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EFFECTS OF LONG-CHAIN FATTY AMINES ON THE GROWTH OF *RAS*-TRANSFORMED NIH 3T3 CELLS

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Abstract—A number of aliphatic primary amines were tested for their effects on the growth of *ras*-transformed NIH 3T3 cells (PAP2 cells), as measured by incorporation of tritiated thymidine into DNA. Long-chain, saturated amines (C_{12} to C_{18}) were growth inhibitory, whereas short-chain amines (C_6, C_8) were not. Farnesylamine, a branched-chain, unsaturated amine (C_{15}), had an IC_{50} of 6.9 μM compared to IC_{50} values of 13.1 to 45.8 μM for straight-chain, saturated amines. Oleylamine, with an IC_{50} of 0.1 μM, was the most potent inhibitor. The long-chain amines, but not the short-chain amines, were also effective inhibitors of protein kinase C, assayed *in vitro* in a cell-free system. In addition, studies with indo-1-loaded PAP2 cells showed that long-chain amines induced a reversible rise in intracellular free Ca^{2+} concentration. Growth inhibition by the amines was positively correlated with this effect, suggesting that factors other than protein kinase C may be involved in the inhibition of growth of PAP2 cells by long-chain amines.

Key words: fatty amines; protein kinase C; *ras*-transformed cells; growth inhibition; Ca^{2+} mobilization

More than 30 years ago, one of us observed the toxic effects of sphingosine and other long-chain amines on rats [1]. In recent years, the cytotoxic effects of sphingolipids and their breakdown products on cells have been reported from several laboratories [2–4]. Various pharmacological responses, such as inhibition of platelet and neutrophil activation [5–7], modulation of receptor function [8], and inhibition of responses induced by phorbol esters [9–11], were also observed when sphingosine and other derived products were added to cells. Recent studies in our laboratory have shown that a diet containing 0.1% octadecylamine sulphate inhibits body weight gain as well as the yield of mammary tumors induced in rats by DMBA‖ [12].

It is not yet clear how sphingolipids and their derived products inhibit growth of cells, but it is thought that one of the mechanisms is through inhibition of PKC [13–16], an enzyme that has been implicated in cell replication, tumor promotion, oncogenesis and signal transduction [17]. A long hydrophobic chain and a free amino group are considered to be important structural features for this particular class of inhibitors of PKC [18]. Many other compounds that are structurally different from sphingosine have also been shown to be potent PKC inhibitors [19].

Derivatives of sphingosine have been observed to mediate a rapid and profound translocation of Ca^{2+} from intracellular stores of a smooth muscle cell line [20]. Sphingosine has also been found to inhibit the Ca^{2+}/calmodulin-dependent protein kinase of GH_2 pituitary cells [21], and the Na^+, K^+-ATPase of purified rat brain synaptosomes [22]. It seems possible that such effects may play a role in growth inhibition by long-chain amines. The use of sphingosine and its metabolites as pharmacological agents for controlling cell growth is limited by the fact that they are converted rapidly into inactive compounds *in vivo* [19]. We are therefore exploring the possible use of other long-chain fatty amines for this purpose. Like sphingosine, they have a long hydrophobic chain and a free amine group, but they may be metabolized in different ways and at different rates.

In this paper, we report the effects of various long-chain amines on the growth of *ras*-transformed cells in culture, as well as their ability to inhibit PKC *in vitro* and to affect the level of intracellular free Ca^{2+} in *ras*-transformed cells.

MATERIALS AND METHODS

Materials. [^{3}H]Thymidine (6.7 Ci/mmol) was obtained from ICN, Irvine, CA. Tissue culture medium and calf serum were purchased from GIBCO, Burlington, Ontario. Most of the amines were purchased from the Aldrich Chemical Co. Inc., Milwaukee, WI. Their purity is listed as follows: hexylamine (C_6) 99%, octylamine (C_8) 99%, dodecylamine (C_{12}) 99%, tridecylamine (C_{13}) 98%, tetradecylamine (C_{14}) 96%, hexadecylamine (C_{16})

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‖ Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene; DMF, dimethylformamide; DTT, dithiothreitol; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; and PKC, protein kinase C.

90%, octadecylamine (C_{18}) 99% and oleylamine ($C_{18:1}$) 80% (98% primary amine). Oleylamine of 97% purity was later obtained from Fluka Chemie AG, Buchs, Switzerland, and inhibited proliferation of PAP2 cells much like the less pure form obtained from Aldrich. Farnesylamine was synthesized as described previously, and its structure was confirmed by GC-MS and NMR spectroscopy [23]. [γ-^{32}P]ATP was obtained from Amersham, Oakville, Ontario. Phosphatidylserine and diolein were obtained from Serdary Research Laboratories Inc., London, Ontario. Rat brains were used as a source of PKC. DEAE cellulose and Phospho cellulose P II paper were from the Whatman Co., Clifton, NJ. Indo-1 acetoxymethyl ester was obtained from Molecular Probe, Portland, OR. Phenyl sepharose and all other chemicals were purchased from the Sigma Chemical Co., St. Louis, MO.

Cell culture. PAP2 cells (malignant T24 H-*ras* transformed NIH 3T3 cells) [24, 25] were maintained at 37° in Dulbecco's modified Eagle's medium containing 3.27 g $NaHCO_3$/L supplemented with 10% bovine serum. The medium was equilibrated with a humidified atmosphere of 5% CO_2. Stock cultures were seeded at a density of 2×10^5 cells/mL and allowed to multiply for 48–72 hr.

Incorporation of [^{3}H]*thymidine into DNA.* PAP2 cells were plated 2×10^4 cells/well in flat-bottomed culture plates in a total volume of 200 μL of medium and incubated at 37° for 24 hr with or without various test compounds. [^{3}H]Thymidine (0.5 μCi/well) was added and after 4 hr the cells were harvested onto a glass fibre filter paper using a semiautomatic 12-well harvester (Skatron Inc., Sterling, VA). Radioactivity on the filter paper was counted in a liquid scintillation counter.

Viability of cells in the presence of fatty amines. Viability of cells was measured by the MTT assay [26]. In this assay, a tetrazolium salt, MTT, is converted to a blue formazan product by dehydrogenases that are active in living cells. The intensity of the blue colour developed is a measure of cell viability. PAP2 cells (8×10^4/well) were seeded with various concentrations of the amine in a 96-well plate in a total volume of 200 μL of medium. MTT (25 μL of 5 mg/mL) was added to each well. After 3 hr, 100 μL of extraction buffer consisting of 20% SDS dissolved in a 50% DMF, 50% water solution at pH 4.0, was added. The blue colour formed was measured at 590 nm.

Purification of PKC. Brains of five rats were homogenized in a buffer containing 20 mM Tris–HCl, pH 7.6, 10 mM DTT, 50 μg/mL leupeptine and 1 mM $CaCl_2$. The homogenate was centrifuged at 40,000 *g* for 15 min, and the pellet was resuspended in 20 mM Tris–HCl buffer containing 10 mM DTT, 50 μg/mL leupeptine, 5 mM EDTA and 2 mM EGTA, stirred gently for 1 hr at 4°, and centrifuged at 140,000 *g* for 1 hr. The supernatant was applied on a DEAE cellulose column, which was equilibrated with a 20 mM Tris–HCl buffer, pH 7.6, containing 1 mM EDTA and 1 mM DTT. The column was then eluted with 0 to 0.3 M NaCl (NaCl dissolved in the same buffer). Active fractions were pooled and an equal volume of 4.5 M NaCl was added and applied through a phenyl Sepharose column. After washing with 1.5, 1.0 and 0.5 M NaCl, the enzyme was eluted with 20 mM Tris–HCl, pH 7.6, containing 1 mM EDTA and 1 mM DTT. Active enzyme fractions were pooled and stored at −80° in ethylene glycol and 1% polyethylene glycol (PEG) until further use.

PKC assay. Stock solutions of oleic acid and all amines were first prepared by dissolving them in dimethyl sulfoxide. The required concentrations of the compounds were obtained by mixing the appropriate stock solution with water and thoroughly sonicating followed by vigorous vortexing.

PKC was assayed as described by Ogita *et al.* [27] with slight modifications. Incorporation of ^{32}P into histone (Type IIIS) was measured. The standard reaction mixture (50 μL) contained 20 mM Tris–HCl, pH 7.5, 0.5 mM $CaCl_2$, 10 μM [γ-^{32}P]ATP, (sp. act. 200 cpm/pmol), Histone Type III (0.2 mg/mL), phosphatidylserine (16 μg/mL) and diolein (1.6 μg/mL). In some assays, oleic acid was added instead of phospholipid and diolein. Various fatty amines were added to the assay mixtures as described in Results. The reaction was started by adding PKC. After a 3-min incubation at 30°, the reaction was terminated by spotting the reaction mixture on P II paper. The paper was washed three times with 75 mM phosphoric acid and finally with ethanol. After drying, the radioactivity remaining on the paper was counted.

Measurement of $[Ca^{2+}]_i$. PAP2 cells grown to a density of 1.5×10^4 cells/cm^2 were detached following treatment with 0.05% trypsin in citrate saline. The detached cells were suspended in Dulbecco's modified Eagle's medium at 4×10^6 cells/mL and loaded with indo-1 acetoxymethyl ester (2 μM) for 30 min at 37°. After loading, cells were washed with HEPES-buffered minimum essential medium and resuspended at approximately 0.5×10^6 cells/mL in a solution containing 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, and 20 mM sodium-HEPES, adjusted to pH 7.3, and 290 mosmol/L. Aliquots (2 mL) of this cell suspension were used for $[Ca^{2+}]_i$ measurement in a fluorometric cuvette.

Relative fluorescence intensity was monitored using a Hitachi F-4010 fluorescence spectrophotometer at 331 nm excitation and 398 nm emission, with slit width settings of 3 nm (excitation) and 10 nm (emission) [28]. The cell suspension was maintained at 37° and stirred continuously during the measurement. At the end of each experiment, digitonin was added to give a final concentration of 50 μM to permeabilize cells and saturate indo-1 with Ca^{2+}, giving maximum fluorescence (F_{max}), while the autofluorescence (F_{auto}) value was measured in the presence of 1 mM $MnCl_2$. Intracellular free calcium concentration, corresponding to fluorescence intensity, F, was calculated using the relation: $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F_{auto})$ [29].

To determine the effect of fatty amines on $[Ca^{2+}]_i$ of indo-1-loaded PAP2 cells, aliquots (50–100 μL) of fatty amine solution were added to 2 mL of the continuously stirred cell suspension after a 2-min equilibration period. Fatty amine samples were made up in aqueous DMSO solution; the final DMSO concentration attained in the cell suspension was kept below 0.01%.

Table 1. Effects of fatty amines on proliferation of PAP2 cells

Compound	IC_{50}* (μM)
Dodecylamine (C_{12})	22.9 ± 4
Tridecylamine (C_{13})	13.1 ± 0.3
Tetradecylamine (C_{14})	19.9 ± 0.4
Hexadecylamine (C_{16})	14.9 ± 3
Octadecylamine (C_{18})	45.8 ± 1
Farnesylamine (C_{15})	6.9 ± 0.8
Oleylamine ($C_{18:1}$)	0.1 ± 0.01

* The concentration of drug required to inhibit cell proliferation by 50%. The experiments were done using triplicate values, and the results are averages of three experiments. Values are given as means ± SEM.

RESULTS

Various short-chain and long-chain amines were tested for their ability to inhibit growth of *ras*-transformed cells, as measured by incorporation of [^{3}H]thymidine into DNA. Short-chain amines (C_6, C_8) did not inhibit at concentrations up to 100 μM, whereas long-chain amines (C_{12} to C_{18}) were potent growth inhibitors. A branched-chain unsaturated amine, farnesylamine, was a more effective inhibitor than straight-chain saturated amines of comparable chain-length, and a monounsaturated, unbranched amine, oleylamine, was the most effective inhibitor (Table 1). Nearly all of the cells were viable at the IC_{50} in each case, as illustrated for tridecylamine and oleylamine in Fig. 1. Although the longer-chain amines inhibited the incorporation of [^{3}H]thymidine at higher concentrations, they slightly stimulated incorporation at low concentrations. This is evident in the chart for oleylamine (Fig. 1B)

All of the long-chain amines inhibited the activity of PKC isolated from rat brain, whereas the short-chain amines had little effect (Fig. 2A). Oleylamine was only slightly more effective than the straight-chain saturated amines and farnesylamine was less effective. When the phosphatidylserine and diolein in the assay mixture were replaced by 20 μM oleic acid, stimulation of PKC was observed in the presence of low concentrations of oleylamine (Fig. 2B).

This phenomenon was explored further with oleylamine and other long-chain amines (Fig. 3). In the presence of 20 μM oleic acid, the addition of amines at the 60 μM level inhibited PKC activity in much the same way as when phosphatidylserine and diolein were used (Fig. 2A). However, the inhibition could be reversed by increasing the concentration of oleic acid (Figs. 3 and 4), and a stimulation was observed in some cases. Oleic acid had little effect on the results with farnesylamine (Fig. 3). In the absence of any amine, increasing the concentration of oleic acid above 20 μM caused an inhibition of PKC. Conversely, in the presence of 60 μM C_{18} amine or $C_{18:1}$ amine, PKC was stimulated at higher concentrations of oleic acid (Fig. 4).

As shown in Fig. 5, C_{13} amine and C_{14} amine produced biphasic elevations of $[Ca^{2+}]_i$ in indo-1-loaded PAP2 cells. The initial rise reached peak values of greater than 500 nM within 1 min of treatment and returned to baseline within approximately 2 min; this was followed by a smaller elevation which lasted for 3–4 min. It is evident that the effects of long-chain aliphatic amines on cellular calcium metabolism were dependent on their chain length (Fig. 5). C_{13} amine and C_{14} amine were most active; C_{12} amine was less effective, while C_8 amine was devoid of any observable effects even at the highest concentration examined (20 μM, data not shown). On the other hand, lengthening the hydrocarbon chain from C_{14} to C_{18} altered the characteristics of the cellular effect of aliphatic amines on calcium homeostasis: C_{18} amine elicited only the second and more delayed rise in $[Ca^{2+}]_i$.

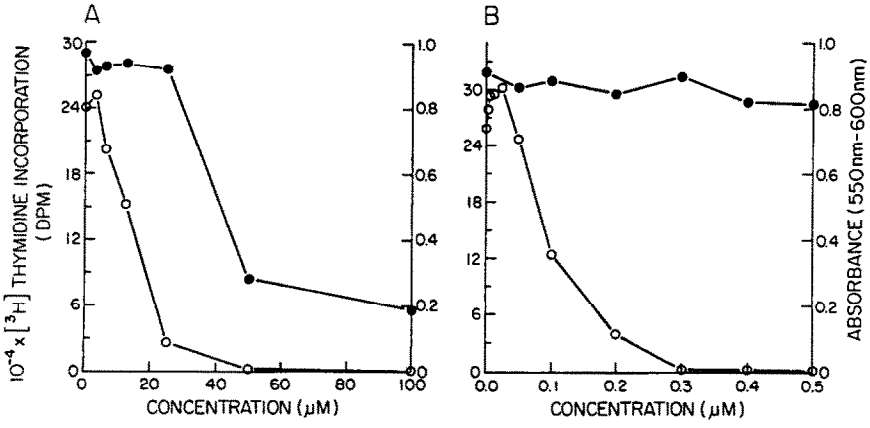

Fig. 1. Effects of (A) tridecylamine and (B) oleylamine on the proliferation (○) and viability (●) of PAP2 cells. Cell proliferation was measured by incorporation of [^{3}H]thymidine into DNA, as described in Materials and Methods. Each point represents triplicate values and is an average of three experiments. Viability of cells was measured by the MTT assay as described in Materials and Methods.

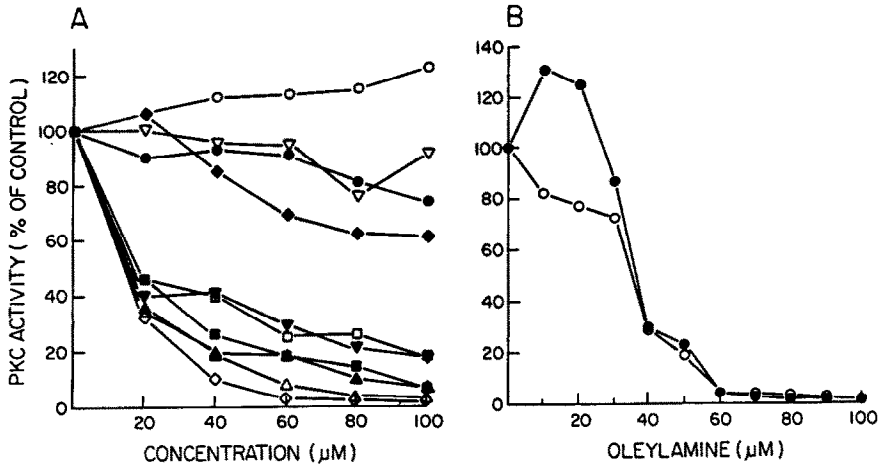

Fig. 2. Effects of fatty amines on PKC activity *in vitro*. (A) Comparison of the various fatty amines listed below. PKC activity was measured as described in Materials and Methods. Phosphatidylserine (16 μg/mL) and diolein (1.6 μg/mL) were added in the assay. Various fatty amines were dissolved in DMSO and added to the assay mixture. Key: (○) DMSO, (▽) C_6 amine, (●) C_8 amine, (◆) farnesylamine, (▼) C_{12} amine, (□) C_{13} amine, (■) C_{14} amine, (△) C_{16} amine, (▲) C_{18} amine, and (◇) oleylamine. (B) Effects of oleylamine. PKC activity was measured at various concentrations of oleylamine in the presence of either phosphatidylserine and diolein (○) or 20 μM oleic acid (●). Values in these panels are averages of three different assays (in duplicate), which differed by no more than 10%. Control PKC activity (100%) = 31,840 ± 1150 dpm/assay (mean ± SEM).

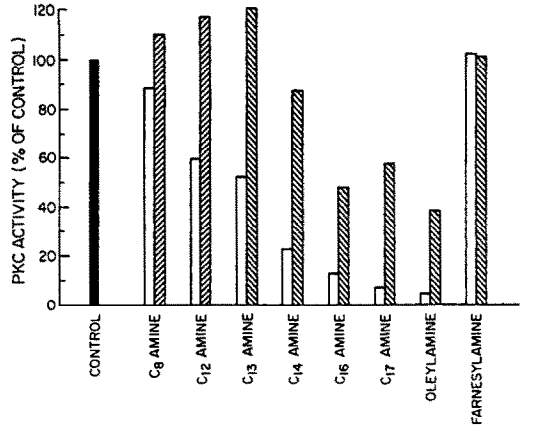

Fig. 3. Reversal of inhibition of PKC activity by oleic acid. PKC activity was measured at a 60 μM concentration of various fatty amines in the presence of either 20 μM oleic acid (open bars) or 40 μM oleic acid (striped bars). Values are averages of three different assays (in duplicate), which differed by no more than 10%. PKC activity (mean ± SEM = 33,040 ± 1010 dpm/assay) in the presence of 20 μM oleic acid, without any added amine, was taken as 100%.

The presence of an olefinic linkage in the C_{18} molecule changed its activity on cellular Ca^{2+} homeostasis, as evidenced by the induction of a rapid and transient rise in $[Ca^{2+}]_i$ by $C_{18:1}$ amine (Fig. 6) in contrast to the more delayed and prolonged elevation associated with C_{18} amine (Fig. 5). In addition, the presence of the olefinic linkage enhanced the potency of the molecule to elevate $[Ca^{2+}]_i$; in fact, $C_{18:1}$ amine was most active when compared with all other compounds examined in this study. It is interesting to note that the effect of farnesylamine, a fatty amine with three olefinic linkages, on $[Ca^{2+}]_i$ was similar to that of $C_{18:1}$ amine (Fig. 6), and its potency was comparable to those of C_{13} amine and C_{14} amine (Fig. 5).

DISCUSSION

Following the observation that sphingosine and other long-chain amines inhibit PKC, it was logical to think that this may account for their growth inhibitory properties, since PKC is known to play an important role in signal transduction and tumor promotion [17]. Besides acting as cell-growth inhibitors, sphingosine and its derived products have also been reported to have stimulating activities, such as activation of the tyrosine kinase of epidermal growth factor receptor in human lung fibroblasts and in epidermoid carcinoma cells [30–32].

The present experiments have been concerned with the growth-inhibitory properties of a number of other long-chain amines that are not known to occur naturally. These showed considerable variation in their ability to inhibit proliferation of PAP2 cells in culture. For example, $C_{18:1}$ amine, which contains one double bond, inhibited cell proliferation at a concentration that was two orders of magnitude less than C_{18} amine, the corresponding saturated amine (Table 1). The unsaturated, branched-chain amine, farnesylamine, was also more effective than saturated, straight-chain amines ranging from C_{12} to C_{18} in length (Table 1).

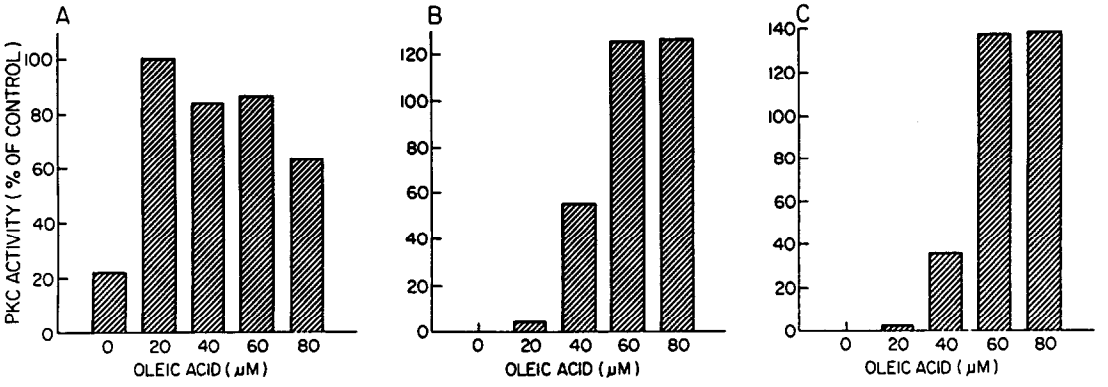

Fig. 4. Effect of various concentrations of oleic acid on PKC activity in the absence or presence of fatty amines. (A) PKC activity in the presence of different concentrations of oleic acid only. (B) PKC activity in the presence of 60 μM octadecylamine and different concentrations of oleic acid. (C) PKC activity in the presence of 60 μM oleylamine and different concentrations of oleic acid. Values are the averages of three different assays (in duplicate), which differed by no more than 10%. PKC activity (mean ± SEM = 31,660 ± 610 dpm/assay) in the presence of 20 μM oleic acid was taken as 100%.

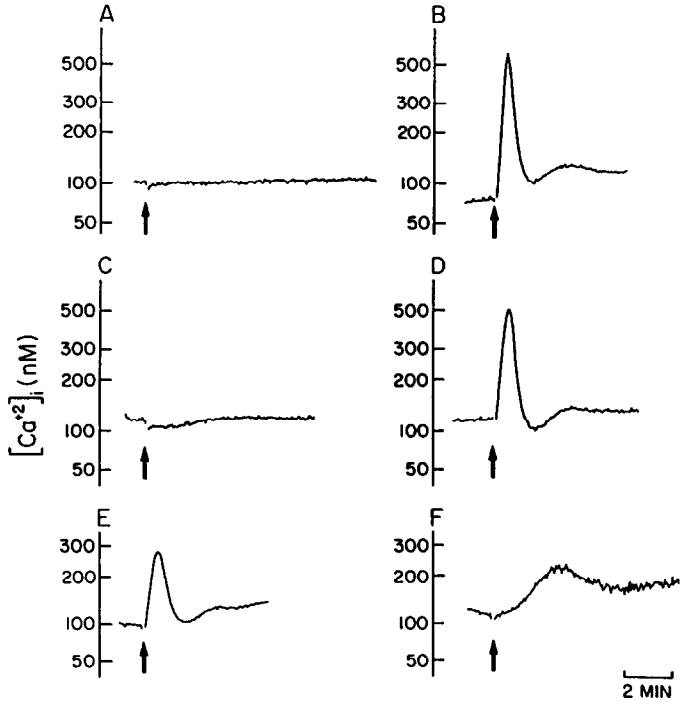

Fig. 5. Changes in $[Ca^{2+}]_i$ in indo-1-loaded PAP2 cells following treatment with various amines. PAP2 cells in suspension were loaded with indo-1 as described in Materials and Methods. These cells were resuspended at approximately 0.5×10^6 cells/mL in Na^+-HEPES buffer at 37°, and the fluorescence signal at 331 nm excitation and 398 nm emission was recorded continuously. Additions of aliphatic amines (10 μM) or vehicle are indicated by arrows. Each spectrofluorometic tracing is representative of responses from three to five separate cell preparations. The concentration of $[Ca^{2+}]_i$ was measured as described in Materials and Methods. Key: (A) Control, (B) C_{13} amine, (C) C_8 amine, (D) C_{14} amine, (E) C_{12} amine, and (F) C_{18} amine.

Whereas higher concentrations of the long-chain amines were inhibitory in each case, there was some indication that they were stimulatory at low concentrations, as illustrated for $C_{18:1}$ amine in Fig. 1B. In earlier studies in our laboratory, it was also observed that a low concentration (0.01%) of C_{18} amine in the diet enhanced the yield of mammary tumors induced in rats by DMBA, whereas a higher

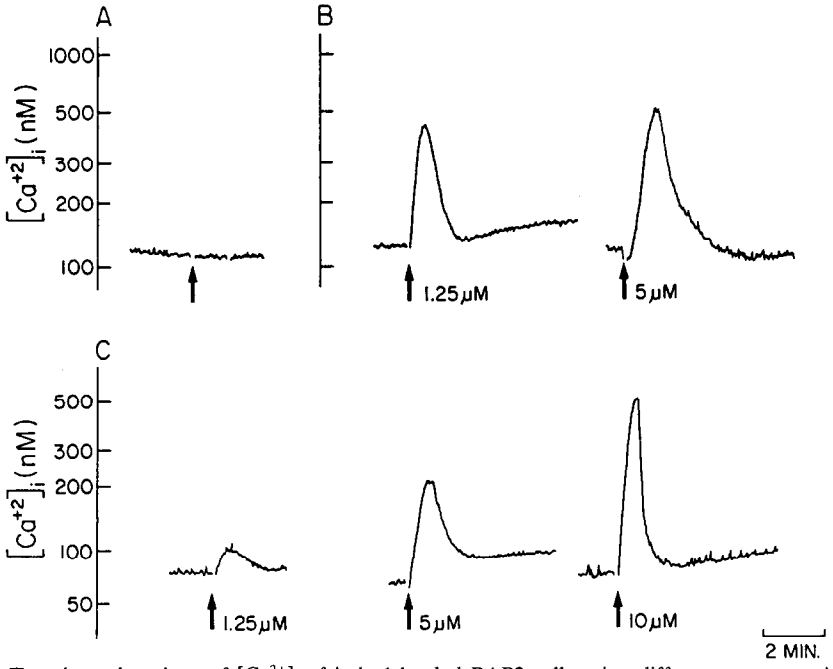

Fig. 6. Transient elevations of $[Ca^{2+}]_i$ of indo-1-loaded PAP2 cells using different concentrations of fatty amines. The experimental conditions were similar to those described in Fig. 5. Key: (A) control without amines, (B) oleylamine at the concentrations shown in the figure, and (C) farnesylamine at the different concentrations shown in the figure.

concentration (0.1%) inhibited both body growth and tumorigenesis [12].

In the present experiments, effects of the long-chain amines on PKC *in vitro* were also investigated (Fig. 2A). The results of these experiments indicated that the ability of the different amines to inhibit PKC was not strongly correlated with their ability to inhibit proliferation of PAP2 cells. For example, farnesylamine was a relatively poor inhibitor of PKC and $C_{18:1}$ amine was not much more effective than the various long-chain saturated amines (Fig. 2A). It remains to be determined whether a better correlation could be obtained if inhibition of PKC by aliphatic amines were carried out in intact PAP2 cells, since cellular uptake, subcellular distribution and metabolism of the amines, in addition to their intrinsic biological reactivity, may play a role in determining their biological potency.

Other studies in our laboratory indicated that farnesylamine may inhibit proliferation of PAP2 cells by interfering with farnesylation of *ras* protein [23]. This inhibition could be reversed by farnesol but not by a number of other related compounds, including geranylgeraniol. Both farnesylamine and $C_{18:1}$ amine inhibited farnesyl protein transferase *in vitro*, but the inhibitory effect of $C_{18:1}$ amine on proliferation of PAP2 cells was not affected by farnesol or by any other compounds tested, including oleic acid (unpublished data).

It is interesting to note that oleic acid has been observed to reverse the inhibitory effect of sphingosine on PKC *in vitro* [33]. In the present study, a series of experiments were carried out in which phosphatidylserine and diolein were replaced by oleic acid in the PKC assay. In the presence of oleic acid, low concentrations of the amines actually stimulated PKC in some cases (Fig. 3). In general, oleic acid reversed the inhibitory effects of the amines (Fig. 3), and complete reversal of the effects of C_{18} amine and $C_{18:1}$ amine was achieved with higher concentrations of oleic acid (Fig. 4). As noted above, oleic acid did not reverse the inhibitory effect of $C_{18:1}$ amine on proliferation of PAP2 cells.

Metabolic products of sphingosine have been reported to induce a rapid release of Ca^{2+} from IP_3-sensitive and -insensitive intracellular stores in permeabilized smooth muscle cells [20]. Moreover, it was reported that sphingosine 1-phosphate is a potent Ca^{2+}-mobilizing agent in viable 3T3 fibroblasts [34]. The long-chain amines examined in the present studies also caused a rapid and transient increase in $[Ca^{2+}]_i$ in intact PAP2 cells. Interestingly, the Ca^{2+} response to aliphatic amines with intermediate chain-length, C_{12-14}, was distinctly biphasic in nature (Fig. 5). Our recent study has shown that the second but not the initial rise was abolished in Ca^{2+}-free medium, suggesting that mobilization of Ca^{2+} from intracellular stores is responsible for the initial rise [35]. These findings indicate that these long-chain aliphatic amines resemble sphingosine and related compounds with respect to their Ca^{2+}-mobilizing effects.

It is important to note that the Ca^{2+} response to different amines corresponded to some extent with their ability to inhibit incorporation of [^{3}H]thymidine by PAP2 cells, with the exception of C_{18}. Calcium

mobilization and a transient increase in $[Ca^{2+}]_i$ are involved in signal transduction and activational activity in cells; it is not clear how the amine-induced Ca^{2+} responses may be linked to their inhibition of cell proliferation.

Exposure to high levels of some cytotoxic agents is known to lead to sustained elevation of $[Ca^{2+}]_i$; this has been linked to Ca^{2+} deregulation and cellular necrosis as well as an increase in nuclear Ca^{2+}, activation of endonuclease, and cell death by apoptosis [36, 37]. It is not certain whether the amine-induced reversible rise in $[Ca^{2+}]_i$ reflects Ca^{2+} deregulation; however, the concentrations of amines that were effective in eliciting the Ca^{2+} response were below their respective IC_{50} values, and they were not cytotoxic under those experimental conditions (Fig. 1). In fact, the magnitudes of the amine-mediated Ca^{2+} response were very similar to those observed in 3T3 fibroblasts following treatment with physiological agonists, such as ATP and platelet-derived growth factor [38]. It is likely that the reversible elevation of $[Ca^{2+}]_i$ observed in the present study represents a specific cellular response to amine exposure rather than a cytotoxic reaction.

From these experiments it appears that long-chain amines are able to exert multiple biological activities at sublethal concentrations, some of which are similar to those associated with sphingosine and related products of cellular lipid metabolism. Moreover, the ability of these amines to inhibit cellular proliferation and alter calcium metabolism is highly dependent on their structural characteristics. Our data suggest that long-chain amines may inhibit cell proliferation by mechanisms that are not necessarily dependent on inhibition of PKC.

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