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ω-6 Polyunsaturated Fatty Acid-Stimulated Cellular Internalization of Phosphorothioate Oligodeoxynucleotides

EVIDENCE FOR PROTEIN KINASE C-ζ DEPENDENCY

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ABSTRACT. The rate of cellular internalization of phosphorothioate oligodeoxynucleotides is determined predominantly by adsorptive plus fluid-phase endocytosis. Internalization of a 5′-fluoresceinated phosphorothioate 15mer homopolymer of thymidine (FSdT15) in K562 cells in medium containing lipid-depleted albumin was reduced consistently versus nondepleted albumin. Treatment of K562 and several other cell lines with ω-6 polyunsaturated fatty acids (ω-6 PUFAs; e.g. arachidonic and linoleic acids) but not saturated fatty acids dramatically increased FSdT15 internalization in a concentration-dependent manner and over a wide albumin concentration range. The rate of efflux of FSdT15 from K562 cells was not affected by the ω-6 PUFA, implying that an increase of cellular fluorescence was due to an increase in the in-rate. These data were consistent with the observation that the binding of FSdT15 to the cell surface was also increased in the presence of ω-6 PUFAs. ω-6 PUFAs are stimulators of protein kinase C (PKC) activity. Inhibition of PKC activity in K562 cells by Go6976, an inhibitor of the classical PKC isoforms, did not block the linoleic acid-induced stimulation of FSdT15 internalization. On the other hand, treatment of cells with Ro318220, which has considerably less isoform specificity, almost totally blocked the effect of linoleic acid on FSdT15 internalization, implying the involvement of a nonclassical PKC isoform in the process. Finally, since the only PKC isoform expressed in K562 cells that also is activated by ω-PUFAs is PKC-ζ, we obtained NIH 3T3 cells expressing a doxycycline-repressible dominant negative PKC-ζ mutant. Expression of the mutant blocked the stimulation of FSdT15 internalization by linoleic acid. Stimulated internalization also was blocked by wortmannin and LY 294002, which are relatively specific inhibitors of phosphatidylinositol 3-kinase (PI 3-K). Taken together, our data suggest that ω-6 PUFA stimulation of fluoresceinated phosphorothioate oligomers may be PKC-ζ dependent, and perhaps PI-3K dependent as well. BIOCHEM PHARMACOL **58**;3:411–423, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. phosphorothioate oligodeoxynucleotide; internalization; PKC-ζ; polyunsaturated fatty acid

The property of Watson-Crick base pair hybridization has allowed oligodeoxynucleotides to be used as sequence-specific inhibitors of gene expression [1]. They offer the potential not only for a therapeutic modality, but for a rapid, high through-put screening technology for the validation of gene function. At the present time, the most widely used and successful class of oligomers is the phosphorothioates [1–3]. These nuclease-resistant compounds elicit RNase H activity, and have been studied extensively both *in vitro* and *in vivo*. For example, in an elegant series of experiments, Gewirtz and colleagues [4] evaluated a 24mer phosphorothioate oligomer targeted to a complementary region of the c-myb 2–9 codons in K562 human erythroid leukemia cells. Sequence-specific inhibition of gene function was observed [4], but at a relatively high concentration of naked oligomer (10 μM). However, micromolar concentrations of phosphorothioate oligodeoxynucleotides can affect cellular biology in a manner that is not sequence-specific, predominately because of their ability to bind to cell-surface heparin-binding proteins [5].

Due to size and charge considerations, phosphorothioate oligodeoxynucleotides cannot passively diffuse across the cell membrane and must be internalized by active processes. The two mechanisms that probably account for the bulk of oligomer internalization within diverse cell types are adsorptive endocytosis and fluid-phase endocytosis (pinocytosis) [6].

In some cell types, various cell surface proteins can mediate the endocytosis rate via the adsorptive process. One is Mac-1 (CR3, CD11b/CD18; the $\alpha M\beta_2$ integrin); phosphorothioate oligomers bind with high affinity to this protein and are internalized with it [7]. However, the

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expression of this protein is restricted to polymorphonuclear leukocytes, macrophages, and natural killer cells [7]. Another cell surface binding protein (M_r = 110 kDa) has been found in hepatocytes [8]. Oligonucleotides also have been reported to bind to a variety of other cell-surface proteins on K562 cells [9]. Subsequent to adsorption of oligodeoxynucleotide, the plasma membrane then is internalized as vesicles that deliver their contents to early endosomes. Oligodeoxynucleotide present in the fluid phase also is trapped in the forming endosome. However, the cellular determinants of the rate of internalization are very poorly characterized. Furthermore, this trapping sequesters oligodeoxynucleotide in intracellular compartments that are irrelevant for antisense activity. Submicromolar concentrations of phosphorothioate oligodeoxynucleotides can produce antisense effects [3], but usually only in the presence of cationic lipids [10] or other vehicles [11] that appear to permit penetration of the endosomal membrane.

Serum albumin plays an important role in governing the oligonucleotide internalization rate, apparently by reducing oligomer adsorption to the cell surface and thus internalization. This reduction, it has been thought, occurs presumably because of low affinity/high capacity oligonucleotide binding sites on the protein. Other factors in serum can also stimulate oligonucleotide internalization, but they have not been characterized yet.

In previous studies, the rate of internalization of oligodeoxynucleotides in HL60 cells appeared to be PKC* dependent [12]. However, these earlier data were obtained solely by the use of PKC inhibitors (e.g. staurosporine) that now are known not to be highly PKC-specific. At the time, no conclusions could be drawn about isoform specificity.

In this work, initially we sought to further characterize the role of albumin in the internalization process. We demonstrate that the rate of intracellular accumulation of fluoresceinated phosphorothioate oligodeoxynucleotides in K562 and other cell lines is under partial control of the fatty acids that are bound to albumin. Specifically, high concentrations of ω-6 PUFAs, e.g. linoleic and arachidonic acids, which are known to activate various isoforms of PKC [13], strikingly stimulated fluoresceinated oligodeoxynucleotide internalization. Saturated fatty acids, which do not affect PKC activity, were inactive. Additionally, by the use of an NIH 3T3 cell line stably transfected by a dominant negative PKC-ζ mutant, we further suggest that the ω-6 PUFA stimulation of fluoresceinated oligomer internalization may be PKC-ζ dependent. We also present data that suggest that the ω-6 PUFA-stimulated internalization process may be strongly dependent on PI 3-K, whose catalytic product, phosphoinositol-3,4,5-trisphosphate, stimulates PKC-ζ activity [14]. These results provide novel information about some of the complex cellular pathways that regulate the rate of intracellular antisense oligodeoxynucleotide internalization, and point to physiological ways of increasing the rate of this process.

* *Abbreviations*: PKC, protein kinase C; HBSS, Hanks' Balanced Salt Solution; FBS, fetal bovine serum; PUFA, polyunsaturated fatty acid; PI 3-K, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol 3-phosphate; PMA, phorbol-12,13-myristyl acetate; FSdT15, 5′-fluoresceinated phosphorothioate 15mer homopolymer of thymidine; and SdC28, phosphorothioate 28mer homopolymer of cytidine.

MATERIALS AND METHODS

Cells

All cell lines were obtained from the American Type Culture Collection. K562, HL60, and Jurkat cells were grown and maintained in RPMI 1640 medium (Gibco BRL), containing 10% (v/v) heat-inactivated (56°) FBS (Gibco BRL), to which was added 2 mM glutamine, 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acids, 100 U/mL of penicillin G sodium, and 100 μg/mL of streptomycin sulfate. RD and MCF-7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Gibco BRL) + 10% heat-inactivated FBS + 0.1 mM nonessential amino acids. Stock cultures were maintained at 37° in a humidified 5% CO_2 incubator.

NIH 3T3 cells were grown and maintained in DMEM containing 10% (v/v) calf serum (Gibco BRL) and 400 μg/mL of G418 (Sigma), 150 μg/mL of hygromycin, and 2 mM glutamine.

To obtain a PKC-ζ dominant negative mutant, NIH 3T3 cells were cotransfected with pUHD15–1 (which encodes a tetracycline-controlled transactivator, tTA, under the control of PhCMV, the human cytomegalovirus promoter IE) and pSV2neo. Clones resistant to G418 were assayed for transactivation of PhCMV by transient transfection with pUHC13–3, a luciferase reporter. Then one of the positive clones was cotransfected with a plasmid carrying the hygromycin-resistance gene and pUHD-10–3-PKC-Z mut. This construct contains mutated rat brain PKC-ζ and was obtained by inserting the corresponding *Eco*RI fragment from pBluescript-PKC-ζ-mut (rat) into the *Eco*RI site of pUHD-10–3. NIH 3T3-pUHD-10–3-PKC-Z mut cells were maintained in 2 ng/nL of doxycycline (Sigma). Twenty-four hours before initiation of ω-6 PUFA treatment and oligodeoxynucleotide internalization, the doxycycline was withdrawn. These cells were provided as a gift by J. Moscat.

Synthesis of FSdT15

FSdT15, a 5′-fluorescein-labeled 15mer phosphorothioate homopolymer of thymidine, was synthesized in two steps. First, the oligomer was synthesized by standard phosphoramidite chemistry and the TETD sulfurizing reagent, and a 5′ free amino group was added via the Aminolink Reagent (Applied Biosystems), as described previously [12]. 5′-Fluorescein labeling then was accomplished by reaction with fluorescein isothiocyanate. The final compound was purified by dialysis, reverse-phase HPLC, and polyacrylamide gel electrophoresis as described by Stein *et al.* [12]. Concentrations were determined by UV spectroscopy.

Reagents

All ω-6 PUFAs, PMA, sphingomyelinase, and wortmannin were obtained from Sigma. LY294002 was a gift of Eli Lilly. Ro318220 was a gift of R. Narayanan (Hoffmann-La-Roche). Go6796 was obtained from Calbiochem, and CGP 41251 from Dr. I. B. Weinstein. DOTAP [*N*-(2,3-dioleyl-oxy-1-propyl)trimethylammonium methyl sulfate] was obtained from Sigma, and DOPE (dioleyl phosphatidyleth-anolamine) was purchased from Avanti Polar Lipids. Aqueous solutions of the sodium salts of all ω-6 PUFAs and oleic acid were prepared immediately before use, and then added at the appropriate concentration to cells in complete medium.

Flow Cytometric Analysis of the Internalization of Fluorescent Oligodeoxynucleotide

Cells (10^5/mL; 3×10^4/mL adherent cells) in log-phase growth in 96-well microtiter plates were incubated in 200 μL of complete medium at 37° for the stated times with FSdT15 (5 μM) and the appropriate concentration of fatty acid. At the end of the stated time, the medium was removed, and the cells were treated for 5 min with 5 μM SdC28 in PBS + 2.5% BSA (PBS/BSA) at 4° in order to remove cell-surface bound FSdT15 [12]. Subsequently, cells were washed twice with PBS/BSA and resuspended in 300 μL of PBS/BSA containing 0.3 μg/mL of propidium iodide. The mean fluorescence intensity of a population of 5000 cells was determined on a Becton-Dickinson FACscalibur dual-laser flow cytometer using Cell Quest software, as previously described [7]. The background cellular autofluorescence, which was always < 10% of the measured fluorescence, was subtracted. Mean fluorescence channel intensities were expressed as the average of these measurements ± the SEM (N = 3). The mean fluorescence intensity for a population of 10,000 cells was determined and expressed as a mean channel number ± the SEM. Following analysis, monensin (20 μM final concentration) was added to each tube (15–30 min) when appropriate, and the mean fluorescence intensity for the population of 5000 cells was determined.

For cell surface binding experiments, plated cells were treated with fatty acid at 37° for 4 hr, and then washed. Next cells were placed at 4°, treated with 5 μM FSdT15 for 5 min, washed again, and analyzed by flow cytometry as above.

Efflux Experiments

These studies were done by the method of Tonkinson and Stein [15]. Cells were plated as described above, and incubated for at least 16 hr. FSdT15 (5 μM) and linoleic (150 μM) or arachidonic (160 μM) acid were added to the cells, which were incubated at 37° for an additional 3 hr. Then the medium was removed, 5 μM SdC28 was added as above, and the cells were resuspended in prewarmed medium and placed in the incubator for the stated efflux times (5 min to 3 hr). Following efflux, the cells were washed two times in cold PBS/BSA, resuspended in 200 μL of PBS/BSA, and added to 100 μL of PBS/BSA with 0.3 μg/mL of propidium iodide. Mean fluorescence was determined by FACS as described above in the absence and presence of monensin.

Data Analysis

The efflux data obtained in the manner described above were fit to an exponential function by DeltaGraph Professional. Each curve was assumed to represent a biexponential function of the form $C_T = Ae^{-\alpha t} + Be^{-\beta t}$ [15], where A and B represent the proportion of oligomer in each compartment at $t = 0$, and α and β are the rate constants for the loss of oligomer from each compartment. The parameters were determined by multiple iterations so as to minimize the residuals, the only restriction being A, B, $\alpha, \beta > 0$.

Where indicated, data are presented as the means ± SEM of the independent experiments. Statistical significance was determined by one-way ANOVA and Fisher's PLSD test (StatView 4.01; Brain Power, Inc.) A P value of < 0.05 was considered statistically significant between the means.

Synthesis of Triglyceride Rich Particles (TGRP)

Neutral lipids were purchased from Nuchek; phospholipid was obtained from Avanti, and [^{3}H]cholesteryl ester from NEN. Intermediate density lipoprotein size TGRP was generated by mixing neutral lipid (trilinolein plus cholesteryl oleate at 1:1 weight ratio, or trilinolein alone) and phospholipid (egg yolk phosphatidylcholine) at 1:1 weight ratio with the addition of [^{3}H]cholesteryl hexadecyl ether (cholesteryl-1-2-3H), a nondegradable marker (2 μCi/mg neutral lipid). Sonication and ultracentrifugation steps were performed as previously described [16]. Cells were incubated with TGRP in the presence or absence of linoleic acid at 37° for 4 hr. The cells then were washed, and TGRP uptake was determined as previously described [16].

RESULTS

Fatty Acids Bound to Albumin and Oligodeoxynucleotide Internalization

FSdT15 was employed as a model oligomer for two reasons: (a) it most likely exists as a random coil under physiologic conditions, and thus aptameric and specific sequence motif effects are minimized; and (b) its length is sufficient for cell-surface adsorption and antisense activity, but not long enough for maximal non-sequence-specific effects.

The 524-nm fluorescent emission of fluorescein is quenched as a function of diminishing pH. In K562 cells, similar to HL60 cells, 15mer phosphorothioate oligodeoxynucleotides are acidified after internalization. Therefore, before assessment of fluorescence by flow cytometry, cells were treated with a 20 μM concentration of the sodium ionophore monensin, which eliminates the pH gradient between endosome and cytoplasm. The monensin-

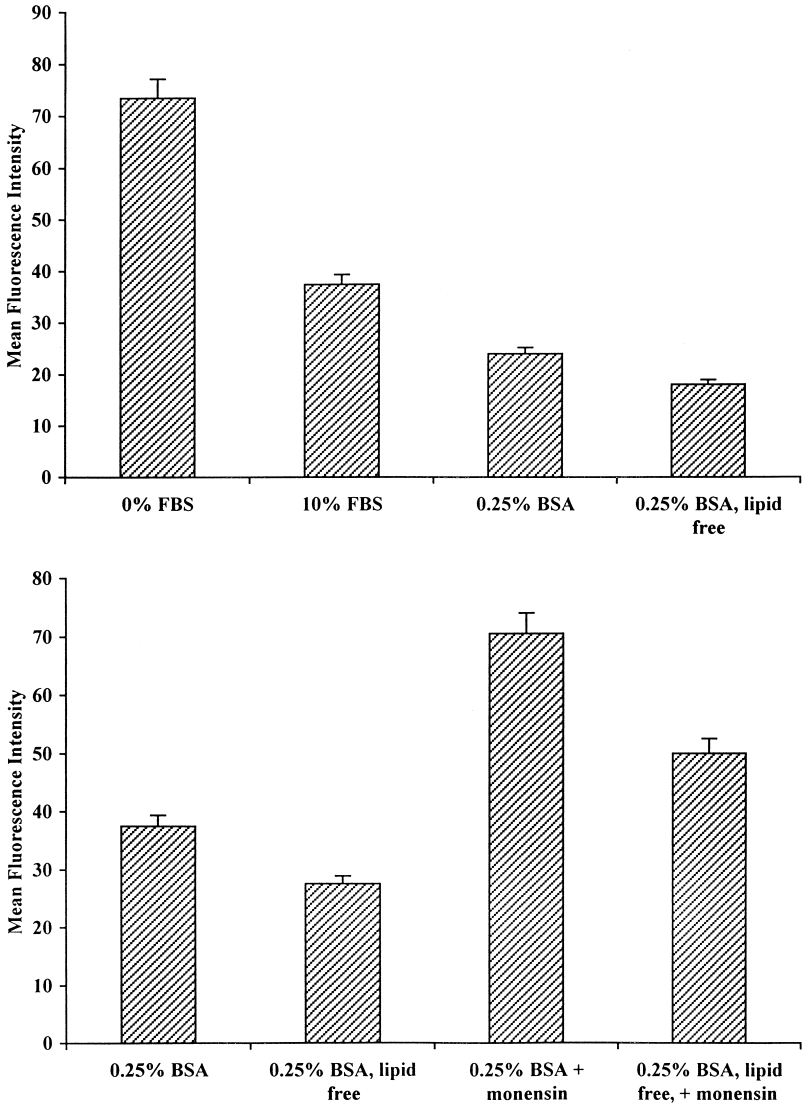

FIG. 1. Effects of serum and serum components on FSdT15 internalization in K562 cells. Top panel: Cells were incubated with oligomer and analyzed by flow cytometry as described in the text. Immediately before the data were collected, cells were treated with 20 μM monensin to disrupt the pH gradient between endosome and cytoplasm, and thus dequench fluorescein fluorescence. Bottom panel: Effects of 20 μM monensin and lipid-free (i.e. methanol-washed) BSA on FSdT15 internalization. Methanol washing of the albumin caused a highly reproducible 30% decrease in oligomer internalization. The results in both top and bottom panels are shown as mean values ± SEM (N = 3).

dequenched fluorescence of FSdT15 (5 μM) after 4 hr of internalization (in complete medium) in K562 cells is depicted in the top panel of Fig. 1. Fluorescence was maximal when internalization occurred in HBSS, and decreased as a function of increasing albumin concentration (not shown). In HBSS + 10% FBS, the mean fluorescence channel was approximately 50% of what was observed in HBSS alone. We then suspended BSA for 12 hr in methanol to remove any fatty acids bound to it. When internalization of FSdT15 in K562 cells in HBSS + 0.25% methanol-stripped BSA was examined (N = 9), an additional 25–30% decrease in internalization was consistently observed versus HBSS + 0.25% unstripped BSA (Fig. 1, bottom). This decrease was observed in both pH-quenched and monensin-dequenched K562 cells, and in other cell types, including HL60 promyelocytic leukemia, RD sarcoma, Jurkat T-cells, and MCF-7 breast cancer cells. No consistent change in internalization was observed with ion-free BSA versus BSA. In addition, when ω-6 fatty acids were added back to the methanol-stripped albumin, internalization increased to baseline and above, depending on the PUFA concentration. These experiments suggest a role (albeit small) of albumin-bound fatty acids in increasing the net oligomer internalization rate.

ω-6 Polyunsaturated Fatty Acids and Cellular Internalization of Oligodeoxynucleotides

The removal of the fatty acids bound to albumin decreased FSdT15 internalization in K562 cells. In sharp contrast, the addition of several ω-6 polyunsaturated fatty acids (Fig. 2, top) stimulated FSdT15 internalization in these cells in complete medium (no methanol stripping). The ω-6 PUFAs (150 μM) included linoleic acid (*cis*, *cis*-octadecadieneoic acid), linolelaidic acid (*trans*, *trans*-octadecadienoic acid), and arachidonic acid. The behavior of the monounsaturated fatty acid oleic acid was similar. Saturated fatty acids (e.g. palmitic and steric acids), as well as the first oxidation products of linoleic acid, 9*S*- and 13*S*-hydroxyoctadecanedienoic acid, did not increase the internaliza-

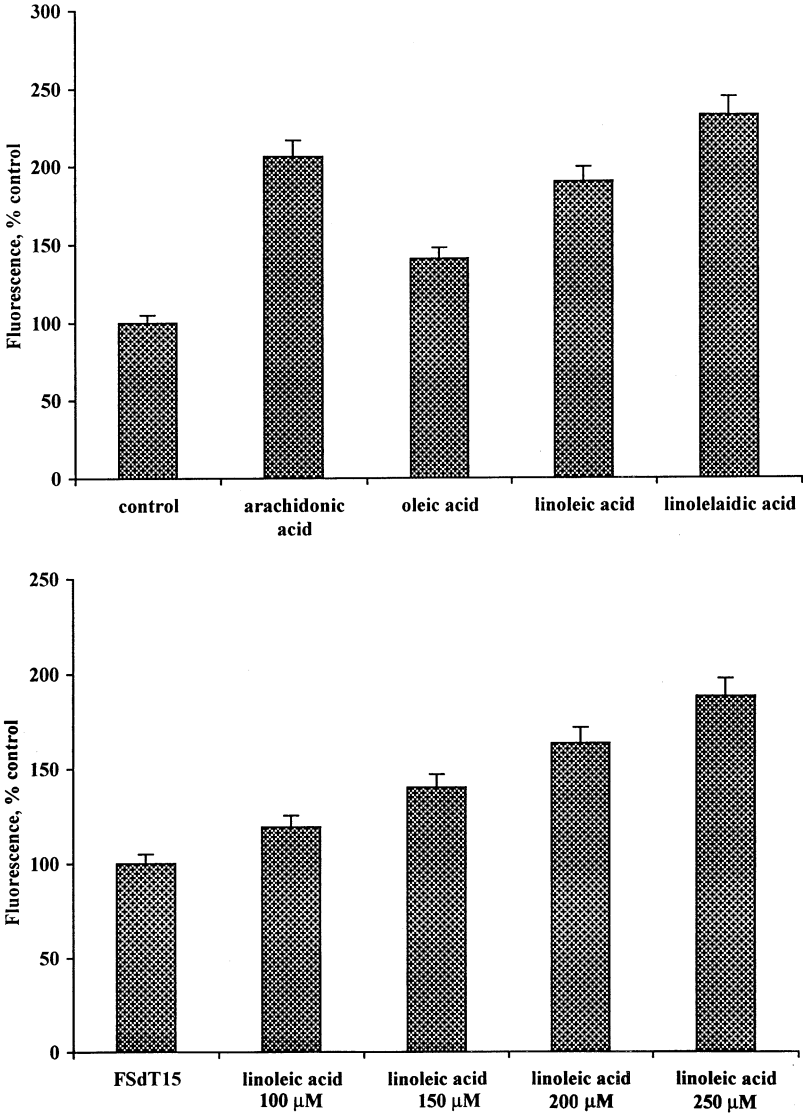

FIG. 2. Effects of ω-6 PUFAs and oleic acid on internalization of FSdT15 in K562 cells. Top panel: K562 cells were co-incubated for 4 hr with 5 μM SdT15 and 150 μM fatty acid. The cells then were washed, stripped with SdC28 to remove cell surface fluorescence, treated with 20 μM monensin, and analyzed by flow cytometry as described in the text. All unsaturated fatty acids (as opposed to palmitic and stearic acids) increased FSdT15 internalization. Control: no fatty acid added. Bottom panel: Concentration-dependence of the linoleic acid-induced stimulation of FSdT15 (5 μM) internalization in K562 cells. Cells were treated as above. At the highest fatty acid concentration, cellular toxicity, as observed by propidium iodide staining, was observed. The results in both top and bottom panels are shown as mean values ± SEM (N = 3).

tion of FSdT15. The stimulation of fluoresceinated oligodeoxynucleotide internalization in K562 cells depended on the ω-6 PUFA concentration (Fig. 2, bottom). Concentrations of ω-6 PUFAs of 150–200 μM were not toxic to the cell lines used in these experiments, as evaluated by propidium iodide staining. At higher concentrations, however, some cytotoxicity was observed.

ω-6 PUFA stimulation of net FSdT15 internalization requires the presence of albumin, as it does not occur in HBSS alone. Stimulation was observed in many cell lines, including HL60, K562, Cos-7, and RD (Fig. 3, top). ω-6 PUFA-stimulated internalization occurred over a wide range (Fig. 3, bottom) of albumin concentrations (0.25 to 4.0%), and a wide fatty acid/albumin ratio (1.8 to 8.0; however, most experiments were performed at a ratio of 4:1, as albumin has approximately 4–5 fatty acid binding sites per molecule). Furthermore, methanol treatment to remove the pre-bound fatty acids from the albumin prior to treatment with ω-6 PUFAs was not necessary for maximal stimulation of internalization.

The time dependence of the linoleic acid-induced augmentation of net FSdT15 internalization in K562 cells is shown in the top panel of Fig. 4. The majority of the rate increase appeared to occur within approximately the first 30 min of internalization. After that, the rates of net internalization appeared to be similar in linoleic acid-treated or untreated cells. The increase in initial rate may be due to the fact that ω-6 PUFAs also increased the binding of FSdT15 at 4° to K562 cells by 5- to 7-fold (Fig. 5). (Because even at 4° all endocytosis does not cease, binding was taken as the difference in fluorescence between treated K562 cells in the absence and presence of 5 μM SdC28, which removes all cell membrane-bound fluorescence [7, 15].)

ω-6 PUFAs and Oligodeoxynucleotide Efflux from K562 Cells

Previously, we demonstrated in HL60 cells that 5′-fluorescein-labeled phosphorothioate oligodeoxynucleotides ef-

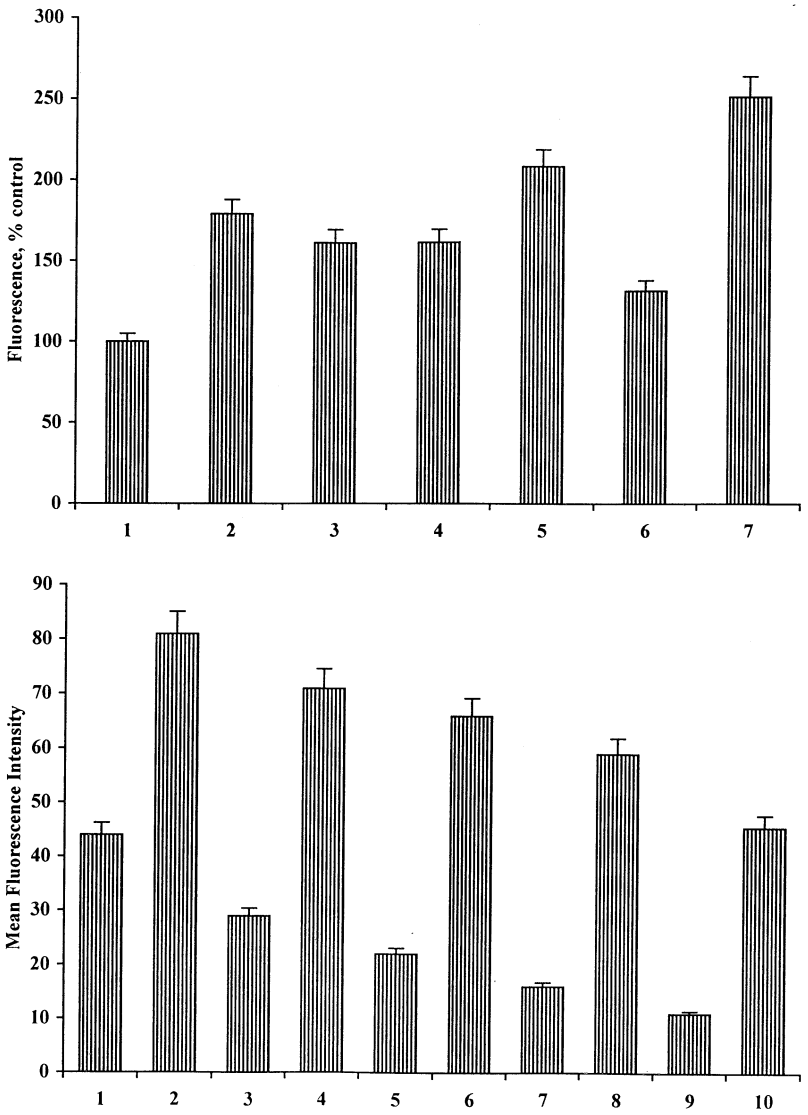

FIG. 3. Top panel: linoleic acid-stimulated internalization of FSdT15 in diverse cell lines. Cells were coincubated for 4 hr with 5 μM FSdT15 and 150 μM linoleic acid. The cells then were washed, stripped with SdC28 to remove cell surface fluorescence, treated with monensin, and analyzed by flow cytometry as described in the text. (1) Control HL60 cells (no added fatty acid); (2) + 160 μM arachidonic acid; (3) + 200 μM oleic acid; (4) + 150 μM linoleic acid; (5) K562 cells + 160 μM arachidonic acid; (6) Cos-7 cells + 150 μM linoleic acid; and (7) RD cells + 150 μM linoleic acid. Bottom panel: linoleic acid-induced stimulation of internalization as a function of albumin concentration range. The fatty acid to albumin ratio was a constant 4:1. K562 cells were incubated in HBSS + BSA for 4 hr with linoleic acid + FSdT15, washed, stripped with SdC28, treated with monensin and analyzed by flow cytometry as described in the text. (1) 0.25% BSA; (2) + 150 μM linoleic acid; (3) 0.5% BSA; (4) + 300 μM linoleic acid; (5) 1% BSA; (6) + 600 μM linoleic acid; (7) 2% BSA; (8) + 1.2 mM linoleic acid; (9) 4% BSA; and (10) + 2.4 mM linoleic acid. The results in both top and bottom panels are shown as mean values ± SEM (N = 3).

flux from two intracellular compartments [15]. One was shallow ($T_{1/2}$ of efflux = 5 min) and the other, deep ($T_{1/2}$ of efflux = 45 min). Approximately 80% of internalized phosphorothioate oligodeoxynucleotides resided in the deep, acidified compartment.

Phosphorothioate oligodeoxynucleotides also underwent efflux from K562 cells. Therefore, the net internalization curve described by the internal mean fluorescence channel of internalized FSdT15 versus time depicted in the top panel of Fig. 4 reflects the sum of the in-rate and the out-rate of the oligomer. To determine if ω-6 PUFA-stimulated net intracellular accumulation is a function of the oligomer in- or out-rate, we studied the rate of FSdT15 efflux from K562 cells in the presence and absence of linoleic (150 μM) or arachidonic (160 μM) acid. The concentration of FSdT15 was 5 μM, and the internalization time was 4 hr. The efflux data are depicted in the bottom panel of Fig. 4. The data points were best fit to a two-compartment model by Delta Graph Professional. In both the presence and absence of linoleic and arachidonic acids, the values of the α and β kinetic rate constants of efflux [15] were equal [$\alpha = 5$; $\beta = 0.06$ hr^{-1} with or without linoleic acid; $\alpha = 4.5$; $\beta = 0.07$ hr^{-1} with or without arachidonic acid ($r^2 > 0.94$ in all cases)]. Furthermore, the values of A (0.16 to 0.20) and B (0.80 to 0.84) were also essentially equivalent. The values of the kinetic efflux parameters determined here for FSdT15 were essentially identical to those previously found in HL60 cells [15] for many phosphorothioate oligodeoxynucleotides. These data further suggest that the ω-6 PUFAs affect intracellular internalization by preferentially affecting the oligodeoxynucleotide in-rate rather than the out-rate.

However, ω-6 PUFAs were not general up-regulators of either receptor-mediated or fluid-phase endocytosis. The rates of cellular internalization of rhodamine or fluorescein-labeled transferrin or [^{3}H]sucrose were not altered in K562 cells by treatment with linoleic acid (150 μM). However, the uptake of [^{3}H]trilinolein (200 μg/mL), which is internalized predominately by adsorptive endocytosis, also is increased dramatically in K562 cells after linoleic acid treatment.

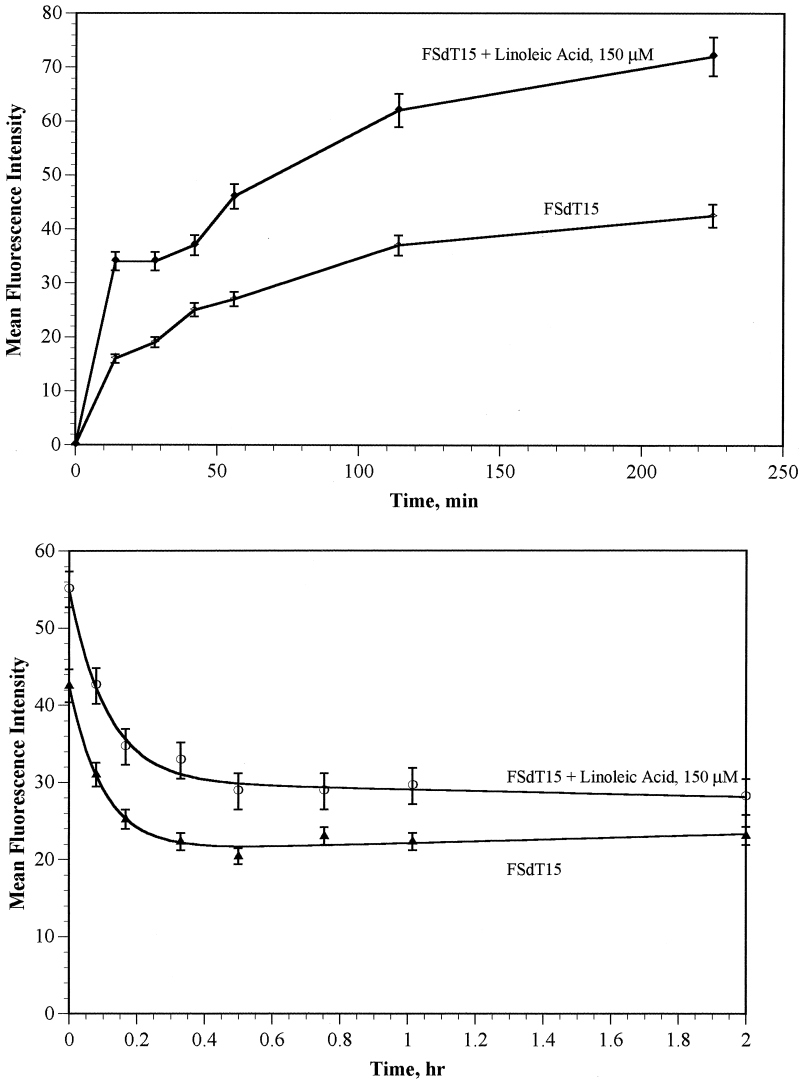

FIG. 4. Top panel: Time dependence of internalization of 5 μM FSdT15 in K562 cells in the presence or absence of 150 μM linoleic acid. Cells were treated with both oligomer and fatty acid for the indicated times, washed, SdC28 stripped, and analyzed by flow cytometry after monensin dequenching as described in the text. Bottom panel: Time dependence of the efflux of 5 μM FSdT15 in K562 cells. Efflux rates were determined via flow cytometry by the method of Tonkinson and Stein [15] in the presence (circles) or absence (triangles) of 150 μM linoleic acid as described in the text. The efflux data were fit to the biexponential function $C_T = Ae^{-\alpha t} + Be^{-\beta t}$ by DeltaGraph Professional. The results in both top and bottom panels are shown as mean values ± SEM (N = 3).

In summary, ω-6 PUFAs appear to affect the early in-rate of oligomer internalization only, possibly by increasing cell membrane adsorption. However, because they do not appear to affect the rate of internalization of other markers, it is also possible that these fatty acids are modulating vesicle sorting processes.

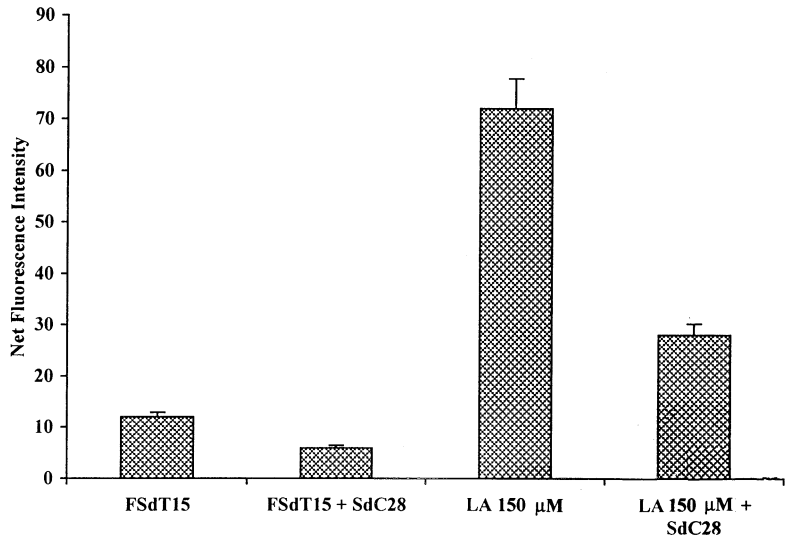

FIG. 5. Effects of linoleic acid on the cell surface binding of FSdT15. K562 cells were treated with 150 μM linoleic acid (LA) for 4 hr, washed, and then treated at 4° with 5 μM FSdT15 for 5 min. The extent of total cellular bound fluorescence was determined via flow cytometry, as described in the text. Cell surface bound fluorescence is taken as the difference between total cellular bound fluorescence in the absence and presence of 5 μM SdC28. The results are shown as mean values ± SEM (N = 3).

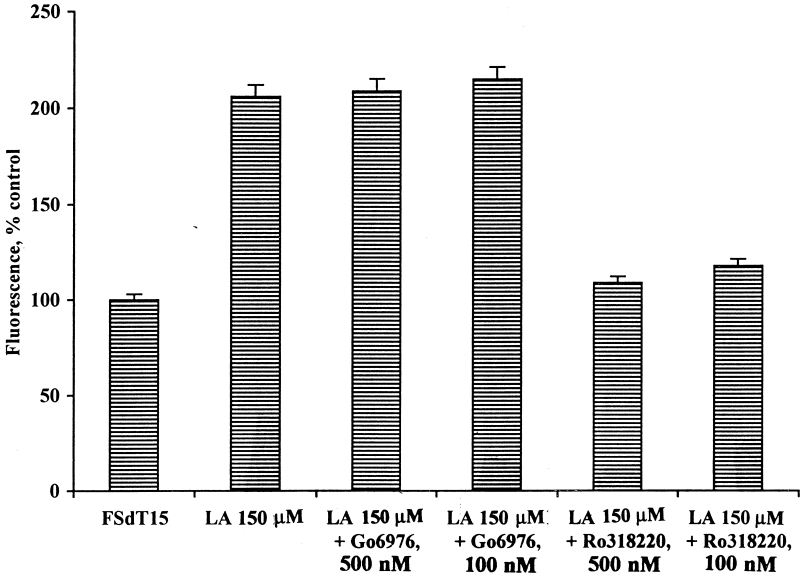

FIG. 6. Effect of inhibitors of PKC activity on the linoleic acid-stimulated internalization of FSdT15 in K562 cells. Cells were co-incubated with 150 μM linoleic acid (LA) and the stated PKC inhibitor for 4 hr. The cells were then washed, stripped with SdC28, dequenched with monensin, and analyzed by flow cytometry as described in the text. The inhibitor Go6976 appears to be highly selective for the classical PKC isoforms. Ro318220 does not have a similar degree of isoform specificity. The results are shown as mean values ± SEM (N = 3).

ω-6 PUFA-Stimulated Oligodeoxynucleotide Internalization and PKC

All of the ω-6 PUFAs employed in this study, but not the saturated fatty acids, are known activators of the kinase activity of several PKC isoforms [13]. These include all of the calcium-dependent classical isoforms (α, βI, βII, γ) as well as the nonclassical isoforms δ and ζ. Given our previous data [12] showing that PKC inhibitors were also inhibitors of baseline 5′-fluorescein-labeled oligodeoxynucleotide internalization, we tested several PKC inhibitors for their ability to block ω-PUFA augmented internalization. Go6976 is a staurosporine analog that selectively inhibits the classical PKC isoforms [17]. However, this compound was completely ineffective at inhibiting the effects of either arachidonic or linoleic acid (150 μM) on oligomer internalization in K562 cells (4-hr incubation) (Fig. 6). In fact, high concentrations of Go6976 killed the cells, as measured by propidium iodide uptake, without affecting stimulated oligomer internalization. Similar results were obtained with the staurosporine analog CGP 41251. However, the PKC inhibitor Ro318220 [18], which is far less selective than Go6976, effectively blocked ω-6 PUFA-augmented internalization with an $IC_{50} < 100$ nM. These experiments suggested that the ω-6 PUFAs were interacting with a nonclassical PKC isoform. However, it has been demonstrated previously that in proliferating K562 cells, only PKC-ζ, and not PKC-δ, is expressed [19]. These data, when taken together, suggest that the interaction of ω-6 PUFAs with PKC-ζ induces augmentation of FSdT15 internalization, at least in K562 cells. Interestingly, Ro318220, but not Go6976, also blocked the ability of the cationic lipid DOTAP (7 μM) and of an equimolar DOTAP/DOPE combination (7 μM each) to stimulate FSdT15 (5 μM; 3 hr) internalization in K562 cells. In this case, however, the block was only partial (50%), and was maximal at a Ro318220 concentration of 0.5 μM. However, as evaluated by confocal microscopy (not shown), the DOTAP/DOPE combination promoted endosomal egress and nuclear accumulation of FSdT15, a property not shared by ω-6 PUFAs. Thus, these data suggested that the cationic lipid-induced stimulation of phosphorothioate oligodeoxynucleotides is, perhaps at least in part, related to PKC-ζ activity.

Oligodeoxynucleotide Internalization and Ceramide, PMA, and Inhibitors of PI 3-K

When PKC is activated, translocation of the enzyme to the cell membrane classically occurs. However, we could not consistently demonstrate linoleic acid-induced translocation of PKC-ζ to the particulate fraction after fractionation experiments (not shown).

If PKC-ζ is involved in the process of oligodeoxynucleotide internalization, then other processes that activate the enzyme should increase internalization. Ceramide is an activator of PKC-ζ activity [20], and we examined its ability to increase FSdT15 internalization in K562 cells. Treatment of cells with bacterial sphingomyelinase is a commonly recognized method of generating intracellular ceramide. Thus, we treated K562 cells in complete medium with bacterial sphingomyelinase (10–50 mU/mL, 4 hr). In multiple experiments, a small but consistent, statistically significant increase in FSdT15 internalization of 35.8 ± 10.7% (N = 9; $P = < 0.0001$ by Fischer's test) was observed after sphingomyelinase treatment versus control (i.e. no sphingomyelinase; N = 6; mean channel fluorescence = 99.7 ± 1.9%). These data are consistent with the idea that activators of PKC-ζ (e.g. ω-6 PUFAs and ceramide) increase oligodeoxynucleotide internalization.

Previous data have demonstrated that PKC-ζ also is stimulated by PIP3 [14]. PIP3 is produced by PI 3-K, an enzyme that is inhibited by the fungal product wortmannin (EC_{50} = 2–4 nM). While wortmannin was thought to be specific for PI 3-K [21], more recent evidence indicates that this is not the case [22]. However, the linoleic acid-induced

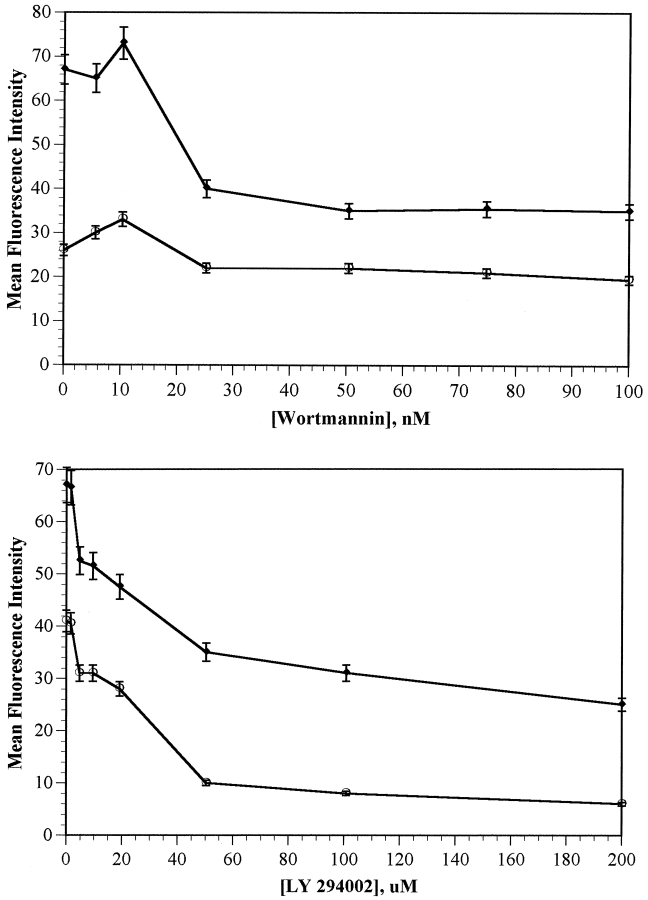

FIG. 7. Top panel: The effects of wortmannin on the linoleic acid-induced stimulation of 5 μM FSdT15 internalization in K562 cells. Cells were co-incubated with (triangles) or without (circles) 150 μM linoleic acid and the indicated concentration of wortmannin for 4 hr. The cells were then washed, stripped with SdC28, dequenched with monensin, and analyzed by flow cytometry as described in the text. Bottom panel: The effects of the PI 3-K inhibitor LY294002 on internalization of FSdT15 in the presence or absence of 150 μM linoleic acid. The experiments were performed as above. The results in both top and bottom panels are shown as mean values ± SEM (N = 3).

augmentation of FSdT15 internalization in K562 cells was inhibited by wortmannin with an IC_{50} value of approximately 25 nM (Fig. 7, top), a concentration that suggests that PI 3-K inhibition is responsible for the inhibition. (Interestingly, FSdT15 internalization was stimulated reproducibly by 15% by 10 nM wortmannin.) Maximal inhibition was observed at 50 nM. Moreover, the only other enzyme known to be inhibited by wortmannin at low nanomolar concentration is phospholipase A_2 [22]. However, neither treatment of K562 cells with exogenous phospholipase A_2 (18–200 U/mL; 6 hr) nor treatment with its inhibitor melittin (500–5000 ng/mL; 6 hr) affected FSdT15 internalization. In addition, the quercetin analog LY294002 [2-(4-morpholinyl-8-phenylchromone)], which is believed to be a specific inhibitor of PI 3-K (EC_{50} = 1.4 μM) [23], also inhibited linoleic acid-stimulated FSdT15 internalization. The IC_{50} in unstimulated and stimulated cells was 14 μM. Maximal inhibition in stimulated cells was at 50–100 μM, and LY294002 inhibited virtually all net oligomer internalization at maximal inhibition, unlike wortmannin, which inhibited only about 50% (Fig. 7, bottom). These data strongly suggest that PI-3 kinase-independent pathways also can modulate oligonucleotide internalization. However, since LY294002 inhibited baseline (i.e. non-linoleic acid-induced) FSdT15 internalization, whereas wortmannin did not, this may be a non-specific effect of the LY294002 unrelated to PI 3-K inhibition.

We then examined the ability of PMA to modulate ω-6 PUFA-induced internalization. PMA binds to PKC-ζ [24], but in contrast to ceramide it does not affect enzyme activity. Consistent with our previous data, no change in linoleic acid-stimulated internalization was observed after treatment of K562 cells for various times (4, 6, 12, 24 hr) with PMA over a wide concentration range (10–50 ng/mL). In summary, when taken together, all the various lines of evidence accumulated using both stimulators and inhibitors of PKC-ζ activity point in precisely the same direction: PKC-ζ apparently modulates the ω-PUFA-induced stimulation of FSdT15 internalization in several cell lines. However, additional non-inhibitor data are necessary to further demonstrate the point.

A Dominant-Negative PKC-ζ Mutant and Oligodeoxynucleotide Internalization

To further demonstrate dependence of stimulated internalization on PKC-ζ activity, we used a dominant negative mutant strategy.

Control, wild-type NIH 3T3 cells were allowed to internalize oligomer in the presence of ω-6 PUFA. Treatment of these cells with linoleic acid (4 hr; 150 μM) caused a 2-fold increase in internalization of FSdT15 (5 μM; not shown). Furthermore, the stimulated internalization was inhibited by PKC inhibitors in the same manner as previously demonstrated in the human cells. The NIH 3T3 cells then were stably transfected with a kinase-defective rat brain PKC-ζ mutant produced by mutagenesis of lysine-281 into tryptophan [25]. This lysine is located in the catalytic domain of the enzyme and diminishes its ability to bind ATP [26]. Thus, the enzyme is inactivated, but, as has been observed for other kinases [27], a dominant negative phenotype is produced.

The rat brain PKC-ζ is highly homologous (98% at the amino acid level) to human PKC-ζ. The plasmid vector pUHD10–3 is repressible by doxycycline (2 ng/mL), and produces a many-fold higher level of expression of the cloned gene versus the wild type in eukaryotic cells [25, *].

Similar to the human cells, when NIH 3T3 cells transfected by the PKC-ζ dominant negative mutant were treated with linoleic acid (4 hr, 150 μM) in the presence of doxycycline in the culture medium, stimulation of FSdT15

* Moscat J and Diaz-Meco M, personal communication. Cited with permission.

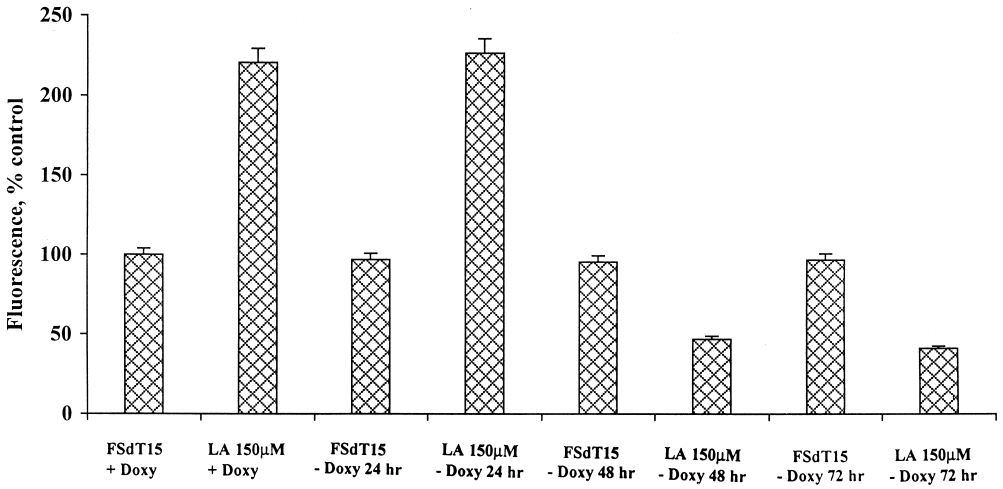

FIG. 8. Effects of the expression of a dominant negative PKC-ζ mutant on the linoleic acid-induced stimulation of FSdT15 internalization in NIH 3T3 cells. Cells were transfected with a plasmid containing mutant rat brain PKC-ζ, and maintained in 2 ng/mL of doxycycline. Twenty-four hours before initiation of the experiment, the doxycycline was withdrawn. The mutant cells were then treated for 4 hr with 150 μM linoleic acid (LA) and 5 μM SdT15 at 24, 48, and 72 hr after withdrawal of the doxycycline. Next cells were washed, stripped with SdC28, dequenched with monensin, and analyzed by flow cytometry as described in the text. The results are shown as mean values ± SEM (N = 3).

internalization was observed (Fig. 8). In contrast, in the absence of doxycycline in the culture medium, which derepresses expression of the dominant negative phenotype, the stimulatory effect of linoleic acid (150 μM) and arachidonic acid (160 μM) on FSdT15 internalization was abolished virtually completely at 48 and 72 hr after removal of the doxycycline.

Thus, in combination with the data obtained from inhibitor studies, the above data are also consistent with the hypothesis that the ω-6 PUFA stimulation of FSdT15 internalization may be dependent on the activity of PKC-ζ. The data also suggest that this process may be dependent on the activity of PI 3-K as well.

DISCUSSION

One of the major obstacles to the facile use of the antisense oligodeoxynucleotide technology is the problem of cellular internalization. The rate of cellular uptake appears to be modulated by PKC activity. PKC is actually a family of serine/threonine kinases that have been linked to the regulation of many cellular functions, including proliferation and differentiation. The family can be divided into three general classes. The classical forms include α, βI, βII, and γ, and are stimulated by ω-6 PUFAs, phosphatidylserine, diacylglycerol, PMA, and calcium. The novel (n) isoforms (δ, ϵ, η, and θ) are calcium- and phosphatidylserine-independent, but can be stimulated by PMA. The atypical isoforms are ζ, λ, μ, and τ. PKC-ζ is stimulated by ω-6 PUFAs [28], phosphatidylinositol-3,4,5-trisphosphate [14], and ceramide [20, 29]. It is not stimulated by PMA [28] and is not down-regulated after prolonged exposure to PMA [30].

Previously, though internalization of fluoresceinated oligodeoxynucleotides in HL60 cells appeared to be PKC dependent, the inhibitors employed (e.g. staurosporine, H4) were not highly specific. Isoform specificity was not examined. To the best of our knowledge, no direct relationship between PKC-ζ and endocytosis has been demonstrated. However, a relationship was implied in U937 cells overexpressing PKC-ζ, where lysosomes, which also concentrate phosphorothioate oligodeoxynucleotides, were found to be discretely concentrated in the perinuclear area, instead of being dispersed in the cytoplasm [31]. Other data also imply a relationship between PKC-ζ and endocytosis. Gomez *et al.* [32] have provided evidence that after activation by interleukin-2 in a murine T-cell line, PKC-ζ, unlike the classical isoforms (e.g. PKC-β), translocates to the actin cytoskeleton, as opposed to the plasma membrane. These data may explain why we were unable to detect translocation of PKC-ζ to the plasma membrane of hematopoietic cells that were activated by ω-6 PUFAs. In addition, inhibition of PKC-ζ expression (and thus translocation to the actin cytoskeleton) by antisense oligodeoxynucleotides caused collapse of the actin architecture. These data thus suggest that PKC-ζ has a role in maintaining the organization of the cytoplasm and membrane. It is thus interesting to note the fact that treatment of mouse fibroblasts with cytochalasin B (50 μg/mL), an inhibitor of actin polymerization, reduced oligonucleotide internalization by 75% [6].

ω-6 PUFAs, and possibly cationic lipids as well, stimulate the intracellular accumulation of FSdT15 in what appears to be a PKC-ζ-dependent fashion. Our data supporting this assertion are (a) ω-6 PUFAs are known activators of PKC-ζ; (b) Go6976, an inhibitor of only classical PKC isoforms, did not affect ω-PUFA-stimulated internalization in K562 cells, whereas Ro318220, an inhibitor with broader

activity, was dramatically inhibitory; (c) treatment of K562 cells with bacterial (*Bacillus cereus*) sphingomyelinase caused a relatively small (35%) but statistically significant increase in phosphorothioate oligodeoxynucleotide internalization, possibly due to generation of intracellular ceramide, an activator of PKC-ζ. However, PMA, which does not affect PKC-ζ activity, did not affect internalization; and (d) a dominant negative PKC-ζ mutant blocked the ω-6 PUFA stimulation of internalization in NIH 3T3 cells.

A possibility exists that all of these results are due to mutually independent mechanisms (though this interpretation violates Ockham's razor). Regardless, since multiple lines of evidence with stimulators and inhibitors all point in the same direction, and especially given the data in (d) above, multiple mutually independent mechanisms seem unlikely.

The data obtained using the inhibitors of PI 3-K (which have been employed in literally dozens of recent studies) suggest that stimulation is dependent on this enzyme as well. This is consistent with the hypothesis that PKC-ζ and PI 3-K are intimately associated. Previous data supporting this idea are: insulin caused a parallel translocation of PI 3-K and PKC-ζ to the membrane of rat-1 cells stably overexpressing the human insulin receptor [33]; PI 3-K associates with PKC-ζ via its p110 subunit after interleukin-2 stimulation in a mouse T cell line. This appears to stimulate phosphoserine PI 3-K kinase activity [34]; and purified bovine kidney PKC-ζ can be activated by PIP3, which is almost as activating for this enzyme as phosphatidylserine [14].

PI 3-kinase activity has been implicated in vesicular trafficking in a number of studies [35, 36]. Fluid phase (horseradish peroxidase) and receptor-mediated (transferrin) endocytosis have been shown to be inhibited but not abolished by treatment of cells with wortmannin [36]. In another study [37], wortmannin slowed the entry of transferrin receptors into the recycling compartment and the efflux of receptors out of this compartment. This resulted in a net decrease of internalized transferrin. Half-maximal depletion was observed at 30 nM, which was the concentration of wortmannin necessary to achieve half-maximal inhibition of PI 3-K activity in intact CHO cells. Maximal inhibition was seen at 100 nM. These effects appear to occur at the level of endosome fusion and Rab 5 function, both of which are virtually abolished by wortmannin [36]. Trafficking of endocytosed platelet-derived growth factor receptor to the lysosomal pathway also was diminished markedly by wortmannin [38], and was clearly dependent on receptor binding to a site on PI 3-kinase [39]. These previous results on inhibition of PI 3-K activity are opposite to those observed in our study when cells were stimulated with ω-6 PUFAs, and similar to those observed when cells were treated with wortmannin. As described above, phosphorothioate oligodeoxynucleotides bind to cell surface receptors such as Mac-1, which then is internalized along with the oligomer. These compounds can also bind to other cell surface receptors, such as epidermal growth factor receptor, and flk-1, a receptor for vascular endothelial growth factor [40]. As with platelet-derived growth factor receptor, it is possible, indeed likely, that internalization and trafficking of such receptors are under the partial control of PI 3-K.

Therefore, a scenario that partially explains our data is that ω-6 PUFA, and possibly DOTAP/DOPE, activates PKC-ζ, which then activates PI 3-K. PKC-ζ can then be activated further by PIP3, a catalytic product of PI 3-K. This mechanism is similar to that proposed by Liu [41]. PKC-ζ itself may affect actin filaments, and both it and PI 3-K may affect receptor and vesicular trafficking, which together modulate the adsorptive endocytosis rate of oligodeoxynucleotides.

However, important qualifications to our interpretations must be considered. First, PKC-τ is highly homologous to PKC-ζ in the catalytic and C-terminal domains [29, 42]. Thus, it is possible that at least some experiments that ascribe biological effects to PKC-ζ also are observing modulation of PKC-τ activity as well. In addition, wortmannin is not specific for PI 3-K, but affects PI 4-K as well [37]. This may explain the $> 75\%$ inhibition of internalization by wortmannin, but the only 50% maximal inhibition by LY294002.

The concentrations of ω-6 PUFAs and the albumin/fatty acid ratios used in our *in vitro* experiments are similar to what can be achieved in humans infused with intravenous triglyceride emulsion [43]. Unfortunately, unlike cationic lipids, the ω-6 PUFAs do not by themselves appear to improve antisense efficacy. This is because while net internalization is increased by ω-6 PUFAs, transit out of the endosome, as assessed by confocal microscopy, is probably not increased. In the absence of this critical process, the vesicular system can still be a sequestering dead end for antisense oligodeoxynucleotides. However, in combination with novel delivery vehicles that permit endosomal penetration, use of ω-6 PUFAs may ultimately dramatically increase nuclear delivery of antisense oligodeoxynucleotides, and hence their efficacy.

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