Use of D-erythro-sphingosine as a pharmacological inhibitor of protein kinase C in human platelets

Wasiuddin A. KHAN,* S. Wayne MASCARELLA,† Anita H. LEWIN,† Chris D. WYRICK,† F. Ivy CARROLL† and Yusuf A. HANNUN*‡

*Department of Medicine and Cell Biology, Duke University Medical Center, Durham, NC 27710, U.S.A., and

†Chemistry and Life Sciences, Research Triangle Institute, Research Triangle Park, NC 27709, U.S.A.

Sphingosine is a naturally occurring long-chain amino diol with potent inhibitory activity against protein kinase C *in vitro* and in cell systems. The use of sphingosine as a pharmacological tool to probe the activity of protein kinase C has been hampered by its amphiphilicity, possible contamination of its commercial preparations, and the existence of other targets for its action. To address these problems, high-purity D-erythro-sphingosine was prepared and employed to develop an approach for the use of sphingosine as a pharmacological agent. The addition of synthetic D-erythro-sphingosine to intact human platelets resulted in quick uptake and preferential partitioning into the particulate fraction. It was rapidly metabolized by intact platelets, 60% being degraded within 1 min after addition. Sphingosine was found to be a potent inhibitor of γ -thrombin-induced aggregation and secretion of washed human platelets. Multiple criteria indicated that this effect is probably mediated through the inhibition of protein kinase C: (1) sphingosine inhibited protein kinase C activity in intact platelets with a similar dose/response to its inhibition of platelet aggregation and secretion; (2) sphingosine inhibited phorbol binding to intact platelets under identical conditions and with a similar dose-dependence; (3) exogenous dioctanoylglycerol overcame sphingosine's inhibition of platelet activation. The effectiveness of sphingosine in inhibiting platelet activation was primarily determined by the ratio of sphingosine to total number of platelets. These data are discussed in relation to a general approach for the use of sphingosine and other parameters for determining biological activities of protein kinase C.

INTRODUCTION

Protein kinase C, a Ca^{2+} - and phospholipid-dependent protein kinase, has been shown to play an important role in signal transduction, hormone action and cell growth and differentiation (Nishizuka, 1986). Protein kinase C exists as a family of seven isoenzymes (Nishizuka, 1988) that are primarily activated by diacylglycerol (DAG) generated from membrane phospholipids. This has implicated protein kinase C as an essential component of the phosphatidylinositol cycle of cell regulation (Nishizuka, 1986). Protein kinase C has been also demonstrated to be the intracellular receptor for phorbol diesters (Niedel *et al.*, 1983), which activate the enzyme by interacting at the DAG site (Nishizuka, 1986). Protein kinase C is therefore assumed to mediate the pleiotropic biological activities of phorbol esters.

A central issue in molecular cell biology is the determination of specific signal-transduction pathways mediating the actions of various extracellular agents. Because of the redundancy and overlap of different signalling pathways, it has been difficult to assign precise roles to different components of these pathways. Thus the use of inhibitors has assumed an important role in dissection of specific signalling pathways. A number of putative inhibitors for protein kinase C have been evaluated, but none has emerged as an ideal agent because of lack of specificity or poor activity *in vivo*.

Sphingosine has been shown to be a potent and specific inhibitor of protein kinase C *in vitro* (Hannun *et al.*, 1986) and in cell systems (Hannun & Bell, 1989; Merrill & Stevens, 1989). Sphingosine has unique properties that offer advantages in its use as an inhibitor for protein kinase C. Unlike most other inhibitors, its mechanism of action involves direct interaction with the regulatory domain of protein kinase C and competitive inhibition

with DAG/phorbol ester (Hannun *et al.*, 1986). Because of its amphiphilic nature, sphingosine can be easily delivered to cell systems where it is assumed to partition in membranes (the relevant site of action for protein kinase C inhibition). In fact, sphingosine has been shown, almost uniformly, to inhibit protein kinase C-mediated effects in various cell systems (Hannun & Bell, 1989). This property allows the use of sphingosine for exclusion of protein kinase C-mediated events, in that biological activities not inhibited by sphingosine are unlikely to be mediated by protein kinase C.

Problems, however, have been noted with the use of sphingosine in cellular systems. These appear predominantly to derive from its amphiphilic nature. Sphingosine's ability to insert into membranes imparts cytotoxic action (Pittet *et al.*, 1987), although cytotoxicity in certain cases has been attributed to direct inhibition of protein kinase C (Stevens *et al.*, 1990) or to the vehicle used to deliver sphingosine (Lambeth *et al.*, 1988). As with other lipid molecules, the effective concentration of sphingosine *in vitro* is its surface concentration, which is primarily determined by the ratio of sphingosine to total available surface (Hannun *et al.*, 1986). This property has resulted in difficulty in determining the relevant concentration of sphingosine in cellular studies. Sphingosine also has targets of action other than protein kinase C. These include tissue factor (Conkling *et al.*, 1989), thyrotropin receptor (Winicov & Gershengorn, 1988), epidermal-growth-factor receptor (Faucher *et al.*, 1988), phospholipase D (Lavie & Liscovitch, 1990), and calmodulin kinases (Jefferson & Schulman, 1988). Although, for the most part, these are affected at concentrations higher than required to inhibit protein kinase C, the range over which sphingosine is used has to be carefully determined. Moreover, and especially for long-term studies, sphingosine's effectiveness may be diminished by metabolic

Abbreviations used: DAG, *sn*-1,2-diacylglycerol; PDBu, phorbol dibutyrate.

‡ To whom all correspondence should be sent.

degradation or incorporation into sphingolipids (Hannun & Bell, 1989). Finally, questions have been raised concerning the validity of use of commercial sphingosine as a source for sphingosine, and whether sphingosine indeed is the active species in such preparations (Igarashi *et al.*, 1989).

In this study we have utilized synthetic *D-erythro*-sphingosine to delineate the activity of this agent against protein kinase C in intact platelets. We also develop an approach for the evaluation of sphingosine as a pharmacological inhibitor of protein kinase C, using platelets as a model system.

EXPERIMENTAL

Materials

Human γ -thrombin was kindly provided by Dr. John Fenton II (Division of Laboratories and Research, New York State Department of Health, Albany, NY, U.S.A.). Phorbol dibutyrate (PDBu) and prostaglandin I_2 were purchased from Sigma Chemical Co. Reagents for SDS/PAGE were obtained from Bio-Rad. [32 P]P_i and [3 H]PDBu were from New England Nuclear.

Synthetic *D-erythro*-sphingosine was prepared by a modification of three independently published methods (Garner *et al.*, 1988; Herold, 1988; Nimkar *et al.*, 1988). The synthetic material was purified by flash chromatography, dried *in vacuo* at 40 °C for 8 h and stored in a freezer under argon. 3 H-labelled *D-erythro*-sphingosine was prepared by addition of [3 H]trialkylstannane to the triple bond of a protected alkynyl sphingosine precursor [1,1-dimethylethyl-2,2-dimethyl-4-(1-hydroxy-2-hexadecynyl)-3-oxazolidine carboxylate] (Garner *et al.*, 1988). The resulting vinylstannane was converted into [3 H]-*D-erythro*-sphingosine by acidic hydrolysis of the protecting and stannyl groups. This material (sp. radioactivity 8 mCi/mmol) was purified and stored as described above for *D-erythro*-sphingosine.

Methods

Preparation of human platelets. Washed human platelets were prepared from blood drawn from healthy drug-free volunteers as described by Siess *et al.* (1983) with a slight modification. Briefly, blood (1 vol.) was drawn in a tube containing acid-citrate-dextrose (9 vol.) as anticoagulant. Platelet-rich plasma was separated by centrifuging at 200 *g* for 20 min at room temperature. Prostaglandin I_2 was added to platelet-rich plasma to a final concentration of 5 ng/ml, and platelets were separated by further centrifuging at 800 *g* for 15 min. The platelets were suspended in modified Tyrode/Hepes buffer, pH 7.4, containing 134 mM-NaCl, 12 mM-NaHCO₃, 2.9 mM-KCl, 5 mM-Hepes, 5 mM-glucose and 300 ng of prostaglandin I_2 /ml. Platelets were counted after 1:10 dilution with Tyrode buffer. The platelets were suspended in Tyrode buffer to a final concentration of $(2.5-3) \times 10^8$ /ml and used for whole-platelet phosphorylation and aggregation and secretion studies.

Platelet aggregation and ATP secretion. Aggregation and ATP secretion of washed platelets was measured by adding 10 μ l of luciferin/luciferase (Chronolume) to 390 μ l of platelet suspension and measuring percentage light transmission and ATP-induced luminescence as described by Hannun *et al.* (1987), by using a Chronolog aggregometer.

Substrate phosphorylation in whole platelets. The 40 kDa-substrate phosphorylation induced by 8 nM- γ -thrombin was studied as described by Hannun *et al.* (1986). The phosphorylated proteins were separated on SDS/PAGE. Gels were stained, destained and dried under vacuum. The phosphorylated bands were made visible by autoradiography.

[3 H]PDBu binding to human platelets. [3 H]PDBu binding to whole platelets was carried out as described by Hannun *et al.* (1986).

Platelet uptake of [3 H]sphingosine. Platelets (1 ml of 2.5×10^8 /ml) in Tyrode buffer were added to a tube containing 74 nCi of [3 H]sphingosine (sp. radioactivity 7 mCi/mmol) in 2 μ l of ethanol. The tubes were gently shaken, and the contents were centrifuged immediately at 800 *g* for 15 min to separate platelets and supernatant. The pellets were resuspended in 1 ml of Tyrode buffer. Total lipids were extracted from the pellet suspension and the supernatant as described by Bligh & Dyer (1959). Uptake of sphingosine was determined as the ratio of radioactivity in the pellet to total added radioactivity.

Metabolism of [3 H]sphingosine. Platelets (5×10^8) in 5 ml of Tyrode buffer were incubated with 10 μ Ci of [3 H]sphingosine (at either 10 or 50 μ M). At the indicated time points, lipids were extracted as described by Bligh & Dyer (1959). Lipids in the organic phase were separated by t.l.c. using chloroform/methanol/2 M-NH₃ (40:10:1, by vol.). Sphingosine spots were scraped and counted for radioactivity in scintillation fluid with an LKB counter.

RESULTS

The chemical identity and purity of synthetic *D-erythro*-sphingosine were verified by comparison of its 250 MHz proton n.m.r. spectrum with published spectra of *D-erythro*-sphingosine, h.p.l.c. analysis of the *o*-phthalaldehyde derivative (Merrill *et al.*, 1988) and t.l.c. analysis. H.p.l.c. analysis was particularly important in verifying that the product was free of contamination from other sphingosine diastereomers. The water content of the dried product was determined to be less than 0.1 % by differential thermogravimetric analysis. Once dried to this level, the synthetic material can be stored for extended periods without difficulty, whereas slightly moist sphingosine is degraded quickly, particularly in contact with air.

The purity, position of the label and specific radioactivity of [3 H]-*D-erythro*-sphingosine were determined by 250 MHz proton n.m.r., h.p.l.c. analysis employing radiochemical detection, and t.l.c.-radioscan analysis. A representative h.p.l.c. profile is shown in Fig. 1.

Metabolism

A potential problem in the use of sphingosine arises from its metabolism. Human platelets contain sphingosine kinase, which is able to metabolize sphingosine to sphingosine 1-phosphate (Stoffel *et al.*, 1973) which may be further degraded to ethanolamine phosphate and hexadecenal. When added to intact platelets, [3 H]-*D-erythro*-sphingosine is rapidly metabolized, with

Fig. 1. H.p.l.c. analysis of synthetic *D-erythro*-sphingosine as its *o*-phthalaldehyde (OPA) derivative

Sample: OPA derivative of synthetic *D-erythro*-sphingosine.
Solvent: 8 % water/methanol. Flow rate: 1 ml/min. Column:
4.6 mm $\times$ 25 cm; 5 μ m C₁₈.

Fig. 2. (a) Metabolism of [4-³H]-D-erythro-sphingosine by intact platelets, and (b) effectiveness of sphingosine as a function of preincubation time

(a) Platelet suspension (5 ml; 5×10^9 total) were incubated with 10 μCi of [³H]sphingosine with (●) 10 or (■) 50 μM synthetic D-erythro-sphingosine. Radioactivity in sphingosine was determined as described in the Experimental section. (b) Platelets (2.5×10^8 /ml) were preincubated with 20 μM-sphingosine for the indicated time, then treated with 8 nM-γ-thrombin. To facilitate comparison with (a), results are expressed as percentage inhibition relative to the initial time point.

50–60% being degraded in 1 min (Fig. 2a) and approx. 90% degraded in 1 h. This rapid metabolism modulates the effectiveness of sphingosine. The ability of sphingosine to inhibit platelets is progressively lost with increasing preincubation time (Fig. 2b).

Monitoring responses to sphingosine

Multiple criteria should be evaluated when determining whether an effect of sphingosine on a cellular response is mediated by protein kinase C. A comparison of dose-dependence of inhibition of protein kinase C *in vitro* and in intact cells, inhibition of phorbol binding and inhibition of the biological response should be evaluated under nearly identical experimental conditions, a common problem being the determination of these different activities of sphingosine under different conditions (for example, using different cell numbers, cell concentration or in the presence of interfering substances such as serum albumin).

Using high-purity synthetic D-erythro-sphingosine and human platelets as a model system, we illustrate an approach for evaluating sphingosine as a pharmacological inhibitor of protein kinase C.

Effects of sphingosine on 40 kDa-substrate phosphorylation in intact human platelets

Agonist-induced activation of human platelets results in phosphorylation of a 40 kDa protein, known to be a selective protein kinase C substrate (Sano *et al.*, 1983). Phosphorylation of multiple other substrates (22–44 kDa) is also noted in response to γ-thrombin (Fig. 3, lane 2) and in response to activation of

Fig. 3. D-erythro-Sphingosine inhibition of 40 kDa-substrate phosphorylation in intact platelets

³²P-labelled platelets (2.5×10^8 /ml) were preincubated with the indicated concentration of sphingosine (SPH) for 30 s and stimulated for 2 min with 8 nM-γ-thrombin. The reaction was stopped by adding an equal volume of 2-fold-conc. sample buffer, and phosphorylated proteins were separated by SDS/PAGE as described in the Experimental section. The 40 kDa band was excised and its radioactivity was counted. The values are expressed as percentage of phosphorylation without added sphingosine after subtraction of the radioactivity of a blank sample (first lane).

protein kinase C by phorbol esters (results not shown). Synthetic D-erythro-sphingosine caused significant inhibition of 40 kDa-substrate phosphorylation in response to the action of 8 nM-γ-thrombin on human platelets, with complete inhibition occurring at 20 μM (Fig. 3). Inhibition of other, less well-characterized, substrates of protein kinase C is also noted. These data show that synthetic D-erythro-sphingosine inhibits protein kinase C-mediated substrate phosphorylation in intact platelets, and they confirm previous results obtained with commercial preparations of sphingosine (Hannun *et al.*, 1986). They are in contrast with the data of Krishnamurthi *et al.* (1989), although no quantitative results were presented in that study. The effects of sphingosine on protein phosphorylation provide a necessary and sufficient requirement to establish the ability of sphingosine to inhibit cellular protein kinase C activity.

Effects of sphingosine on platelet aggregation and secretion

In earlier studies, commercial sphingosine was shown to inhibit platelet aggregation when studied in platelet-rich plasma (Hannun *et al.*, 1987). To eliminate the effects of albumin and other serum proteins in these preparations that may bind sphingosine and thus decrease its potency, we evaluated the activity of sphingosine against platelet aggregation and secretion, using washed human platelets. Synthetic D-erythro-sphingosine inhibited platelet aggregation in a dose-dependent manner, with near-total inhibition occurring at approx. 20 μM (Fig. 4). It also displayed a similar dose-response in inhibiting platelet secretion of ATP (Fig. 4).

Inhibition of [³H]PDBu binding to intact platelets

Under nearly identical conditions (with the same number and concentration of cells and the same buffer), synthetic D-erythro-sphingosine inhibited [³H]PDBu binding to intact platelets in a dose-dependent manner (Fig. 5). This dose-dependence was similar to the concentration of sphingosine that is required for inhibition of 40 kDa-substrate phosphorylation (Fig. 3).

The dose-dependence for inhibition of endogenous substrate phosphorylation and PDBu binding in intact cells closely resem-

bles the concentration-dependence for inhibition of platelet aggregation in response to agonists (Fig. 4). From these studies it can be concluded that inhibition of platelet aggregation by sphingosine occurs in parallel with its ability to inhibit protein kinase C, as judged by the inhibition of endogenous substrate phosphorylation and PDBu binding.

Over-ride of sphingosine inhibition by diacylglycerol or phorbol esters

Although the above studies suggest a role for protein kinase C in mediating platelet aggregation, they do not prove such a role. Further proof requires the demonstration that sphingosine effects could be selectively overcome by the action of exogenous diacylglycerol or phorbol ester. Since sphingosine is a competitive inhibitor of protein kinase C with respect to DAG (or phorbol esters) (Hannun *et al.*, 1986), it is expected that addition of these activators should overcome inhibition of protein kinase C by sphingosine in platelets. The addition of dioctanoylglycerol or PDBu can specifically overcome the ability of sphingosine to inhibit platelet aggregation (Fig. 6). The data in Fig. 6 indicate that 30 μM synthetic *D-erythro*-sphingosine completely inhibited the aggregation and secretion induced by 8 nM- γ -thrombin (Figs. 6a and 6b). When DiC₈ (dioctanoylglycerol; 40 μM) was added to platelets pretreated with sphingosine and γ -thrombin, full aggregation and secretion was observed (Fig. 6c). DiC₈ by itself at this concentration caused only minimal response (results not shown). These results clearly demonstrate that protein kinase C activators can overcome sphingosine inhibition of platelet activation.

Estimation of effective concentration of sphingosine

One of the difficulties encountered when evaluating sphingosine in cell systems is the determination of its effective concentration. This problem primarily arises from the amphi-

Fig. 5. *D-erythro*-Sphingosine inhibition of [^3H]PDBu binding to intact human platelets

Platelets (0.4 ml; $2.5 \times 10^8/\text{ml}$) were incubated for 1 min with the indicated concentration of synthetic *D-erythro*-sphingosine, and [^3H]PDBu binding was carried out in the absence or presence of 100-fold excess of unlabelled PDBu. Specific binding was determined by subtracting non-specific binding from total binding. The data are expressed as percentage of binding without added sphingosine (B_{max}).

philic nature of sphingosine and its predominant surface localization (Slife *et al.*, 1989). The potency *in vitro* of sphingosine against protein kinase C is primarily determined by its surface concentration (i.e. its molar ratio to other membrane components). In cell systems, it is expected that the potency of sphingosine would be determined by the partitioning of sphingosine into lipid bilayers and by the ratio of mass of sphingosine to total mass of lipids. In washed human platelets (that is, in the

Fig. 4. *D-erythro*-Sphingosine inhibition of human platelet aggregation and secretion

Platelets (0.4 ml; 2.5×10^8 platelets/ml) were treated with 8 nM- γ -thrombin, and aggregation (upper panels) and secretion (lower panels) were monitored. The effect of sphingosine was evaluated by preincubating the platelets with the indicated concentration of sphingosine (SPH) for 30 s. Arrows indicate the point of γ -thrombin addition.

Fig. 6. Reconstitution of *D-erythro*-sphingosine-inhibited aggregation and secretion by DiC_8

(a) Aggregation and secretion with 8 nM- γ -thrombin; (b) platelets preincubated with 30 μM -*D-erythro*-sphingosine (SPH) followed by 8 nM- γ -thrombin; (c) over-riding the sphingosine inhibition by 40 μM - DiC_8 . The arrows indicate the point of addition of indicated components.

Fig. 7. Effect of cell number on sphingosine inhibition of aggregation

Platelets were diluted to either $1.25 \times 10^8/\text{ml}$ (●) or $2.5 \times 10^8/\text{ml}$ (□), and aggregation was monitored as described in the Experimental section, (a) as a function of sphingosine concentration, and (b) as a function of ratio of sphingosine to cell number.

remaining in solution. This decreases the problem of determining the effective concentration of sphingosine to that of determining the ratio of sphingosine to membrane lipids. This can be formulated as follows:

$$[\text{Sphingosine}]_{\text{effective}} \propto \text{mass of sphingosine/total cells}$$

or

$$[\text{sphingosine}]_{\text{effective}} \propto [\text{sphingosine}]/[\text{cells}]$$

where [sphingosine] is the molar concentration of sphingosine and [cells] is the cell density expressed as cells/volume.

The validity of this analysis is illustrated by the following experiments. The potency of *D-erythro*-sphingosine in inhibiting platelet aggregation is found to vary depending on cell number. As shown in Fig. 7, synthetic *D-erythro*-sphingosine shows 50% inhibition at around 10 μM and complete inhibition at 20 μM when 2.5×10^8 cells are used. However, when half as many cells are used (1.25×10^8 cells), 50% inhibition is observed at approx. 5 μM and complete inhibition at 10 μM (Fig. 7a). When the data are replotted as aggregation versus the ratio of sphingosine to cells (pmol of sphingosine/ 10^8 platelets), a nearly identical inhibition profile is observed with sphingosine (Fig. 7b) irrespective of the number of platelets used. Therefore the ability of sphingosine to inhibit cellular responses is primarily determined by its surface concentration.

DISCUSSION

The studies reported here illustrate an approach for employing sphingosine as a cellular inhibitor of protein kinase C, by using human platelets as a model. High-purity synthetic *D-erythro*-sphingosine was used to eliminate uncertainty in the quality of experimental data due to possible variations in the quality of commercial sphingosine. As illustrated in Fig. 7, the effective concentration of sphingosine is primarily determined by the ratio of sphingosine to cell number, which is predicted from the preferential partitioning of sphingosine into cellular membranes. Therefore, for comparison of effectiveness of sphingosine between different experiments and different cell systems, an index relating sphingosine's concentration to cell number or total cellular lipids is more appropriate than the bulk (molar) concentration of sphingosine. [It should be noted that expressing the concentration in relation to cellular lipids or cell number provides only an approximation for sphingosine's effective concentration. The effectiveness of sphingosine could also be modulated by partitioning into other membranes of cells, where sphingosine may not interact with protein kinase C, or by the presence of fat droplets in cells, which would act as a sink for sphingosine (Robertson *et al.* 1989).]

Synthetic *D-erythro*-sphingosine inhibited platelet aggregation in a dose-dependent manner. Under identical conditions, it also inhibited phosphorylation of the 40 kDa substrate of protein kinase C and PDBu binding to intact platelets. These parameters, coupled with the ability of sphingosine to inhibit purified platelet protein kinase C (Hannun *et al.*, 1986; Khan *et al.*, 1990), strongly suggest a relation between the inhibition of protein kinase C and inhibition of platelet biology.

Major confirmation for a role for protein kinase C inhibition in mediating the action of sphingosine arises from the use of DAG (or phorbol esters) to overcome the action of sphingosine. This result indicates that sphingosine is either acting at the same site as DAG (i.e. protein kinase C) or at a more proximal site. Therefore, when coupled to the inability of sphingosine to decrease DAG formation in response to platelet activators (Hannun *et al.*, 1987), this criterion provides strong evidence for a role for protein kinase C.

absence of serum proteins), synthetic *D-erythro*-sphingosine is predominantly taken up by platelets, with more than 74% of added material recovered in the platelet pellet and less than 25%

However, because sphingosine is not an exclusive inhibitor of protein kinase C (although most other targets are affected at concentrations higher than those required to inhibit protein kinase C), studies with sphingosine should not be taken as conclusive in demonstrating a role for protein kinase C. Such a role is better ascertained by employing multiple criteria and approaches. For example, it is important to demonstrate that the action of agonists on cells causes an elevation in DAG levels and activation of protein kinase C, as determined by phosphorylation of endogenous substrates. These substrates should be phosphorylated in response to protein kinase C both *in vitro* and in intact cells with similar tryptic maps, and on the same serine/threonine residues (although the concomitant action of other kinases/phosphatases may create discrepancies between cellular phosphorylation patterns and those *in vitro*).

Another parameter indicating activation of protein kinase C relates to the ability of agonists to cause 'translocation' of protein kinase C from cytosolic to particulate fractions (Kraft & Anderson, 1983). However, the validity of this phenomenon (which probably arises from increased affinity of protein kinase C to membranes after/during exposure to DAG) has not been rigorously evaluated (for example, what is the minimum duration and amplitude of the DAG/Ca²⁺ signal that is sufficient for translocation?). Supporting evidence is also provided by demonstrating that phorbol esters (or exogenous DAGs) cause at least part of the spectrum of biological/biochemical effects of natural agonists. Because protein kinase C activation may contribute only part of the natural signalling pathways, its activation may not reconstitute the full biological picture. It is also suggested that other inhibitors be evaluated, such as H7 (Hidaka *et al.*, 1984) and staurosporine (Tamaoki *et al.*, 1986), which may show some specificity toward protein kinase C, and H8 which lacks specificity.

Down-regulation of protein kinase C, in response to prolonged action of phorbol esters (Rodriguez-Pena & Rozengurt, 1984), has also been used to assess its physiological roles, so that responses to agonists that persist following down-regulation are unlikely to be related to protein kinase C, whereas responses that are abolished are presumed to involve protein kinase C. Two major problems limit the usefulness of this approach. First, not all isoenzymes of protein kinase C are down-regulated (Kariya & Takai, 1987); second, the prolonged exposure of cells to phorbol esters that is required to down-regulate protein kinase C may significantly alter cellular events and signal-transduction pathways.

In conclusion, a role for protein kinase C in a biological response requires an integrated approach that investigates multiple parameters such as DAG response, protein kinase C activations *in vivo* and the careful use of inhibitors. In this context, negative results with sphingosine help rule out a role for protein kinase C, while a controlled positive effect adds support to such a role.

We thank Dr. Mark H. Werner for careful review of the manuscript, and Marsha Haigood for expert secretarial assistance. This work was

supported in part by grants from NCI (CA46738) and from NIH (HL43707).

REFERENCES

- Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- Conkling, P. R., Patton, K. L., Hannun, Y. A., Greenberg, C. S. & Weinberg, J. B. (1989) *J. Biol. Chem.* **264**, 18440–18444
- Faucher, M., Girones, N., Hannun, Y. A., Bell, R. M. & Davis, R. (1988) *J. Biol. Chem.* **263**, 5319–5327
- Garner, P., Park, J. M. & Malecki, E. (1988) *J. Org. Chem.* **53**, 4395–4398
- Hannun, Y. A. & Bell, R. M. (1989) *Science* **243**, 500–507
- Hannun, Y. A., Loomis, C. R., Merrill, A. H., Jr. & Bell, R. M. (1986) *J. Biol. Chem.* **261**, 12604–12609
- Hannun, Y. A., Greenberg, C. S. & Bell, R. M. (1987) *J. Biol. Chem.* **262**, 13620–13626
- Herold, P. (1988) *Helv. Chim. Acta* **71**, 354–362
- Hidaka, H., Inagaki, M., Kawamoto, S. & Sasaki, Y. (1984) *Biochemistry* **23**, 5036–5041
- Igarashi, Y., Hakomori, S., Toyokuni, T., Dean, B., Fujita, S., Sugimoto, M., Ogawa, T., El-Ghendi, K. & Racker, E. (1989) *Biochemistry* **28**, 6796–6800
- Jefferson, A. B. & Schulman, H. (1988) *J. Biol. Chem.* **263**, 15241–15244
- Kariya, K. & Takai, Y. (1987) *FEBS Lett.* **219**, 119–124
- Khan, W. A., Dobrowski, R., El Touny, S. & Hannun, Y. A. (1990) *Biochem. Biophys. Res. Commun.* **172**, 683–691
- Kraft, A. S. & Anderson, W. B. (1983) *Nature (London)* **301**, 621–623
- Krishnamurthi, S., Patel, Y. & Kakkar, V. V. (1989) *Biochim. Biophys. Acta* **1010**, 258–264
- Lambeth, J. D., Burnham, D. N. & Tyagi, S. R. (1988) *J. Biol. Chem.* **263**, 3818–3822
- Lavie, Y. & Liscovitch, M. (1990) *J. Biol. Chem.* **265**, 3868–3872
- Merrill, A. H., Jr. & Stevens, V. L. (1989) *Biochim. Biophys. Acta* **1010**, 131–139
- Merrill, A. H., Jr., Wang, E., Mullins, R. E., Jamison, W. C. L., Nimkar, S. & Liotta, D. C. (1988) *Anal. Biochem.* **171**, 373–381
- Niedel, J. E., Kuhn, L. J. & Vandenbark, G. R. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 36–40
- Nimkar, S., Menaldino, D., Merrill, A. H., Jr. & Liotta, D. C. (1988) *Tetrahedron Lett.* **29**, 3037–3040
- Nishizuka, Y. (1986) *Science* **233**, 305–312
- Nishizuka, Y. (1988) *Nature (London)* **334**, 661–665
- Pittet, D., Krause, K.-H., Wollheim, C. B., Bruzzzone, R. & Lew, D. P. (1987) *J. Biol. Chem.* **262**, 10072–10076
- Robertson, D. G., DiGirolamo, M., Merrill, A. H., Jr. & Lambeth, J. D. (1989) *J. Biol. Chem.* **264**, 6773–6779
- Rodriguez-Pena, A. & Rozengurt, E. (1984) *Biochem. Biophys. Res. Commun.* **120**, 1053–1059
- Sano, K., Takai, Y., Yamanishi, J. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 2010–2013
- Siess, W., Siegel, F. & Lapetina, E. G. (1983) *J. Biol. Chem.* **258**, 11236–11242
- Slife, C. W., Wang, E., Hunter, R., Wang, S., Burgess, C., Liotta, D. C. & Merrill, A. H., Jr. (1989) *J. Biol. Chem.* **264**, 10371–10377
- Stevens, V. L., Nimkar, S., Jamison, W. C. L., Liotta, D. C. & Merrill, A. H., Jr. (1990) *Biochim. Biophys. Acta* **1051**, 37–45
- Stoffel, W., Heimann, G. & Hellenbroich, B. (1973) *Hoppe-Seyler's Z. Physiol. Chem.* **354**, 562–566
- Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. & Tomita, F. (1986) *Biochem. Biophys. Res. Commun.* **135**, 397–402
- Winicov, I. & Gershengorn, M. C. (1988) *J. Biol. Chem.* **263**, 12179–12182