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INHIBITION OF PROTEIN KINASE C BY SPHINGOSINE CORRELATES WITH THE PRESENCE OF POSITIVE CHARGE

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The role of the 2-amino group of sphingosine on the in vitro inhibition of protein kinase C was investigated by comparing protein kinase C activity in the presence and absence of sphingosine at various pH's. Inhibition by sphingosine was found to be pH dependent. Above pH 7.75, sphingosine has little or no inhibitory effect. In fact, at pH 8.5, sphingosine slightly enhances enzyme activity above that which occurs when the enzyme is stimulated by diacylglycerol and phosphatidylserine. After correcting for electrostatic repulsion, we find that the intrinsic pK for sphingosine in Triton micelles is 8.5. Inhibition of protein kinase C by sphingosine at physiological pH's therefore correlates with the presence of a positive charge.

Various agonists acting through their specific membrane receptors mediate in vivo effects by generating second messengers, some of which activate protein kinase C. Protein kinase C has attracted much interest because it has been implicated in cell growth and differentiation, as well as cellular regulation and oncogenesis [1]. As a result, many investigations have uncovered a number of compounds, possessing diverse chemical structures which activate or inhibit protein kinase C [2-4]. In particular, charge has been shown to play an important role in modulating enzyme function [3]. A number of positively charged compounds inhibit polycationic substrate phosphorylation by protein kinase C [2,3]. Specific inhibitors are of interest because they can potentially increase our understanding of the mechanism of enzyme action as well as enzyme function in cells. Sphingosine, a constituent of the sphingolipids is a potent protein kinase C inhibitor [for a recent review, see 5]. Much interest has been given to studying sphingosine inhibition of protein kinase C because it is a natural constituent of cells [6]. In fact, it has been proposed that sphingosine may be an important in vivo modulator of signal transduction and further, through the inhibition of protein kinase C, it may be a causative agent in the pathogenesis of the sphingolipidoses [7,8]. Yet, despite numerous investigations, the exact mechanism by which sphingosine inhibits protein kinase C still remains speculative. Recently, a detailed

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study investigating the important structural features of sphingoid bases indicated that maximum inhibition of protein kinase C requires a free amino group and a C-18 aliphatic side chain [9]. The results of our study suggest that sphingosine's inhibitory effect on protein kinase C is dependent on its positive charge.

MATERIALS AND METHODS

Materials: Bovine brain phosphatidylserine was purchased from Avanti Polar Lipids, Birmingham, AL, D-sphingosine and histone (type III-S) were purchased from the Sigma Chemical Company, St. Louis, MO and [γ-^{32}P] adenosine 5'-triphosphate was from NEN, Quebec. Protein kinase C was purified from rat brain as previously described [10]. The enzyme was never frozen and was stored at 4°C at a protein concentration of 115 µg/mL in 25% ethylene glycol.

Mixed micelle assay for protein kinase C: The Triton X-100 assay as previously described by Bell and coworkers was used to measure enzyme activity [11]. Material soluble in organic solvent, including phosphatidylserine and 1,2-diolein with and without sphingosine were combined in a solution of chloroform/methanol (2/1, v/v). The chloroform/methanol solutions were dispensed into appropriate assay tubes. Solvent was evaporated with a stream of nitrogen and last traces removed in a vacuum desiccator at 40°C. The lipid films were then solubilized by the addition of 3% Triton X-100, vortexed vigorously for 30 sec and then incubated at 30°C for 10 min to allow equilibration. A 25 µL aliquot of this solution was used in a final assay volume of 250 µl, containing 20 mM Tris-HCl, 10 mM $MgCl_2$, 200 µg/mL histone III-S, 100 µM $CaCl_2$, 10 µM [γ-^{32}P] adenosine 5'-triphosphate, 2.75 mM Triton X-100 with 8 mol % phosphatidylserine and 2.5 mol % diolein. Where present, the sphingosine concentration was 160 µM. The reaction was carried out at various pH's ranging from pH 7 to pH 8.5. For controls, 25 µL of 2 mM EGTA replaced the $CaCl_2$. To initiate the reaction, 90 ng of protein kinase C was added. After briefly vortexing, the tubes were incubated for 15 min at 30°C. The reaction was terminated by adding 1 mL of cold 0.5 mg/mL BSA and 1 mL of cold 25% trichloroacetic acid. The samples were placed on ice for 15 min, then filtered through Whatman GF/C filters which were then washed 5 times with 2 mL each of ice-cold 25% trichloroacetic acid. After drying, the filters were counted with 6 mL ACS scintillation fluid.

Estimation of the intrinsic pK of sphingosine: The intrinsic pK of sphingosine in the Triton micelle assay was estimated using [^{1}H] NMR. NMR spectra were recorded on a Bruker-AM500 spectrometer. A sample for NMR was prepared by taking 1 mL of a 10% Triton solution made up in 5% ^2H_2O, 95% deionized distilled water, and vortexing vigorously with 10 mg of D-sphingosine until homogeneous. Sphingosine-containing Triton micelles were titrated with 0.01 M NaOH and 0.01 M HCl, taking a pH reading before and after the NMR spectra were recorded. In an independent experiment, the same result was confirmed by potentiometric titration after correcting for the amount of base required to alter the pH of a solution of the detergent alone.

RESULTS AND DISCUSSION

This study assayed protein kinase C activity in the presence and absence of sphingosine over the pH range of 7 to 8.5. The pH range for optimal enzyme activity in the absence of sphingosine is between 7.5 and 8.0, agreeing with previous findings [10]. When sphingosine is present in Triton micelles, inhibition of protein kinase C occurs in a pH dependent manner (Fig. 1). We find that between pH's 7 and 7.5, inhibition appears to remain constant; the

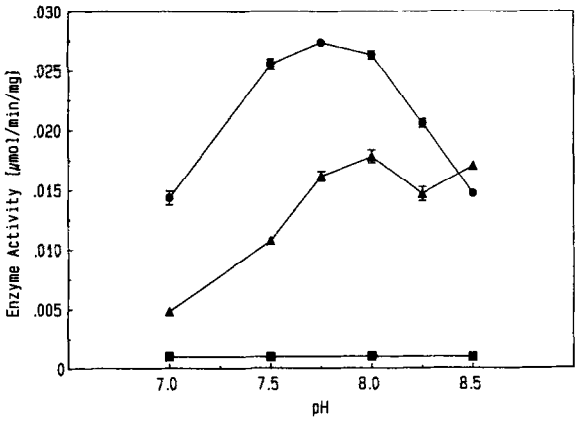

Figure 1. The pH dependence of diolein-stimulated protein kinase C activity in Triton X-100 micelles in the absence of sphingosine (●) and in the presence of 160 μM sphingosine (▲). Control in Triton X-100 micelles with EGTA and with no other additives (■).

curves of the inhibited and uninhibited enzyme are roughly parallel. However, at pH's above 7.75, sphingosine is not as potent an inhibitor; both curves begin to converge. At pH 8.5 sphingosine actually activates the enzyme to a small extent. We were unable to extend this study to lower or higher pH's because of loss of buffering capacity or loss of enzyme activity, respectively.

The decreasing inhibition with increasing pH suggested an effect resulting from a change in the state of ionization of sphingosine either in the Triton micelle or bound to protein kinase C. To assess the state of protonation of sphingosine in Triton micelles at various pH's, a titration measuring the NMR spectra as a function of pH was carried out. The chemical shift of the CH proton adjacent to the amino group in sphingosine is sensitive to the protonation state of the amino group and was displaced from 4.24 to 3.88 ppm between the pH's 4.38 and 8.95. We could not generate a complete titration curve at higher pH's because the CH proton signal of interest merged with a resonance generated by the detergent. Nevertheless, at extremely high pH's, the chemical shift was not displaced beyond this resonance to a lower chemical shift thus allowing us to approximate the upper limit of the titration curve. In an independent experiment, a potentiometric titration gave a curve that was superimposable with that which was obtained by NMR [Fig. 2]. From the titration curve, the apparent pK of sphingosine in Triton micelles is 7.7. This is in contrast to a recent study which reported the pK to be 6.7 [9]. This may be due to our use of a 20 fold greater concentration of sphingosine and Triton X-100. Although the sphingosine to Triton ratio is the same in both studies, more acid and base is required for titrating the 20 fold greater quantity of sphingosine present in our Triton micelles. Consequently the ionic strength of the solution would be increased. This

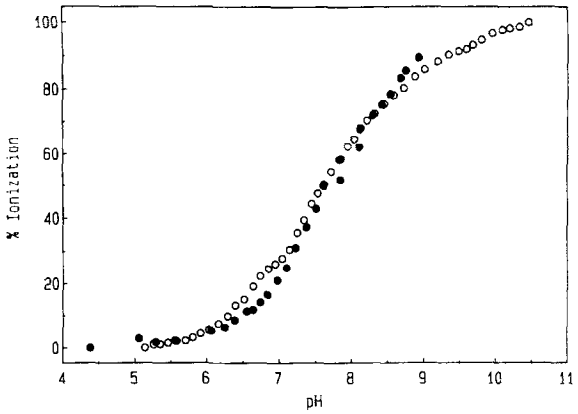

Figure 2. pH titration curves of sphingosine in Triton X-100 measured potentiometrically (○) and with [^{1}H] NMR (●) as described under methods.

would suppress electrostatic repulsion and as a result, raise the apparent pK to higher values probably contributing to the observed discrepancy. Regardless of the precise details of the ionization properties of sphingosine, it is clear that inhibition of protein kinase C by sphingosine correlates with its state of protonation.

An unusual feature of the sphingosine titration curve is that unlike the titration curves of small, soluble molecules, the titration curve obtained for sphingosine in Triton micelles is broad. This can be explained by electrostatic interaction among sphingosine molecules within a detergent micelle [13]. Figure 3 shows the data for the titration curve plotted to take into account electrostatic repulsion [13]. In cases where electrostatic interactions do not occur, pH-log[α/(1-α)] is independent of pH. However, this is not the case for sphingosine in Triton micelles, suggesting that the

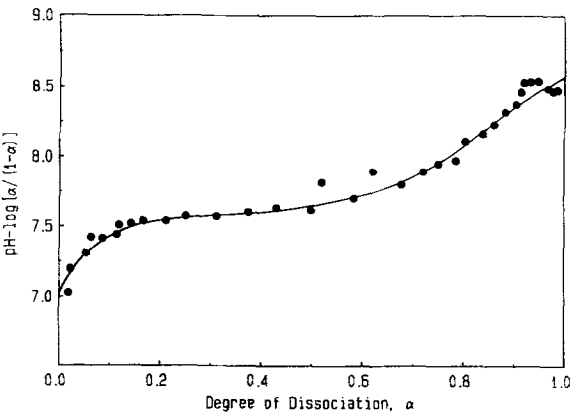

Figure 3. Dependence of pH-log[α/(1-α)] on the degree of dissociation of sphingosine (α). The data used are from Figure 2. Because the NMR titration method does not require correction for a blank, the data from NMR is considered more accurate and is used to generate this analysis. However, above pH 9, we used the potentiometric titration curve to complete this plot.

apparent pK is affected by electrostatic repulsion in the micelle. When the net charge of sphingosine is zero (i.e. when the degree of dissociation, α is 1), the intrinsic pK can be obtained from the value of the ordinate. We find that the intrinsic pK for sphingosine is 8.5.

We suggest that the inhibition by sphingosine requires the presence of positive charge. A number of previous studies have noted the necessity of the free amino group of sphingosine for the inhibition of protein kinase C, implicating the importance of a positive charge. N-acetyl sphingosine, an analog of sphingosine does not alter the activity of isolated protein kinase C nor the effect of this enzyme in human platelets [14]. Neither does N,N dimethyl sphingosine, a derivative without a dissociable proton, affect protein kinase C activity [9]. The mechanism of inhibition of protein kinase C by sphingosine is complex. It has been shown to be a competitive inhibitor with respect to Ca^{2+} [15], which could implicate it binding to an anionic site on the protein. However, sphingosine is also competitive with respect to diolein and to phorbol dibutyrate [15]. It has also been suggest that sphingosine inhibits protein kinase C by interacting with and neutralizing the charge of anionic lipids necessary for protein kinase C activation [16]. This type of interaction was shown to inhibit histone translocation to the membrane [16]. In addition to charge, it has been shown that the effect of membrane additives on lipid phase propensity determines their effect on protein kinase C activity [3,17]. Since sphingosine is a hexagonal phase-promoter [3], it would be expected to be an activator of protein kinase C, if it were not for its positive charge. At pH 8.5, where the positive charge on sphingosine is almost completely removed (Fig. 2), sphingosine becomes a weak activator of protein kinase C (Fig. 1), as predicted on the basis of its effect on lipid polymorphism [3,17]. Unfortunately, protein kinase C did not exhibit activity at higher pH values required for the complete deprotonation of sphingosine. Nevertheless, it is clear that the presence of a substantial fraction of the positive charge on sphingosine is required for its inhibitory effect on protein kinase C.

REFERENCES

1. Nishizuka, Y. (1986) Science 233, 305-312.
2. Weinstein, I. B. (1988) Mutation Research 202, 413-420.
3. Epand, R. M. (1987) Chem.-Biol. Inter. 63, 239-247.
4. Epand, R. M., Stafford, A. R., Bottega, R. and Ball, E. H. (1989) Bioscience Reports, in press.
5. Merrill Jr., A. H. and Stevens, V. L. (1989) Biochim. Biophys. Acta 1010, 131-139.
6. Merrill Jr., A. H. and Wang, E. (1986) J. Biol. Chem. 261, 3764-3769.
7. Bell, R. M., Loomis, C. R., and Hannun, Y. A. (1988) In Protein Kinase C Regulation, Vol. LIII, pp. 103-110, Cold Spring Harbor Symposiua on Quantitative Biology.

8. Hannun, Y. A. and Bell, R. M. (1987) Science 235, 670-674.
9. Merrill Jr., H. A., Nimkar, S., Menaldino, D., Hannun, Y. A., Loomis, C., Bell, R. M., Tyagi, S. R., Lambeth, J. D., Stevens, V. L., Hunter, R., and Liotta, D. C. (1989) Biochemistry 28, 3138-3145.
10. Litchfield, D. W. and Ball, E. H. (1987) J. Biol. Chem. 262, 8056-8060.
11. Hannun, Y. A., Loomis, C. R., and Bell, R. M. (1985) J. Biol. Chem. 260, 10039-10043.
12. Kikkawa, U., Minakuchi, R., Takai, Y., and Nishizuka, Y. (1983) In Methods in Enzymology, Vol. 99, pp. 288-298. Academic Press, Inc.
13. Tanford, C. (1961) In Physical Chemistry of Macromolecules, pp. 526-586, John Wiley and Sons, Inc.
14. Hannun, Y. A., Greenberg, C. S., and Bell, R. M. (1987) J. Biol. Chem. 262, 13620-13626.
15. Hannun, Y. A., Loomis, C. R., Merrill, A. H., Jr., and Bell, R. M. (1986) J. Biol. Chem. 261, 12604-12609.
16. Bazzi, M. D. and Nelsestuen, G. L. (1987) Biochem. Biophys. Res. Commun. 146, 203-207.
17. Epand, R. M., Stafford, A. R., Cheetham, J. C., Bottega, R. and Ball, E. H. (1988) Bioscience Reports 8, 49-54.