified PI-PLC I. The substrate kinetics of PI-PLC I suggest that manoalide does not inhibit purified PI-PLC I by simple competitive or noncompetitive inhibition. Enzyme activity was not recovered after dialysis of manoalide-treated PI-PLC I, indicating that inactivation of PI-PLC I was irreversible. To determine whether manoalide inhibited PI-PLC in cells, the effects of manoalide on norepinephrine (NE)-stimulated phosphoinositide hydrolysis and calcium mobilization were investigated in a smooth muscle-like cell line, $DDT_1MF$-2. Manoalide inhibited NE-induced inositol 1,4,5-trisphosphate and inositol 1-phosphate formation in a concentration-dependent manner. The $IC_{50}$ for inhibition of inositol 1-phosphate formation was 1.5 $\mu$M. Manoalide also inhibited NE-induced calcium transients in $DDT_1MF$-2 cells, exhibiting an $IC_{50}$ of 2 $\mu$M. These data suggest that inhibition of PI-PLC may account, in part, for the anti-inflammatory actions of manoalide.

Manoalide is a nonsteroidal sesterterpenoid isolated from the sponge *Luffariella variabilis* (1). The pharmacological properties of manoalide include analgesic activity in the mouse phenylquinone writhing model and inhibition of phorbol 12-myristate 13-acetate-induced inflammation in the mouse ear, but not arachidonic acid-induced inflammation (2, 3). Preliminary studies concerning the mechanism of action of manoalide demonstrated that manoalide was a potent inhibitor of venom $PLA_2$s (3–5). The inhibition of $PLA_2$ by manoalide was pH-dependent and irreversible, possibly forming a covalent adduct with lysine residues (4, 5). These studies, based on venom $PLA_2$, suggested that manoalide may exert its anti-inflammatory activity through inhibition of intracellular $PLA_2$. However, we and others recently reported that manoalide was 300- to 1000-fold less active against mammalian $PLA_2$s than against $PLA_2$s isolated from venoms (6, 7), suggesting that $PLA_2$ may not be the principal intracellular target for manoalide.

Various hormones, neurotransmitters, growth factors, and proinflammatory stimuli are known to promote a rapid turnover of the phosphoinositides in their respective target tissues (reviewed in Refs. 8–10). The signal initiated by the binding of ligand to the receptor is transduced through a guanine nucleotide-binding protein (8–14), resulting in the activation of a PI-PLC. Hydrolysis of $PIP_2$ by PI-PLC generates two proposed second messengers, $IP_3$ and 1,2-diacylglycerol (8–10). The initial transient increase in intracellular calcium associated with calcium-mobilizing hormones is attributed, in part, to $IP_3$ promoting the release of calcium from intracellular stores (8–10, 15, 16). The increase in cytoplasmic calcium activates a variety of calcium-dependent enzymes. The second product of $PIP_2$ hydrolysis, 1,2-diacylglycerol, has been proposed to activate a calcium- and phospholipid-dependent protein kinase, protein kinase C (17, 18). Protein kinase C, in turn, may regulate some of the enzymes involved in the stimulus response pathway or, alternatively, may regulate phosphoinositide turnover via feedback inhibition (18–23).

**ABBREVIATIONS:** $PLA_2$, phospholipase $A_2$; PI-PLC, phosphoinositide-specific phospholipase C; PI, phosphatidylinositol; PLC, phospholipase C; PIP, phosphatidylinositol 4-phosphate; $PIP_2$, phosphatidylinositol 4,5-bisphosphate; $IP_1$, inositol 1-phosphate; $IP_3$, inositol 1,4,5-trisphosphate; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis($\beta$-aminoethyl ether)-*N*, *N*, *N'*, *N'*-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; BSA, bovine serum albumin; FCS, fetal calf serum; KRH buffer, Krebs-Ringer-Henseleit buffer; NE, norepinephrine; SDS, sodium dodecyl sulfate; fura 2 AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5-methylphenoxy)-ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid, pentaacetoxymethyl ester.

Because many inflammatory mediators utilize phosphoinositide turnover as part of their signal transduction pathway (8, 9), we speculated that inhibition of PI-specific phospholipase C could mediate some of the observed pharmacological activities of manoalide. To test this hypothesis we examined the ability of manoalide to inhibit PI-PLCs in crude cystolic preparations and a purified PI-PLC from guinea pig uterus (24). We found that manoalide inhibited PI-PLC enzyme activity in a concentration-dependent manner. Furthermore, inhibition of agonist-induced phosphoinositide hydrolysis and calcium mobilization in intact cells was demonstrated.

## Experimental Procedures

**Materials.** Female guinea pigs were obtained from Hazelton Research. Bovine serum albumin, phenylmethylsulfonyl fluoride, aprotinin, leupeptin, PIP, phosphatidylinositol 4,5-phosphate, and NE were obtained from Sigma Chemical Co. (St. Louis, MO). Glycerol, Tris, and sucrose were purchased from Bethesda Research Laboratories (Gaithersburg, MD). FCS was purchased from Hyclone Laboratories (Logan, UT). HEPES, bis-Tris, sodium cholate, fura-2 AM, and sodium deoxycholate were purchased from Calbiochem (San Diego, CA). Bovine liver PI was purchased from Avanti Polar Lipids (Birmingham, AL). Eagle's minimal essential medium was purchased from GIBCO (Grand Island, NY). 1-Stearolyl-2-[1-$^{14}$C]arachidonyl phosphatidylinositol (60 mCi/mmol) was purchased from Amersham (Arlington Heights, IL). L-$\alpha$-[*myo*-inositol-2-$^{3}$H(N)]phosphatidylinositol (8.4 Ci/mmol), L-$\alpha$-[inositol-2,3-$^{3}$H(N)]phosphatidylinositol 4,5-bisphosphate (2.0 Ci/mmol), L-$\alpha$-[inositol-2-$^{3}$H(N)]phosphatidylinositol 4-phosphate (1.5 Ci/mmol), and L-*myo*-[1,2-$^{3}$H]inositol (40–60 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Silica gel G thin layer chromatography plates were purchased from Analtech (Newark, DE). Manoalide was purified from *Luffariella variabilis* by the Synthetic Chemistry Department, Discovery Research, Allergan Inc./Herbert Laboratories.

**Enzyme source.** Total cytosolic proteins were isolated from cells in culture or from tissue by homogenization in 0.25 M sucrose/5 mM $MgCl_2$/1 mM EDTA/10 mM Tris-HCl, pH 7.4 at 4°. Tissues were homogenized with a Polytron homogenizer (Kinematica, Switzerland) and tissue culture cells were homogenized with a tight-fitting Dounce homogenizer. The following protease inhibitors were added at the indicated final concentration just before homogenization: phenylmethylsulfonyl fluoride (0.5 mM), leupeptin (0.1 mM), and aprotinin (10 $\mu$g/ml). All subsequent manipulations were carried out at 4°. The homogenate was centrifuged for 10 min at 2,000 × *g*; the supernatant was then centrifuged at 100,000 × *g* for 60 min. Between 70 and 95% of the total PI-PLC enzyme activity was found in the 100,000 × *g* supernatant which was used for all assays utilizing total cytosolic proteins. Protein concentrations were determined as described by Bradford (25). Polyacrylamide gel electrophoresis was performed as previously described (26).

A PI-PLC was purified greater than 1400-fold from guinea pig uterus cytosol (24) using a modification of the procedure described by Hoffmann and Majerus (27). The enzyme used for these studies resembles PLC I from sheep seminal vesicle (27) as determined by its elution from AH-Sepharose, molecular weight, calcium sensitivity, and substrate preference (24).

**Phospholipase assays.** $PLA_2$ activity was quantitated by the release of $^{14}$C-arachidonic acid from 1-palmityl-2-[1-$^{14}$C]arachidonyl phosphatidylcholine as previously described (6). PLC activity was quantitated by the release of $^{14}$C-1,2-diacylglycerol from 1-stearoyl-2-[1-$^{14}$C]arachidonyl phosphatidylinositol. The phospholipid substrate was dried under argon, dissolved in a solution containing 5 mg/ml deoxycholate, and sonicated 10 min in a sonicating water bath. The reaction mixture (50 $\mu$l final volume) contained 100 mM Tris-HCl, pH 8.0, 5 mM $CaCl_2$, 50 mM KCl, 2.4 mM deoxycholate, 5% glycerol, and 10 $\mu$M labeled substrate (30,000 dpm) unless otherwise indicated. Reactions were initiated by the addition of substrate and incubated from 5 to 30 min at 37° as indicated. The reactions were stopped and the lipids extracted by the sequential addition of 50 $\mu$l of chloroform/methanol (1:2), 50 $\mu$l of chloroform, and 50 $\mu$l of 4.0 M KCl. Samples were mixed by vortexing followed by centrifugation at 10,000 × *g* for 1 min to separate the aqueous and organic layers. The organic layers were spotted onto thin layer chromatography plates with unlabeled standards and chromatographed using a petroleum ether/diethyl ether/acetic acid (70:30:1) solvent system. The bands corresponding to arachidonic acid and 1,2-diacylglycerol were visualized with iodine vapor, scrapped into scintillation vials, and extracted with 0.5 ml of methanol. Radioactivity was determined using a Beckman model 9800 liquid scintillation counter with 10 ml of Beckman HP/b scintillation cocktail. Alternatively, PLC activity toward phosphoinositides was assayed using $^{3}$H-inositol-labeled PI or polyphosphoinositides. Assays were performed as described above, but lipids were extracted by sequential addition of 250 $\mu$l of chloroform/methanol/concentrated HCl (50:50:0.3) followed by 75 $\mu$l of 1 N HCl containing 5 mM EGTA. A 150-$\mu$l aliquot of the aqueous phase was removed for radioactive determination. The $^{3}$H-phosphoinositides were diluted with the appropriate unlabeled bovine brain PI to a final specific activity of 45.4 mCi/mmol.

All assays were linear with respect to time and protein concentration when less than 25% of substrate was consumed. Enzyme concentrations were adjusted such that between 5 and 20% of substrate was consumed during the incubation period. In cell cytosol preparations the formation of arachidonic acid from PI was less than 8% of the formation of 1,2-diacylglycerol; therefore, possible actions of PI-preferring $PLA_2$ or diglyceride lipase were neglected. Furthermore, similar results were obtained when the formation of 1,2-diacylglycerol or $IP_1$ was measured.

Manoalide was dissolved in ethylene glycol and preincubated with the enzymes for the indicated time period at 37°. The final concentration of ethylene glycol in the reaction mixture was 8.0%, which had no effect on enzyme activity (data not shown). The control reactions also contained 8% ethylene glycol. Following the preincubation period, the radiolabeled substrates were added and the mixture incubated for 5–30 min at 37°. The concentration of manoalide indicated represents the concentration during the preincubation period. Results shown are representative of two to three separate experiments.

**Cell culture.** $DDT_1MF$-2 cells were a kind gift of Dr. James Norris (University of Arkansas). Cells were maintained in Dulbecco's modified Eagle's medium (4.5 g of glucose/liter) supplemented with 10% FCS. The $BC_3H1$ cell line was obtained from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco's modified Eagle's medium supplemented with 20% FCS and 1.0 g of glucose/liter.

**Measurement of $^{3}$H-inositol release from intact cells.** $DDT_1MF$-2 cells grown in suspension culture were labeled with $^{3}$H-inositol (5–100 $\mu$Ci/ml) for 2 hr at a concentration of 6 × $10^6$ cells/ml as indicated. LiCl at a final concentration of 10 mM was added for the final 15 min of labeling. Cells were washed three times in Krebs-Ringer solution (118 mM NaCl, 4.6 mM KCl, 24.9 mM $NaHCO_3$, 1 mM $KH_2PO_4$, 11.1 mM glucose, 1.1 mM $MgCl_2$, 5 mM HEPES, pH 7.4) supplemented with 10 mM LiCl and suspended at a final concentration of 2 × $10^6$ cells/ml. Cells (0.3 ml) were either preincubated with manoalide (dissolved in dimethyl sulfoxide) or vehicle for the indicated times. NE ($10^{-5}$ M) was added and cells were incubated for 30 sec for $IP_3$ measurements or 30 min for $IP_1$ measurements. Water-soluble inositol phosphates were extracted with 0.93 ml of chloroform/methanol (1:2), followed by 0.3 ml of chloroform and 0.3 ml of water. Inositol phosphates were separated by chromatography on Dowex AG1X8 as previously described (28, 29).

**Measurement of intracellular calcium.** $DDT_1MF$-2 cells, cultured as described above, were washed twice with 118 mM NaCl, 4.6 mM KCl, 1.1 mM $MgCl_2$, 5 mM HEPES, 11.1 mM glucose, 24.9 mM $NaHCO_3$, 1 mM $KH_2PO_4$, 1 mM $CaCl_2$, 0.1% BSA, pH 7.4 (KRH buffer). The cells were incubated with 5 $\mu$M fura-2 AM for 30 min at 37° at a concentration of 2 × $10^6$ cells/ml of KRH buffer in a shaking water bath, under 95% $O_2$, 5% $CO_2$. Cells were washed once in KRH buffer

and incubated 15 min at 37° to hydrolyze the fura-2 AM. Cells were washed in KRH buffer without BSA and suspended at a concentration of 4 × $10^6$ cells/ml in KRH buffer without BSA. For fluorometric analysis, 1 × $10^6$ cells/ml were incubated in a total volume of 2 ml at 37° in a fluorometer manufactured by The Johnson Foundation (University of Pennsylvania, Philadelphia, PA), with excitation and emission wavelengths of 340 and 510 nm, respectively. Cytosolic calcium concentrations were calculated as previously described (30).

## Results

**Inhibition of PI-PLC by manoalide.** Previously, we determined that manoalide was relatively inactive in inhibiting mammalian cytosolic and membrane bound $PLA_2$ (6). Subsequent experiments demonstrated that PI-PLC was sensitive to inactivation by manoalide. Inhibition of PI-PLC by manoalide was concentration dependent (Fig. 1). In guinea pig uterus and the mouse smooth muscle cell line, $BC_3H1$ cells, manoalide exhibited an $IC_{50}$ of 4–6 μM for inhibition of cytosolic PI-PLC (Fig. 1). This contrasts with the inhibition of $PLA_2$ from the same tissues, which ranged from 30 to 300 μM (6). Thus, in these mammalian cells, manoalide appears to be a more potent inhibitor of PI-PLC than of $PLA_2$, when assayed under identical conditions.

To examine the inhibition of PI-PLC by manoalide in greater detail, we studied the effects of manoalide against a purified PI-PLC. A PI-PLC purified to homogeneity from guinea pig uterus was used (24). The purified enzyme exhibited an apparent molecular weight of 62,000 on SDS-polyacrylamide gels (Fig. 2A). Guinea pig uterus PI-PLC was similar to form I PI-PLC from sheep seminal vesicles (27) and a PI-PLC isolated from rat liver (31) with respect to its physical and kinetic properties. In keeping with the nomenclature established by Hoffmann and Majerus (27), we will refer to this enzyme as PI-PLC I.

Purified PI-PLC I was inhibited by manoalide in a concentration-dependent manner (Fig. 2B), exhibiting an $IC_{50}$ value of 3 μM. Inactivation of PI-PLC I by manoalide was pH dependent (Fig. 3), displaying an optimum at pH 8–9. At pH values greater than 8.0 the enzyme exhibited less than 20% of maximal activity. Lombardo and Dennis (4) have previously demonstrated that the hemiacetal and/or lactone rings of manoalide open at alkaline pH to form free carbonyls. It was the open ring form of manoalide which was proposed to inactivate cobra venom $PLA_2$. Because inactivation of PI-PLC appears to occur through a mechanism similar to that of inactivation at $PLA_2$, we performed all subsequent experiments at pH 8.0. This pH value was chosen to achieve maximal inactivation of the enzyme yet still maintain enzymatic activity in the absence of inhibitors. Like cobra venom $PLA_2$ (4), inhibition of PI-PLC I by manoalide was also calcium dependent, with maximal inactivation occurring at 10 mM calcium (data not shown). However, calcium concentrations greater than 5 mM markedly inhibited hydrolysis of PI by PI-PLC I.

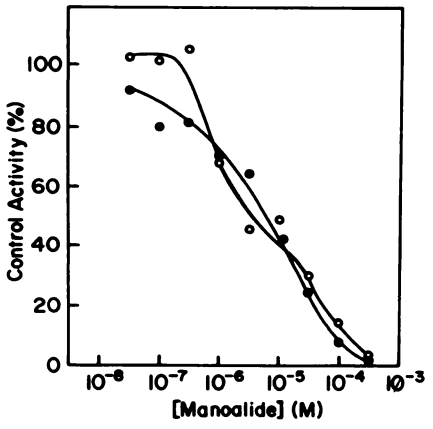


**Fig. 1.** Inhibition of cytosolic PI-PLC by manoalide. Manoalide was preincubated with cytosolic proteins for 20 min at 37°, then assayed for PLC activity. Cytosol was obtained from guinea pig uterus, 1 μg of protein (○) and a smooth muscle-like cell line, $BC_3H1$, 4 μg of protein (●). PLC assays were performed at pH 8.0 with 10 μM PI for 30 min. The results are the mean of four to six data points.

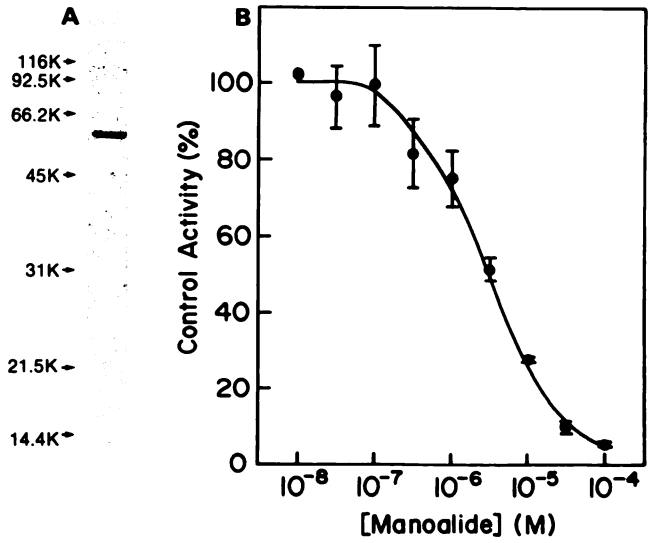


**Fig. 2.** A. SDS-polyacrylamide gel electrophoresis of purified guinea pig uterus PI-PLC I. Purified PI-PLC I (2 μg) was separated on an SDS-polyacrylamide gel containing 10% polyacrylamide and stained with Coomasie brilliant blue R-250. B. Inhibition of purified PLC I by manoalide. Manoalide was preincubated with 15 ng of purified PLC I for 20 min at 37°, pH 8.0. PLC activity was determined with 10 μM PI for 30 min at 37°, at pH 8.0. Data points represent the mean ± standard deviation from three determinations.

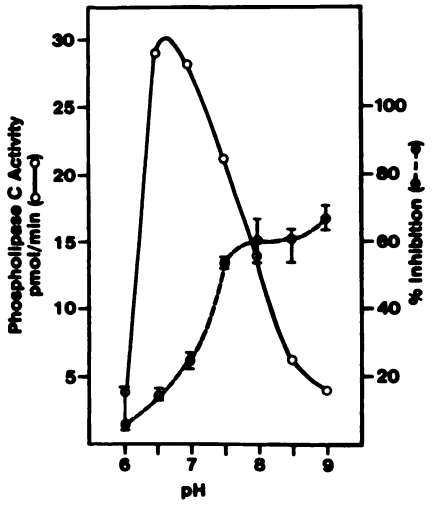


**Fig. 3.** pH-dependent inhibition of PLC by manoalide. Purified PI-PLC I (100 ng of protein) was preincubated with 10 μM manoalide or vehicle (○) for 20 min at 37° at the indicated pH with 1 mM $CaCl_2$. The PLC activity was then determined at the same pH using 20 μM PI and 10 min incubation. Results are the mean ± standard deviation from three data points.

The substrate kinetics of PI-PLC I treated with 5 μM and 30 μM manoalide for 20 min at 37° failed to demonstrate simple competitive or noncompetitive inhibition using computer-assisted analysis (Fig. 4). Previous studies showed that manoalide irreversibly inactivated bee and cobra venom $PLA_2$s (4, 5). To determine if inactivation of PI-PLC was also irreversible, a 2000-fold molar excess of manoalide (100 μM) was incubated with guinea pig uterus PI-PLC I for 20 min at 37°. Aliquots were then assayed for enzymatic activity (40 μM manoalide in enzyme assay), while the remaining enzyme was dialyzed for 4 hr at 4° against 3000 volumes of buffer. There was some loss of enzyme activity during the dialysis step (Table 1); however, the inhibitory effects of manoalide toward PI-PLC were not reversed by dialysis (Table 1). These data suggest that manoalide irreversibly inactivates PI-PLC I. Manoalide inhibited hydrolysis of all three phosphoinositides to a similar extent (Table 2), further suggesting that manoalide was acting on the enzyme and not the substrate. It should be noted that, under these assay conditions, PI and PIP were better substrates than $PIP_2$ (Table 2).

**Inhibition of agonist-induced phosphoinositide hydrolysis.** The effect of manoalide on agonist-induced hydrolysis of phosphoinositides was examined in the smooth muscle cell line $DDT_1MF$-2. Previously, it has been reported that NE promotes the hydrolysis of $PIP_2$ and subsequently increases the concentration of free cytosolic calcium in this cell line (32). As previously reported (32), NE produced a dose-dependent increase in $IP_1$ formation, reaching a maximal effect at 10 μM (data not shown). NE at a concentration of 10 μM produced a time-dependent increase in $IP_3$, reaching maximal levels 30 sec after stimulation, while $IP_1$ increased linearly with time for 30 min after stimulation (data not shown). The production of $IP_3$ in response to NE was measured 30 sec after addition of agonist. NE produced a small, but statistically significant increase in $IP_3$ relative to control values; 71.9 ± 6.5 dpm and 49.0 ± 2.6 dpm, respectively ($n$ = 6). This small increase in $IP_3$ could be due to high levels of intracellular $IP_3$ phosphatase (33, 34). Manoalide at a concentration of 1 μM inhibited $IP_3$ production 30% (65.0 ± 8.5 dpm) while 10 μM manoalide inhibited $IP_3$ production 60% (58.6 ± 3.2 dpm). Because of the small changes in $IP_3$ production in response to NE, it was difficult to obtain accurate concentration-dependent inhibition curves with manoalide. Therefore, we measured changes in $IP_1$ production in the presence of LiCl at later time points, assuming that accumulation of $IP_1$ was the result of stimulation of $PIP_2$ hydrolysis and the subsequent metabolism of $IP_3$ to $IP_1$ (8–10, 33, 34). Manoalide, when added concurrently with NE, inhibited $IP_1$ production in a concentration-dependent manner (Fig. 5). The $IC_{50}$ value for inhibition of $IP_1$ production was 1.5 μM, consistent with the inhibition of the isolated enzyme. Manoalide did not induce any changes in the basal level of $IP_1$ (data not shown).

**Inhibition of norepinephrine-induced increase in cytosolic calcium by manoalide.** Following agonist stimulation, one of the major effects of $IP_3$ is to increase cytosolic calcium (8–10). Therefore, manoalide should inhibit NE-induced increases in intracellular calcium. To examine this question, $DDT_1MF$-2 cells were loaded with fura-2 to monitor NE-induced calcium mobilization. NE produced concentration-dependent increases in cytosolic calcium, reaching maximal values at 10 μM (data not shown), as has been previously demonstrated (32). Pretreatment of the cells with manoalide for 5 min at 37° resulted in a concentration-dependent decrease in calcium mobilization (Fig. 6A). Manoalide inhibited the NE-induced increase in cytosolic calcium by 50% at 2 μM and completely inhibited changes in cytosolic calcium at 5 μM (Fig. 6B). Manoalide failed to inhibit NE-induced increases in cytosolic calcium at concentrations as high as 10 μM when the incubation buffer contained 0.1% BSA (data not shown). These results demonstrate that manoalide inhibits PI-PLC in crude cytosolic preparations, in the purified state, and in intact cells.

## Discussion

The marine natural product, manoalide, antagonized phorbol myristate acetate-induced inflammation in the mouse ear, but not arachidonic acid-induced inflammation (2–5). Thus, manoalide appears to act as an anti-inflammatory agent at a step prior to the release of arachidonic acid from membrane phospholipids. Consistent with this model was the finding that manoalide was a potent inhibitor of bee and snake venom $PLA_2$ enzymes, $IC_{50}$ = 0.05–2.0 μM (3–5). Manoalide was found to irreversibly inactivate $PLA_2$, possibly by forming a covalent

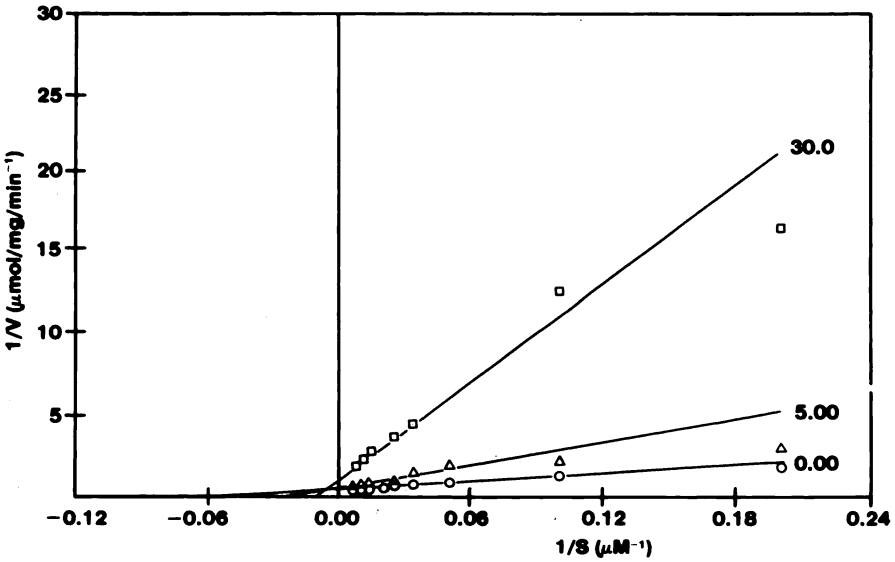


**Fig. 4.** Substrate kinetics of PI-PLC I pretreated with manoalide. PI-PLC I (13.5 ng) was treated with 0 (○), 5 (△), or 30 (□) μM manoalide for 20 min at 37° and then incubated with increasing concentrations of $^3$H-PI for 15 min at 37°. The incubation buffer contained 50 mM Tris·HCl, pH 8.0, 50 mM KCl, 5 mM $CaCl_2$. Results are expressed as the double reciprocal plot of 1/velocity ($V$) vs. 1/substrate ($S$) concentration ($n$ = 3).

TABLE 1

**Irreversibility of manoalide inhibition of PI-PLC I**

Purified PI-PLC I (3.2 μg) was incubated with 100 μM manoalide in 250 μl of 50 mM Tris·HCl (pH 8.0), 50 mM KCl, 5 mM $CaCl_2$, 2% glycerol for 20 min at 37°. Aliquots were assayed for enzyme activity ($n = 4$), while the remaining enzyme was dialyzed against 500 ml of 10 mM Tris·HCl (pH 7.4), 50 mM KCl, 0.5 mM EGTA, 20% glycerol for 4 hr at 4°. Following dialysis, aliquots were assayed for enzyme activity using [$^3$H]PI as a substrate. Results are expressed as the mean ± standard deviation from four determinations.

| | Before dialysis | After dialysis |
|---|---|---|
| | nmol/mg/min | |
| Control | 529 ± 7 | 320 ± 32 |
| Treated | 40 ± 2 | 18 ± 2 |

TABLE 2

**Manoalide inhibition of PI-PLC I using different substrates**

PI-PLC I (26 ng) was preincubated with manoalide for 20 min at 37° in 50 mM Tris·HCl (pH 8.0), 50 mM KCl, and 5 mM $CaCl_2$. Enzyme activity was determined using a 10 μM concentration of each substrate. Results are expressed as the mean ± standard deviation of two experiments performed in triplicate. Control activities for PI, PIP, and $PIP_2$ hydrolysis were 760 ± 52 nmol/mg/min, 762 ± 72 nmol/mg/min, and 162 ± 14 nmol/mg/min, respectively.

| Manoalide | Control activity | | |
|---|---|---|---|
| | PI | PIP | $PIP_2$ |
| | % | | |
| 1 μM | 72.6 ± 1.2 | 68.1 ± 3.8 | 55.9 ± 7.4 |
| 3 μM | 51.4 ± 5.4 | 43.4 ± 4.8 | 38.0 ± 5.1 |
| 10 μM | 26.2 ± 0.5 | 17.5 ± 0.8 | 26.3 ± 1.8 |

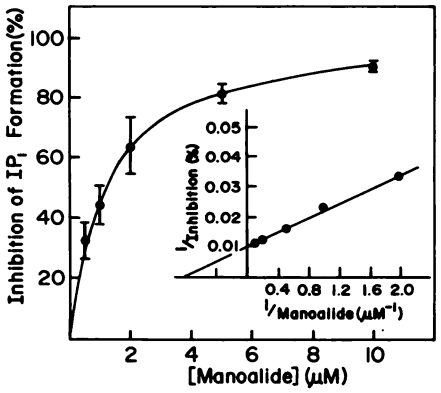


**Fig. 5.** Inhibition of $IP_1$ formation. $DDT_1MF$-2 cells were incubated for 2 hr with 10 μCi/ml [$^3$H]-*myo*-inositol. Cells were treated with 10 μM NE with increasing concentrations of manoalide. The amount of $IP_1$ formed was determined 30 min after stimulation with NE. Results are the average of two experiments each performed in triplicate. Experiment 1, control = 532 cpm, NE stimulated = 2598 cpm; Experiment 2, control = 561 cpm, NE stimulated = 1998 cpm ($n = 3$ data points per experiment).

adduct with lysine residues (4). However, manoalide exhibited weak inhibitory activity toward mammalian intracellular $PLA_2$ enzymes, $IC_{50}$ = 30 to >300 μM (6, 7). These data suggested that the pharmacological activity of manoalide may not entirely be due to inhibition of intracellular $PLA_2$.

PI-PLC has been characterized as a key enzyme in the signal transduction pathway for a variety of inflammatory mediators, including leukotriene $B_4$, histamine, antigen, chemotactic peptides, platelet-activating factor, leukotriene $D_4$, bradykinin, etc. (8–10, 29, 35–37). Therefore, inhibition of PI-PLC would abrogate the action of many pro-inflammatory stimuli. In this report we have demonstrated that manoalide was a potent inhibitor of crude, cytosolic PI-PLC ($IC_{50}$ = 4–6 μM). Comparison of the potency of manoalide against $PLA_2$ and PI-PLC in the same cytosolic extracts, assayed under identical conditions, revealed that manoalide was 15–50 times more potent at inhibiting PI-PLC (Ref. 6 and Fig. 1, this paper).

We have further characterized the mechanism of inhibition in greater detail using a purified PI-PLC from guinea pig uterus (PI-PLC I) which exhibits those properties expected of an enzyme coupled to agonist-induced phosphoinositide turnover (24). PI-PLC I was identified in both cytosolic fractions and tightly associated cell membrane fractions in a variety of tissues (24). The $V_{max}$ displayed by PI-PLC I for each substrate was $PIP_2$ > PIP > PI at 4 μM free calcium, whereas substrate affinities were in the reverse order. At physiologically relevant concentrations of calcium, $PIP_2$ and PIP were the preferred substrates (24). However, at calcium concentrations greater than 1 mM, $PIP_2$ was a poor substrate compared to PI and PIP. The $IC_{50}$ for inhibition of purified PI-PLC I (3 μM) was similar to the $IC_{50}$ value for cobra venom $PLA_2$ (1.7–2.0 μM) (4). Inhibition of PI-PLC by manoalide was not reversed by dialysis and was calcium and pH dependent, as was its inhibitory activity toward cobra venom $PLA_2$ (4). The substrate kinetics of PI-PLC in the presence of manoalide suggested that it did not exhibit simple competitive or noncompetitive inhibition, a result which was consistent with the irreversible nature of its interaction with PI-PLC I. Thus, inhibition of PI-PLC by manoalide appears to occur via a mechanism similar to that of inhibition of cobra venom $PLA_2$ (4).

Recently, Meade *et al.* (38) stated that rabbit polymorphonuclear leukocyte microsomal $PLA_2$ was more sensitive to inactivation by manoalide ($IC_{50}$ = 2–7 μM) than was cytosolic PI-PLC. The discrepancy between the results obtained by Meade *et al.* (38) and our results may be explained in part by differing assay conditions. Meade *et al.* (38) performed the $PLA_2$ assays at pH 8.0, which would favor formation of free carbonyl groups from the hemiacetal or lactone rings, whereas the PI-PLC assays were performed at pH 7.0. Alternatively, we cannot rule out the possibility that different isoenzymes of $PLA_2$ and PI-PLC exhibit different sensitivities to manoalide. The results of Meade *et al.* (38) are also different from those reported by Master and Jacobs (7), who reported that manoalide exhibited an $IC_{50}$ of 94 μM toward mouse liver microsomal $PLA_2$.

To test whether manoalide inhibits PI-PLC in intact cells, the effects of manoalide on NE-stimulated inositol phosphate formation in the hamster smooth muscle cell line, $DDT_1MF$-2, were determined. Antibodies prepared against guinea pig uterus PI-PLC I specifically reacted with a 62-kDa protein in cytosolic and membrane fractions from $DDT_1MF$-2 cells, suggesting that these cells contain a PI-PLC similar to guinea pig uterus PI-PLC I (data not shown). Treatment of intact cells with manoalide inhibited NE-induced formation of $IP_3$ and $IP_1$ in a concentration-dependent manner. The $IC_{50}$ for inhibition of $IP_1$ formation was 1.5 μM, a value similar to the $IC_{50}$ for inhibition of purified PI-PLC I. Although it was difficult to measure NE-induced release of $IP_3$ in $DDT_1MF$-2 cells, it was possible to measure inhibition of intracellular calcium release by manoalide. Difficulty in obtaining a relationship between intracellular calcium release and formation of $IP_3$ has also been reported for A-431 cells, in which it was also reported that manoalide inhibited epidermal growth factor-induced calcium mobilization (39). Manoalide inhibited NE-induced increases in intracellular calcium in a concentration-dependent manner, $IC_{50}$ = 2.0 μM. It was previously demonstrated that BSA or

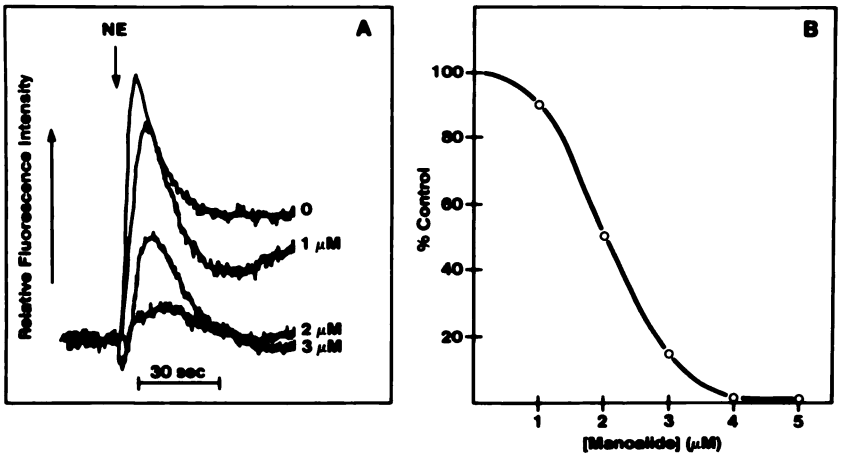


**Fig. 6.** Inhibition of NE increase in cytosolic calcium. DDT$_1$MF-2 cells were labeled with fura-2 as described under Experimental Procedures. Cells were pretreated for 5 min at 37° with either vehicle (dimethyl sulfoxide) or manoalide, then stimulated with 10 μM NE. Changes in cytosolic calcium in response to stimulation with NE in cells pretreated with 0, 1, 2, and 3 μM manoalide are shown in A. Quantitation of the inhibition of NE-induced increase in cytosolic calcium is shown in B. Results are the average of two separate experiments.

poly-L-lysine protected bee venom $PLA_2$ from inactivation by manoalide (6). Thus, serum proteins may protect cellular targets from inactivation by manoalide. In this report we demonstrate that the inhibition of the NE-induced increase in intracellular calcium by manoalide could be blocked by incubating the cells in the presence of BSA. It was also noted that the sensitivity of the cells to manoalide was dependent upon cell density, with cells at a lower density exhibiting greater sensitivity to manoalide. Because of these findings we used cells at the same density to study the effects of manoalide on both inositol phosphate formation and calcium mobilization. Both of these findings were consistent with manoalide forming covalent adducts with lysine residues on proteins. Increasing the concentration of protein in solution, either by adding exogenous proteins or by increasing cell concentration, would effectively reduce the concentration of manoalide which is available for interaction with PI-PLC. The latter effects may explain why previous investigators (39) found that manoalide was more potent in inhibiting calcium mobilization in A-431 cells stimulated with EGF than we report here for NE-induced calcium mobilization in the DDT$_1$MF-2 cells. Alternatively, different isoenzymes of PI-PLC (9, 10, 24, 27) may exhibit differential sensitivity to inactivation by manoalide.

Manoalide appears to be one of the most potent inhibitors of PI-PLC described (40–43). However, manoalide can interact with other cellular enzymes. As previously mentioned, manoalide is known to inhibit venom $PLA_2$s (3–7). The $IC_{50}$ values for venom $PLA_2$s range from 0.05 μM for bee venom $PLA_2$ (5) to 2 μM for *Naja naja* and *Crotalus* venom $PLA_2$s (4, 6). However, since manoalide appears to form a covalent bond with its target enzymes, the $IC_{50}$ value depends upon length of incubation, as well as other incubation conditions. Wheeler *et al.* (39) recently reported that manoalide inhibited $K^+$ depolarization-activated calcium channels and agonist-induced calcium mobilization in a variety of cell types at concentrations of 1 μM or less. It was not clear from these studies whether manoalide interacts directly with the different calcium channels or indirectly prevents activation of the calcium channels. As discussed above, inhibition of a PI-PLC would account for inhibition of agonist-induced mobilization of intracellular calcium. The role played by the inositol phosphates in regulating plasma membrane calcium channels is currently unknown. It was recently reported that $IP_4$ may activate membrane calcium channels in sea urchin eggs (44); thus, inhibition of PI-PLC may also prevent activation of a plasma membrane calcium channel.

Manoalide does appear to exhibit some selectivity in its actions, which may be determined in part by the hydrophobic chain (1). Manoalide did not compete with $^3$H-phorbol 12,13-dibutyrate binding to protein kinase C in guinea pig brain cytosol at concentrations up to 100 μM (data not shown). Manoalide did not effect forskolin (39)- or isoproterenol-stimulated[1] increases in cAMP at concentrations up to 10 μM. Manoalide had no effect on $^3$H-vasopressin binding to $V_1$ receptors in the rat thoracic duct smooth muscle cells, A10.[2] Manoalide had no effect on membrane potential in the $GH_3$ cell line (39), suggesting that it was not a nonspecific membrane perturbant. Further evidence that the hydrophobic chain on manoalide may confer some specificity toward those lysine residues with which it reacts was provided by the finding that treatment of DNase I [an enzyme previously documented to be sensitive to modification of lysine residues (45)] with 100 μM manoalide had no effect on enzymatic activity.[3] These results suggest that manoalide does exhibit some selectivity in its actions and that inhibition of PI-PLC may account, in part, for the observed pharmacological properties of manoalide.

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