Articles

Structural Requirements for Long-Chain (Sphingoid) Base Inhibition of Protein Kinase C in Vitro and for the Cellular Effects of These Compounds[†]

Alfred H. Merrill, Jr.,*[,‡] Sanjay Nimkar,[§] David Menaldino,[§] Yusuf A. Hannun,[‖] Carson Loomis,[‖] Robert M. Bell,[‖] Shiv Raj Tyagi,[‡] J. David Lambeth,[‡] Victoria L. Stevens,[‡] Rosemary Hunter,[‡] and Dennis C. Liotta[§]

Departments of Chemistry and Biochemistry, Emory University School of Medicine, Atlanta, Georgia 30322, and Departments of Medicine and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710

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ABSTRACT: Sphingosine, sphinganine, and other long-chain (sphingoid) bases inhibit protein kinase C in vitro and block cellular responses to agonists that are thought to act via this enzyme. To gain further insight into the mechanism of this inhibition, a series of long-chain analogues differing in alkyl chain length (11–20 carbon atoms), stereochemistry, and headgroup were examined for (a) inhibition of protein kinase C activity in vitro, (b) the neutrophil respiratory burst in response to phorbol myristate acetate (PMA), (c) the PMA-induced differentiation of HL-60 cells, and (d) the growth of Chinese hamster ovary cells. In every instance, the effects were maximal with the 18-carbon homologues, which are the same length as the predominant naturally occurring long-chain base (sphingosine). The lower potency of the shorter chain homologues was partially due to decreased uptake by cells. Small differences were obtained with the four stereoisomers of sphingosine (i.e., D and L forms of *erythro*- and *threo*-sphingosine), with *N*-methyl derivatives of the different sphingosine homologues, and with simpler alkylamines (e.g., stearylamine). The potency of the different headgroup analogues may be affected by the degree of protonation at the assay pH. The pK_a of sphingosine was measured to be 6.7; the pK_a varied among the analogues. These findings establish that the major structural features required for inhibition of protein kinase C and cellular processes dependent on this enzyme are the presence of a free amino group and an aliphatic side chain and that other groups have more subtle effects.

Protein kinase C mediates cellular responses to a growing list of agonists, which include tumor promoters, hormones, and growth and differentiation factors (Nishizuka, 1986; Bell, 1986; Berridge, 1987). Some aspects of the regulation of protein kinase C involve receptor-mediated turnover of phosphoglycerolipids to form *sn*-1,2-diacylglycerol, a potent activator of this enzyme. The participation of protein kinase C in normal cellular functions and its probable contribution to cell transformation and other diseases underscore the importance of understanding how various factors modulate its activity.

During studies of the mechanism of this lipid activation using a mixed micelle assay system (Ganong et al., 1986; Lee & Bell, 1986; Hannun et al., 1986a), Hannun et al. (1986b) discovered that sphingosine[1] and other long-chain bases, the backbone moieties of sphingomyelin, gangliosides, and other complex sphingolipids, are potent inhibitors of protein kinase C in vitro. Sphingosine inhibition is competitive with diacylglycerol, phorbol dibutyrate, and Ca^{2+} and depends on the amounts of the other components present. Sphingosine at equimolar concentrations with 1,2-dioleoylglycerol causes 50%

inhibition; hence, sphingosine is a relatively potent inhibitor of this enzyme. Lysosphingolipids, which retain the polar headgroup, are also inhibitors of protein kinase C in vitro (Hannun & Bell, 1987).

Long-chain (sphingoid) bases were additionally shown to inhibit protein kinase C dependent processes in vivo using platelets (Hannun et al., 1986b; Hannun & Bell, 1987), neutrophils (Wilson et al., 1986, 1987; Lambeth et al., 1988) and HL-60 cells (Merrill et al., 1986). Many laboratories have subsequently expanded the list of cellular events that can be affected by sphingosine.[2] Examples include the inhibition of phorbol ester induced down regulation of protein kinase C (Grove & Mastro, 1988), glutamate-induced translocation of protein kinase C in primary cultures of cerebellar neurons (Vaccarino et al., 1987), and phorbol ester induced and insulin-induced changes in hexose uptake by 3T3-L1 cells (Nelson & Murray, 1986) and isolated adipocytes. Recent studies have suggested that, in addition to inhibition of protein kinase C, sphingosine may stimulate phosphorylation of the EGF receptor (Faucher et al., 1988). These findings illustrate

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* Author to whom correspondence should be addressed.
[‡] Department of Biochemistry, Emory University School of Medicine.
[§] Department of Chemistry, Emory University School of Medicine.
[‖] Departments of Medicine and Biochemistry, Duke University Medical Center.

[1] Throughout this paper, the generic term sphingosine refers to the long-chain base containing the 4-trans double bond (which has also been called 4-*t*-sphingenine). Where not otherwise specified, sphingosine refers to the 18-carbon species. Sphinganine is used for the dihydro species, which has been called dihydrosphingosine or sphinganine.

[2] One report has suggested that the effects of long-chain bases are due to their cytotoxicity (Pittet et al., 1987); however, this has been shown (Lambeth et al., 1988) to be an artifact of the method of addition used by these investigators.

the general efficacy of long-chain bases as modulators of cell functions.

Long-chain bases are of additional interest because they are natural constituents of cells. Free sphingosine has been observed in HL-60 cells (Merrill et al., 1986), neutrophils (Wilson et al., 1988), and liver (Merrill et al., 1988), and for neutrophils, the amounts were found to be modulated by phorbols and other agonists (Wilson et al., 1988). Lysosphingolipids are also present in tissues, although these are mostly associated with sphingolipidoses [see Hannun and Bell (1987) for a discussion of these compounds]. Hence, free sphingosine and lysosphingolipids may be involved in the regulation of protein kinase C under normal and/or pathological conditions.

To obtain more mechanistic information about long-chain base inhibition of protein kinase C in vitro and cellular processes dependent on this enzyme, a series of analogues were synthesized according to a recently developed method (Nimkar et al., 1988) and tested as inhibitors of protein kinase C and three well-characterized cell systems. These studies delineated the role of the amino group, the alkyl chain length, and more subtle features of stereochemistry in the potency of the long-chain bases.

EXPERIMENTAL PROCEDURES

Materials

Compounds. Fatty acid free bovine albumin (BSA) was purchased from Boehringer Mannheim (Indianapolis, IN), and the cell culture media and fetal bovine serum were from Gibco (Grand Island, NY). Phorbol 12-myristate 13-acetate (PMA) was purchased from LC Services Corp. (Woburn, MA). Calf thymus DNA, Hoechst 33258 [bis(benzamide)], stearyl alcohol, stearylamine, *erythro*-dihydrosphingosine (sphinganine), sphingosine, ceramides (from bovine brain sphingomyelin), phytosphingosine, and chloroquine were obtained from Sigma (St. Louis, MO). The other biochemicals were of high quality and have been cited in other publications from these laboratories.

The long-chain base homologues and stereoisomers were synthesized by a recently developed procedure (Nimkar et al., 1988). Each of the compounds was pure on the basis of thin-layer chromatography on silica gel H plates developed with $CHCl_3$ methanol 2 N NH_4OH (40:10:1) with visualization with iodine vapor and ninhydrin; and, where a free amino group was present, they were also certified to be >95% pure by HPLC (Merrill et al., 1988). The stereochemistries were determined on the basis of their physical (Stoffel & Bister, 1973) and spectral (Herold, 1988) properties.

N-Acetylsphinganine was prepared from *erythro*-sphinganine as described by Gaver and Sweeley (1966). [3-^{3}H]-Sphinganine was synthesized by NaB^3H_4 reduction of *N*-acetyl-3-ketosphinganine followed by acid hydrolysis as described previously (Merrill & Wang, 1986).

Preparation of the Bovine Serum Albumin Complex with Sphingosine and Other Long-Chain Bases.

The long-chain bases were dissolved in absolute ethanol with heating to yield a concentrated solution (i.e., 50–100 mM). Stock solutions of the long-chain base–albumin complexes (in a 1:1 mole ratio) were prepared by adding the appropriate aliquots with vigorous mixing to 2 mM fatty acid free bovine serum albumin (BSA) that had been dissolved in phosphate-buffered saline and warmed to 37 °C. The mixtures briefly turned cloudy and then cleared as the long-chain bases bound to albumin. The solution was incubated at 37 °C for 1 h before use and stored frozen (if desired, the mixture can be dialyzed against physiological saline without loss of sphingosine). There was no apparent change in potency in solutions that had undergone several freeze/thaw cycles.

Methods

Mixed Micellar Assay for Protein Kinase C. Protein kinase C was assayed with Triton X-100 mixed micelles as previously described (Hannun et al., 1985, 1986a,b) by using protein kinase C that was purified to apparent homogeneity from rat brain by the method of Kikkawa et al. (1986). The enzyme was greater than 98% pure by sodium dodecyl sulfate–polyacrylamide gel electrophoresis stained with Coomassie Blue R-250 or silver and had a specific activity of 5–8 μmol/(min·mg) according to the mixed micellar assay.

Measurement of Neutrophil Respiratory Burst. The effects of the long-chain bases on PMA-induced superoxide production by human neutrophils were examined as described by Wilson et al. (1986, 1987). In these experiments, approximately 5×10^5 neutrophils/mL were stirred in cuvettes at 37 °C, and superoxide formation was quantitated by the (superoxide dismutase inhibitable) reduction of cytochrome c (extinction coefficient of 21 000 M^{-1} at 550 nm). Varying concentrations of the BSA complex of the long-chain bases were added 2 min before 1 μM PMA was added to stimulate the respiratory burst.

Effects on PMA-Induced Differentiation of HL-60 Cells. The inhibition of the PMA-induced differentiation of HL-60 cells to cells with a more monocyte/macrophage-like phenotype was assessed as described previously (Merrill et al., 1986). This involved incubating suspended HL-60 cells (which were between passages 24 and 30) in RPMI 1640 medium containing 10% Hyclone with 8 nM PMA and varying concentrations of the long-chain base added as the 1:1 complex with bovine serum albumin. After 24 h, the number of viable cells in suspension was estimated with a hemacytometer and the number attached to the Petri dish was determined by assays for DNA (West et al., 1985). The results were expressed as the percent of cells attached.

Measurement of Long-Chain Base Effects on Cell Growth with Chinese Hamster Ovary Cells. Chinese hamster ovary cells (CHO-WT5, a gift of Dr. Louis Siminovitch, Hospital for Sick Children, Toronto, Canada) were grown at 37 °C in Ham's F-12 medium supplemented with 5% fetal bovine serum in an atmosphere of 5% CO_2. Under these conditions the cells grow attached to the plastic culture dishes and divide approximately every 12.5 h.

Cells were released from near-confluent 100-mm Petri dishes with 3 mL of 0.5 mM EDTA in Pucks saline (8 g/L NaCl, 0.4 g/L KCl, 0.36 g/L $NaHCO_3$, and 1 g/L glucose) and were plated (1×10^4 cells/well) in 12-well culture dishes with 3 mL of long-chain base containing medium. The number of cells attached after 3 days was quantitated by assaying DNA by the method of West et al. (1985).

Long-Chain Base Uptake Measurements. Neutrophils (0.56 $\times 10^7$ cells/mL) were suspended in the same medium as used for measuring the respiratory burst, and the 1:1 BSA complexes of the different chain length homologues were added to yield a final concentration of 10 μM. After 5 min at 37 °C, the cells were diluted 10-fold with medium and centrifuged at 2000 rpm for 5 min, the supernatant was removed, and 1 mL of phosphate-buffered saline was added followed by mixing and centrifugation. The cell pellet was analyzed for the amounts of long-chain base that were taken up by using high-performance liquid chromatography as described by Merrill et al. (1988), with correction for the small amount (<5%) that was detected when similar analyses were conducted without adding the cells.

Table I: Effects of Long-Chain Bases on Protein Kinase C Activity in Vitro, on the Activation of Human Neutrophils by Phorbol Ester, and on Cell Growth and Viability Using Chinese Hamster Ovary Cells

long-chain base	concentration for 50% inhibition		
	in vitro (mol %)[a]	neutrophils (μM)[b]	CHO cells (μM)[c]
stereoisomers			
D-*erythro*-sphingosine (2*S*,3*R*)	2.8	1.1	2.8
L-*erythro*-sphingosine (2*R*,3*S*)	3.3	1.3	2.0
D-*threo*-sphingosine (2*R*,3*R*)	2.8	1.3	2.5
L-*threo*-sphingosine (2*S*,3*S*)	2.2	1.9	2.6
D-(+)-*erythro-cis*-sphingosine	7.0		
chain length homologues			
C11-sphingosine	$\gg$10[d]	90	95
C14-sphingosine	7.3	19	8.0
C16-sphingosine	6.8	1.0	3.2
C18-sphingosine	5.0	0.7	3.0
C20-sphingosine	8.6	2.2	3.2
N-methyl homologues			
N-methyl-C11-sphingosine	$\gg$10[e]	100	ND
N-methyl-C14-sphingosine	6.0	17	ND
N-methyl-C16-sphingosine	5.5	1.7	ND
N-methyl-C18-sphingosine	4.0	0.4	ND
N-methyl-C20-sphingosine	4.8	1.9	ND
N,*N*-dimethyl-C18-sphingosine	10	ND	ND
N-methyl-*N*-isopropyl-C18-sphingosine	>10[f]	activates	ND
other			
4,5-dehydrosphingosine	ND	1.3	ND
dihydrosphingosine	ND	ND	2.9
phytosphingosine	ND	ND	5.2
psychosine	ND	ND	32
N-methyl-C18-dihydrosphingosine	ND	ND	2.3
N-acetyl-C18-dihydrosphingosine	ND	ND	70
N-acylsphingosine (ceramide)	>10[g]	>100[h]	>100
nonsphingolipid analogues			
stearylamine	2.4[g]	25[h]	6.8

[a] Assayed in mixed micelles with Triton X-100, phosphatidylserine, etc., as described in the text. [b] Inhibition of PMA induction of superoxide generation by neutrophils as described in the text. [c] Reduction of cell number by growth inhibition and cytotoxicity, as described in the text. [d] 5% inhibition at 10 mol %. [e] 18% inhibition at 10 mol %. [f] 30% stimulation at 10 mol %. [g] From Hannun et al. (1986b). [h] From Wilson et al. (1986).

CHO cells (1 × 10⁵) were grown in glass scintillation vials for 24 h. The medium was removed and replaced with 2 mL of medium containing 1 μM [3-³H]C11- or C18-sphinganine as the 1:1 complex with fatty acid free bovine serum albumin. At various times, the amount of radiolabel taken up by the cells was determined by removing the medium, adding scintillation cocktail, and counting the vials.

Estimation of the pK_a of Sphingosine and Other Analogues. The long-chain bases (1 mg) were mixed with 9 mg of Triton X-100 or octyl β-D-glucopyranoside and dispersed in 2 mL of distilled, deionized water. The detergent alone and the detergent plus long-chain base were titrated with 10 mM HCl followed by 10 mM NaOH with measurement of the pH after each addition upon reaching a stable value. All of the long-chain bases analyzed formed clear solutions; however, the mixed micelles with stearylamine became turbid at approximately pH 8.

Results

Long-Chain Base Inhibition of Protein Kinase C in Vitro. D-(+)-*erythro*-Sphingosine (2*S*,3*R*), prepared synthetically, inhibited protein kinase C activity in the mixed micelle assay system with 50% inhibition at 2.8 mol % (Figure 1 and Table I). This agreed with the previous findings (Hannun et al., 1986b) with commercially available D-sphingosine, which is obtained by hydrolysis of more complex sphingolipids and contains small amounts of sphinganine and various chain-length homologues.

Sphingosine has two asymmetric carbons (2 and 3) and can exist in four stereoisomers, although only the D-(+)-erythro form is thought to occur naturally (Karlsson, 1970). Comparison of the four stereoisomers (Table I) revealed that all
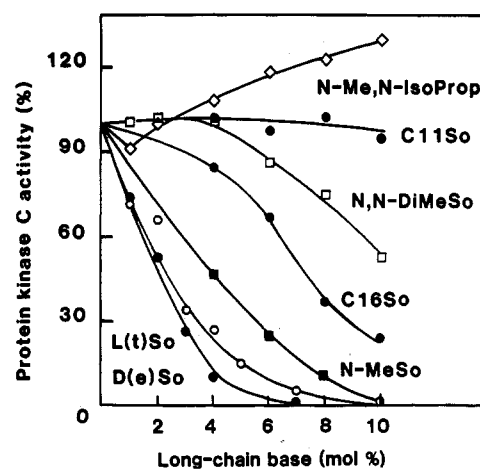

FIGURE 1: Inhibition of purified protein kinase C in mixed micelles containing Triton X-100, phosphatidylserine, diacylglycerol, and varying mole percents of the different long-chain bases. The analyses were conducted as described under Experimental Procedures by using D-*erythro*-sphingosine [D(e)So], L-*threo*-sphingosine [L(t)So, circles], and the chain length homologues of D-*erythro*-sphingosine with 11 and 16 carbon atoms (C11So and C16So, respectively), as well as the *N*-methyl (N-MeSo), *N*,*N*-dimethyl (N,N-DiMeSo), and *N*-methyl-*N*-isopropyl (N-Me,N-IsoProp) derivatives.

had approximately the same potency as inhibitors of protein kinase C in vitro, with a slight preference for the L-threo form (Table I and Figure 1). Another isomer, sphingosine with a cis double bond instead of trans between carbons 4 and 5, was about half as inhibitory (Table I).

Earlier investigations have shown that acetylation of the free amino group essentially abolishes the inhibition of protein kinase C in vitro (Hannun et al., 1986b). This could be due
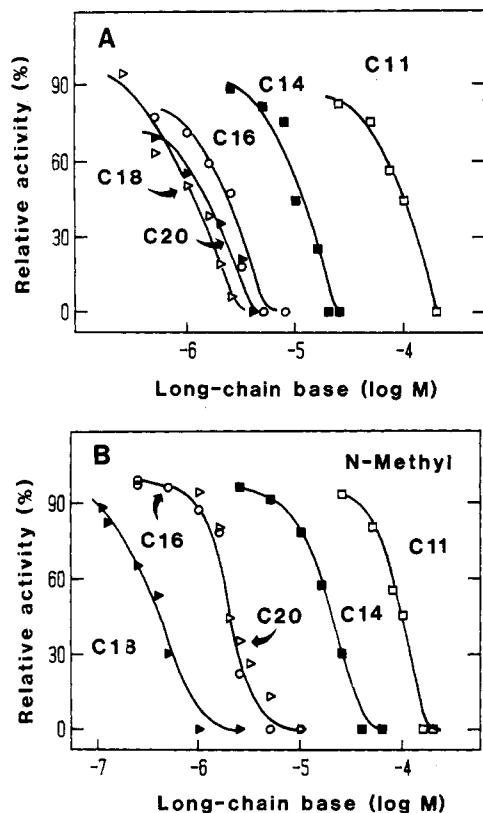
FIGURE 2: Inhibition of the PMA-inhibited respiratory burst in neutrophils by sphingosine (A) and N-methylsphingosine (B) homologues. The effects of the long-chain bases on the inhibition of PMA-induced superoxide production was determined as described under Experimental Procedures by using approximately 5×10^5 neutrophils/mL. The long-chain bases were added 2 min before 1 μM PMA was added to stimulate the respiratory burst. The abbreviations refer to the total number of carbon atoms in the sphingosine (panel A) or N-methylsphingosine (panel B) homologues.

FIGURE 3: Dose response of the inhibition of the PMA-induced respiratory burst in human neutrophils by cis- and trans-sphingosine, N-methylsphingosine (N-MeSo), and N-methyl-N-isopropyl-sphingosine (N-Me,N-IsoPrSo). These experiments were conducted as described in the legend to Figure 2.

Table II: Effects of Long-Chain Base Homologues on PMA-Induced Differentiation of HL-60 Cells

added long-chain base	% adherence with 2.5 μM long-chain base[a]	added long-chain base	% adherence with 2.5 μM long-chain base[a]
none	100 ± 11	C16-sphingosine	73 ± 9
C11-sphingosine	90 ± 6	C18-sphingosine	45 ± 16
C14-sphingosine	81 ± 8	C20-sphingosine	72 ± 3

[a] HL-60 cells were incubated with 8 nM PMA and the long-chain base for 24 h, and then the numbers of suspended and adherent cells were determined as described previously (Merrill et al., 1986). The data are the mean ± SD for $n = 3$.

to steric effects or abolishment of the positive charge that is thought to be required for inhibition. The N-methyl derivative of sphingosine (and of other homologues) was about as inhibitory as the parent compound (Figure 1 and Table I). Addition of a second methyl group (N,N-dimethylsphingosine) decreased the inhibition significantly (Figure 1 and Table I) and the even more bulky substituent N-methyl-N-isopropyl yielded a compound that stimulated protein kinase C activity (Figure 1 and Table I). These findings suggest that protein kinase C inhibition probably requires a relatively unhindered amino group capable of protonation.

The role of the hydroxyl groups is not obvious since stearylamine, which has an alkyl chain length similar to sphingosine but lacks both hydroxyl groups, is as inhibitory as sphingosine for protein kinase C in vitro (Hannun et al., 1986b; Table I). However, differences were found between these compounds, and these will be discussed later.

Another major determinant of the potency of these compounds as inhibitors of protein kinase C was the alkyl chain length (Table I). No inhibition was observed if the alkyl chain was removed and substituted with a phenyl group, and homologues with up to 11 carbon atoms showed insignificant inhibition at as high as 10 mol % (Figure 1 and Table I). Increasing the alkyl chain length resulted in bimodal inhibition—a slight increase in potency as the alkyl chain was increased to 18 carbons followed by a decrease at the 20-carbon homologue. The same pattern was observed for the homologous series of N-methyl compounds. This demonstrates that the maximal inhibition is obtained with long-chain bases of 18 carbon atoms.
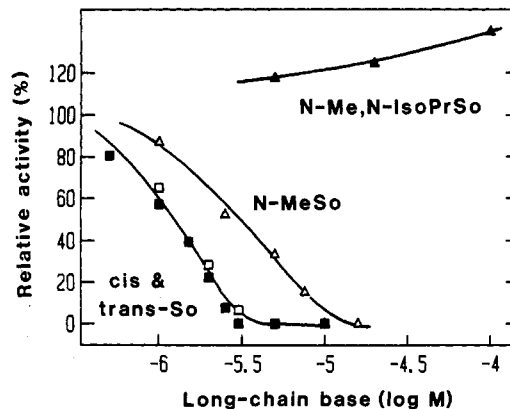
Effects on the Neutrophil Respiratory Burst. All of the analogues that inhibited protein kinase C in vitro blocked the PMA-induced respiratory burst in human neutrophils (Figures 2 and 3 and Table I). Neutrophils (polymorphonuclear leukocytes) generate superoxide anion upon protein kinase C activation by phorbol esters and other agonists and appear to be a straightforward model system for studying cellular responses that involve this enzyme (Wilson et al., 1986, 1987).

As has been seen before (Wilson et al., 1986), sphingosine is a potent inhibitor of superoxide generation, and in this study the natural stereoisomer effected 50% inhibition at 1.1 μM (Figure 2A), a slightly lower concentration than the other isomers. The chain length homologues exhibited different potencies, with C16- and C18-sphingosine being the most inhibitory (Figure 2A). Inhibition was seen with C11-sphingosine, but at very high concentrations. A similar chain length dependence was seen with the N-methyl derivatives (Figure 2B); however, N-methyl-C18-sphingosine was somewhat more inhibitory than sphingosine for neutrophils. These differences may reflect the interposition of additional steps (cellular uptake and metabolism) in the inhibition of protein kinase C by these compounds in intact cells.

N-Methyl-N-isopropylsphingosine did not inhibit the PMA-stimulated oxidative burst and, instead, activated the cells at concentrations comparable to the inhibitory levels of the other long-chain bases (Figure 3 and Table I). This was consistent with the activation of protein kinase C observed in vitro.

Inhibition of PMA-Induced Differentiation of HL-60 Cells. The importance of the alkyl chain length as a factor in the modulation of cellular responses by long-chain bases was also tested with the PMA-induced differentiation of HL-60 cells (Table II). These cells are induced to differentiate into macrophage/monocyte-like cells by phorbol esters, and this
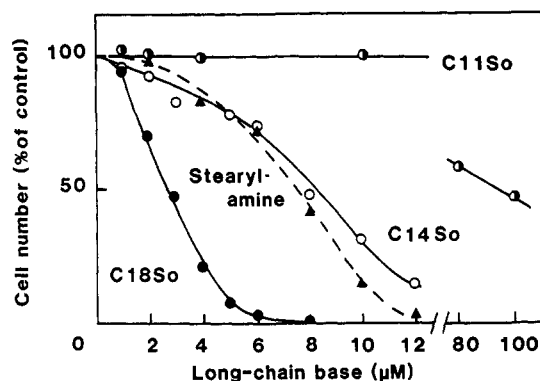
FIGURE 4: Effects of long-chain bases on cell number for Chinese hamster ovary cells. Cells were incubated in 12-well culture dishes with 3 mL of medium with sphingosine (C18So, closed circles), the 11- or 14-carbon homologues of sphingosine (C11So, split circles; C14So, open circles), or stearylamine (triangles). The cell number after 3 days was compared to that for cells grown in medium without sphingosine as described under Experimental Procedures.

FIGURE 5: Uptake of [^{3}H]sphinganines with 11 and 18 carbon atoms by Chinese hamster ovary cells. Chinese hamster ovary cells were incubated with 1 μM [3-^{3}H]C11- (open circles) or C18- (closed circles) sphinganine as the 1:1 complex with fatty acid free bovine serum albumin. At various times, the amount of radiolabel taken up by the cells was determined as described under Experimental Procedures.

Table III: Uptake of Various Sphingosine Homologues by Human Neutrophils

long-chain base	mean retention time[a] (min)	amount of sphingosine associated with the cells[b] (nmol)
C11-sphingosine	2.25	0.053 ± 0.025
C14-sphingosine	3.45	0.27 ± 0.04
C16-sphingosine	5.08	0.48 ± 0.14
C18-sphingosine	7.78	0.72 ± 0.29
C20-sphingosine	12.5	0.74 ± 0.09

[a] The long-chain bases were quantitated as the *o*-phthalaldehyde derivatives on C18 reverse-phase HPLC with methanol/5 mM potassium phosphate (90:10) as described by Merrill et al. (1988). [b] Measured by using 0.56 × 10^7 cells/mL and 10 μM analogues as described under Experimental Procedures.

FIGURE 6: pH titration of long-chain bases in Triton X-100 and octyl β-D-glucopyranoside. Sphingosine (So, open circles), *N*-methyl-sphingosine (*N*-MeSo, closed squares), and stearylamine (open squares) were solubilized with Triton X-100 and titrated with NaOH as described under Experimental Procedures. A similar titration was conducted with sphingosine in octyl β-D-glucopyranoside (closed circles). The microequivalents of base consumed per pH was calculated by subtracting the results with Triton X-100 (or for octyl β-D-glucopyranoside) alone from each analogue.

phenotype is exemplified by conversion of suspended cells to ones adherent to the culture dish. Sphingosine has been shown to be a potent inhibitor of this phenomenon (Merrill et al., 1986). As is shown in Table II, D-*erythro*-sphingosine was inhibitory at low concentrations (i.e., 2.5 μM) and was more inhibitory than the other chain length homologues.

Effects of Long-Chain Bases on Chinese Hamster Ovary Cell Growth. A common concern in the addition of long-chain bases to cells is that they are also growth inhibitory and cytotoxic at high concentrations (Merrill, 1983; Merrill et al., 1986; Lambeth et al., 1988). The effects of the long-chain bases on these parameters were assessed with CHO cells, which are known to be growth inhibited by long-chain bases (Merrill, 1983). The concentration dependence for some of the long-chain base analogues is shown in Figure 4 and Table I and followed basically the same pattern as for protein kinase C inhibition in vitro and for blockage of the PMA-induced respiratory burst in neutrophils. While the exact mechanism for these effects is not known,[3] these results are consistent with an involvement of protein kinase C.

Long-Chain Base Uptake. Since the rate of cellular uptake of the long-chain bases could be an additional contributor to the chain length dependence of their effects with intact cells, uptake was compared by using two systems, neutrophils and CHO cells. For neutrophils, mass measurements of the amounts taken up by the cells during a 5-min incubation
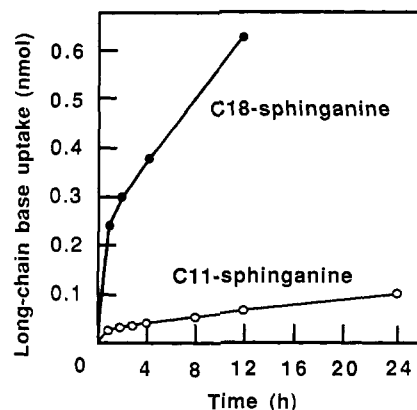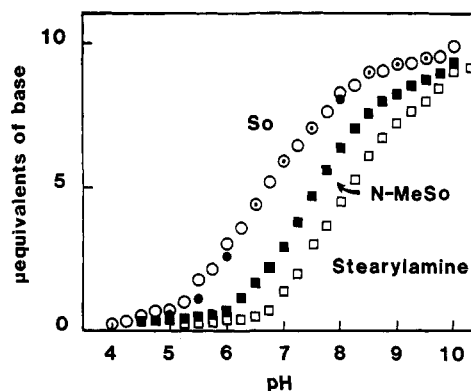
(approximately the same time course used in measuring the effects of these compounds on the neutrophil respiratory burst) were conducted by high-performance liquid chromatography (Table III). There was a marked chain length dependence; however, the magnitude of the differences among the homologues was much less than their relative potencies as inhibitors (cf. C11-sphingosine uptake and inhibition).

The incorporation of [^{3}H]C11-sphinganine and [^{3}H]C18-sphinganine (which were prepared by reduction of the respective sphingosines) into CHO cells over time is shown in Figure 5. The C11-sphinganine was taken up about 12-fold more slowly than the C18 compound (i.e., 0.03 versus 0.48 nmol at 4 h). Thin-layer chromatography of the cell-associated radiolabel revealed that most was still the free long-chain base during this time course (not shown). Thus, differences in uptake undoubtedly contribute to the potency of these compounds in intact cells; however, this is probably not the only explanation since C11-sphingosine is much less potent (i.e., 17- to 32-fold less than C18-sphingosine) than is implied by the uptake data.

Estimation of the pK$_a$ of Sphingosine and Other Analogues. An additional factor worth considering is the pK$_a$ of the amino group since this will influence the concentration of the positively charges species, and the cationic form of sphingosine

probably inhibits protein kinase C (Hannun et al., 1986b). To obtain some insight into this question, the pK_a's of sphingosine and some of the analogues were estimated in mixed micelles (Figure 6). The pK_a of D-*erythro*-sphingosine is 6.7 as a 10% mixed micelle with Triton X-100 (open circles in Figure 6). There was concern that the nature of the Triton X-100 micelles [i.e., the amino group is surrounded by poly(oxyethylene) groups with a low dielectric constant] might be the major cause of the low pK_a; hence, the same determination was made by using octyl β-D-glucopyranoside (closed circles). Both gave the same pK_a; hence, any effects of association of sphingosine with a detergent micelle are not peculiar to Triton X-100. There was clearly a contribution by the neighboring alkyl chain because the pK_a of 2-amino-1,3-dihydroxy-3-phenyl-propanolamine, which is water soluble, was 8.3 (data not shown).

The pK_a's of stearylamine and N-methylsphingosine in Triton X-100 were 8.0 and 7.5, respectively (Figure 6); that of octylamine was 9.8 (not shown). As with sphingosine, the pK_a's were essentially the same with Triton X-100 and octyl β-D-glucopyranoside (not shown). The observed pK_a for octylamine (9.8) was somewhat lower than in water (10.65) (Jencks & Regenstein, 1976), which further illustrates the effect of association of long-chain bases with a hydrophobic environment. We did not observe a dissociable proton with N,N-dimethylsphingosine or N-methyl-N-isopropylsphingosine over this pH range.

DISCUSSION

The major finding of these investigations was that the effects of the long-chain analogues on various cellular functions thought to be mediated by protein kinase C exactly parallelled the relative potency of these compounds as inhibitors of this enzyme in vitro. While this does not prove that inhibition of protein kinase C accounts totally for the effects of these compounds, this is the simplest explanation for this correlation. It warrants comment that sphingosine has recently been reported to inhibit calmodulin-dependent kinases (Jefferson & Schulman, 1988); however, the concentrations were about 50-fold higher than those found to affect neutrophils, HL-60 cells, or CHO cells in our study.

The homologue that was most inhibitory for protein kinase C and the various cell functions was the 18-carbon species (the same chain length dependence was seen among the N-methyl compounds), which is also the predominant long-chain base of most mammalian sphingolipids (Karlsson, 1970) and has been found in free form in liver (Merrill et al., 1988), neutrophils (Wilson et al., 1988), and HL-60 cells (Merrill et al., 1986). The chain length dependence is apparently a combination of the extent to which the homologues partition into the membrane and intrinsic differences in the ability of the compounds to inhibit protein kinase C.

The headgroup also had a significant influence on the inhibition in vitro and in vivo. Whereas there were little differences between the sphingosines and derivatives with a single methyl substituent on the 2-amino group, substitution with an N,N-dimethyl- group resulted in a much lesser potency. N-Methyl-N-isopropylsphingosine was an activator of protein kinase C in vitro and the neutrophil oxidative burst. N-Acetyl- and N-acylsphingosines have not been reported to inhibit or activate (Hannun et al., 1986b; Wilson et al., 1986). Since quaternary amines are known inhibitors in vitro (Hannun et al., 1986b) but not in intact cells (Wilson et al., 1986), it appears that a positive charge is needed for protein kinase C inhibition in vitro but that the amino group must be capable of existing in a neutral form to facilitate entry into the cell.
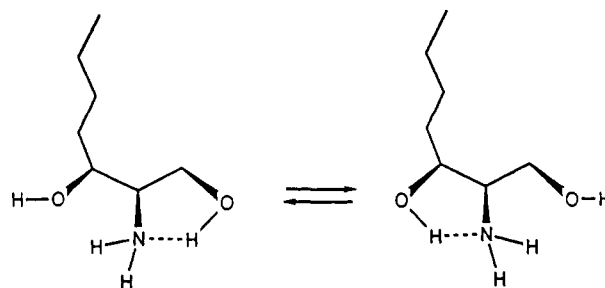

FIGURE 7: Intramolecular hydrogen bonding in sphingosine.

The hydroxyl groups appeared to have smaller effects on the inhibition of protein kinase C, as reflected in the ability of stearylamine to inhibit protein kinase C in vitro and the responses of the various cell types. This may be deceiving, however, since the pK_a of sphingosine is 6.7 versus 8.0 for stearylamine. At the assay pH of 7.5 (or a cytosolic pH of 7.4), only 15% of the sphingosine will be protonated whereas stearylamine will be 75% ionized. Thus, if the ionized species is the inhibitor, a 2-fold difference in the apparent potency of sphingosine really reflects about a 10-fold greater inhibition when corrected for the smaller amount of the protonated form. These numbers should only be regarded as approximations, however, until more detailed studies of the pK_a of these compounds in the same micelles used for assaying protein kinase C can be conducted. We are in the process of synthesizing the suitably labeled compounds for NMR studies of this type.

The basis for this difference in pK_a is shown in Figure 7. The amino group of sphingosine can form two intramolecular hydrogen bonds, which is known to shift the pK_a of an amino group, as illustrated in the pK_a's of ethylamine (10.6), 2-amino-1-propanol (9.5), 2-amino-2-methyl-1,3-propanediol (8.8), and 2-amino-3-phenyl-1,3-propanediol (8.3, this study). An additional 1–2 pK_a units is gained from the localization of the ionizing species adjacent to the hydrophobic alkyl chain (as seen in the pK_a's of 8.0 and 9.8, respectively, for stearylamine and octylamine under these same conditions). These features of the structure of sphingosine have intriguing consequences, since they enable the molecule to exist at physiological pH both in a charged form, which would facilitate movement through an aqueous phase (in analogy to the behavior of fatty acids), and as a neutral species, which can readily traverse biological membranes. Furthermore, about 1% should be in the neutral form even at the acidic pH of lysosomes (ca. 5), allowing the lysosomal degradation product of more complex sphingolipids to exit for further metabolism, which is thought to occur in other subcellular compartments (Sweeley, 1985).

The stereochemistry of the headgroup hydroxyls has little effect on the potency of these compounds in vitro or in vivo. This may indicate that the mechanism of inhibition does not involve specific interactions with protein kinase C or other stereospecific components of the membrane. However, it should be noted that all four stereoisomers can yield the same headgroup conformation (Figure 8) if the orientation of the alkyl chain is not critical. Thus, the data with the stereoisomers do not provide definitive evidence that specific or nonspecific interactions are involved in the inhibition. Some aspects of the orientation of the headgroup relative to the hydrophobic region may be important, however, since *cis*-sphingosine was less inhibitory than *trans*-sphingosine in some of the systems examined.

These findings are consistent with a model for the interaction of protein kinase C with lipid activators and inhibitors in which protein kinase C binds to membranes via interactions with
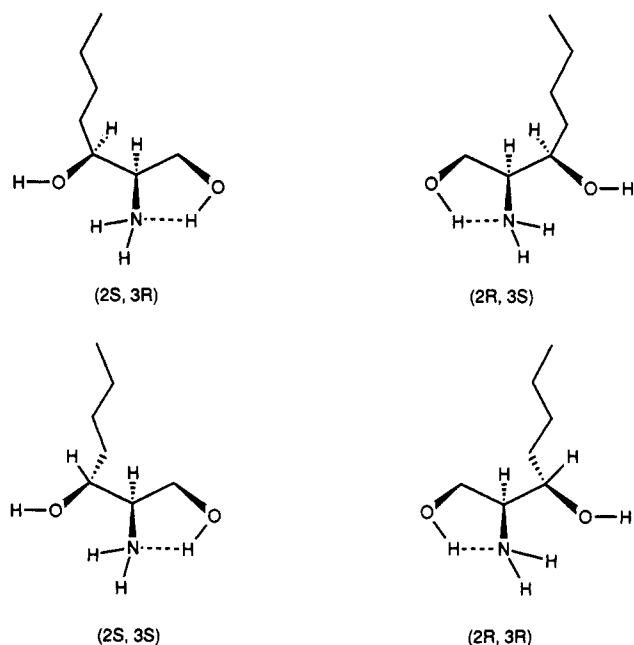
FIGURE 8: Conformations of the four stereoisomers of sphingosine that provide equivalent structural elements in the headgroup region.

diacylglycerol and negatively charged phospholipids (e.g., phosphatidylserine) (Bell, 1986). Sphingosine may localize in the region of the negative phospholipids, blocking formation of the complex required for activation. The inhibition of protein kinase C by charge neutralization in mixed micelles (Bazzi & Nelsestuen, 1987a–c) and erythrocyte membranes (Moruzzi et al., 1987) has been discussed by others. It is often regarded as a nonspecific effect and implied to be a technical artifact of these in vitro assays. Since the inhibition of cellular functions closely parallels their in vitro potency, the inhibition has clear relevance to the function of these compounds in biological systems. This is particularly so since the amounts that are usually taken up by cells when long–chain bases are added exogenously are similar to the levels of free sphingosine that occur naturally in cells (Merrill et al., 1986; Kobayashi et al., 1988; Merrill et al., 1988; Wilson et al., 1988).

Protein kinase C is inhibited by other types of hydrophobic amines, such as acylcarnitines (Katoh et al., 1981; Wise et al., 1983), and some of these may act via the same mechanism as sphingosine. Palmitoylcarnitine is structurally related to long–chain bases, and acylcarnitines exhibit the same dependence on alkyl chain length for inhibition; i.e., acetyl- and octanoylcarnitines are not inhibitory, whereas, palmitoylcarnitine and stearoylcarnitine (as well as hexadecyltrimethylammonium bromide and stearoylcholine) are about equivalent (Nakadate & Blumberg, 1987).

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Posttranslational Modification of Hepatic Cytochrome P-450. Phosphorylation of Phenobarbital-Inducible P-450 Forms PB-4 (IIB1) and PB-5 (IIB2) in Isolated Rat Hepatocytes and in Vivo[†]

John A. Koch and David J. Waxman*

Department of Biological Chemistry and Molecular Pharmacology and Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115

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ABSTRACT: Phosphorylation of hepatic cytochrome P-450 was studied in isolated hepatocytes incubated in the presence of agents known to stimulate protein kinase activity. Incubation of hepatocytes isolated from phenobarbital-induced adult male rats with [^{32}P]orthophosphate in the presence of $N^6,O^{2'}$-dibutyryl-cAMP (diBtcAMP) or glucagon resulted in the phosphorylation of microsomal proteins that are immunoprecipitable by polyclonal antibodies raised to the phenobarbital-inducible P-450 form PB-4 (P-450 gene IIB1). Little or no phosphorylation of these proteins was observed in the absence of diBtcAMP or glucagon or in the presence of activators of Ca^{2+}-dependent protein kinases. Two-dimensional gel electrophoresis revealed that these ^{32}P-labeled microsomal proteins consist of a mixture of P-450 PB-4 and the closely related P-450 PB-5 (gene IIB2), both of which exhibited heterogeneity in the isoelectric focusing dimension. Phosphorylation of both P-450 forms was markedly enhanced by diBtcAMP at concentrations as low as 5 μM. In contrast, little or no phosphorylation of P-450 forms reactive with antibodies to P-450 PB-1 (gene IIC6), P-450 2c (gene IIC11), or P-450 PB-2a (gene IIIA1) was detected in the isolated hepatocytes under these incubation conditions. Phosphoamino acid analysis of the ^{32}P-labeled P-450 PB-4 + PB-5 immunoprecipitate revealed that these P-450s are phosphorylated on serine in the isolated hepatocytes. Peptide mapping indicated that the site of phosphorylation in hepatocytes is indistinguishable from the site utilized by cAMP-dependent protein kinase in vitro, which was previously identified as serine-128 for the related rabbit protein P-450 LM2. Phosphorylation of P-450s PB-4 and PB-5 (as well as immunoreactive P-450 forms PB-1 and PB-2a) was also shown to occur in the liver in vivo in a process that is facilitated by, but not obligatorily dependent on, administration of diBtcAMP + theophylline to stimulate cAMP-dependent protein kinase. In vitro analyses revealed that phosphorylation of P-450 PB-4 leads to a loss of monooxygenase activity, suggesting that the posttranslational modification of this P-450 enzyme by cAMP-dependent protein kinase may play a role in the modulation of P-450-dependent monooxygenase activity in vivo.

C̲ytochrome P-450 (P-450)[1] is comprised of a superfamily of heme protein monooxygenase enzymes that catalyze oxidative metabolism of a broad range of endogenous and exogenous compounds including many steroids, drugs, and chemical carcinogens. P-450 expression in liver and other tissues is modulated by exposure to foreign compounds, many of which can differentially induce and suppress the levels of

individual P-450 enzymes (Guengerich, 1987). Many P-450-dependent activities are also regulated by gonadal and pituitary hormones, which play an important role in the sex-dependent developmental regulation of P-450 gene expression in rodent liver and kidney (Skett, 1987; Waxman, 1988). Hormonal regulation of P-450 enzyme activity is also suggested by studies on the modulation of P-450-dependent mono-

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[1] Abbreviations: P-450, cytochrome P-450; SDS, sodium dodecyl sulfate; cAMP, cyclic AMP; diBtcAMP, $N^6,O^{2'}$-dibutyryl-cAMP; the terms P-450 form and P-450 enzyme are used interchangeably in this report.