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MECHANISM OF PROTEIN KINASE C INHIBITION BY SPHINGOSINE

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Summary. The in vitro mechanism by which sphingosine inhibits protein kinase C (PKC) was investigated by comparing enzyme activity and the physical associations of reaction components. Light scattering intensity measurements showed that sphingosine prevented the association of the substrate, histone, with micelles of Triton plus phosphatidylserine (PS). Addition of phosphatidylinositol (PI) or phosphatidylglycerol (PG) restored histone interaction. In direct correlation, both PI and PG were able to reverse inibition of PKC activity by sphingosine. In Triton mixed micelles, neither PI nor PG alone would support PKC activity or substrate-lipid binding. Inhibition of PKC by positively charged sphingosine appeared to be related to simple charge neutralization of the lipid, thereby preventing interaction with PKC and/or its protein substrate. © 1987 Academic Press, Inc.

The Ca^{2+} and phospholipid dependent protein kinase C (PKC) is a key regulatory enzyme believed to be involved in many cell functions (1,2). In vitro, the activation of PKC requires the presence of phospholipid and Ca^{2+}. Diacylglycerol or phorbol esters activate PKC by reducing the Ca^{2+} requirement. Recently, we reported that the activity as well as the cofactor requirements of PKC were modulated by factors such as the type of substrate, interaction of substrate with enzyme and/or phospholipid, the physical structure of the lipid components, and the ratio of phospholipid to substrates (3). These properties may be related to many in vitro agonists or antagonists of PKC which could have little relevance to in vivo agonists or antagonists of PKC. Understanding the mechanism of modulation by in vitro agonists of PKC is essential to assess the potential for in vivo influence on PKC.

Sphingosine and lysosphingolipid have been reported to inhibit the activity and the phorbol ester binding capability of PKC (4,5). This inhibition is proposed to be physiologically relevant, and to represent the "missing functional link" among many lipid disorders (5). In this study, we examined the mechanism of sphingosine inhibition of PKC. The results suggested that sphingosine exerted its function by decreasing the availability of phosphatidylserine.

Materials and Methods

Unless otherwise indicated, all chemicals were obtained from Sigma Chemical Company. [γ-^{32}P]ATP was from Amersham corporation. Nitrocellulose filters were from Millipore corporation. Highest purity bovine brain PS was from Avanti Polar Lipids.

PKC was purified to apparent homogeneity from bovine brain (6). Light scattering intensity measurements were performed as described previously (3). Large changes in light scattering intensity provide qualitative evidence of protein-lipid interaction. PKC activity was assayed as

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described by Hannun et al. (7). PS-Triton mixed micelles containing sphingosine were prepared by drying the lipid components at molar ratios of (PS:sphingosine) 1:2, 1:1, 1:0.5, and 1:0, followed by suspension of the lipid components with 3% Triton X-100 to give a PS content of either 8 or 5 mole % (mole PS/mole Triton). All other sphingosine compositions were obtained by serial dilutions of these mixtures. In all of the experiments reported here, the micelles contained 2.5 mole % diolein, and the final concentration of Triton X-100 was 0.3% (w/v).

Results and Discussion

The activity of PKC can be supported by phospholipids presented in the form of vesicles or in detergent micelles (7). The PS-Triton mixed micelle system was used for these studies, and the composition of micelles is expressed as mole percent of Triton. However, the state of the phospholipid in the activation assay is uncertain due to aggregation events (3).

The data shown in figure 1 were in agreement with previous results (4) and indicated that sphingosine inhibited PKC in a manner that was dependent on the PS content of the micelles. In fact, there appeared to be a direct relationship between the inhibitory levels of sphingosine needed and the amount of PS in the micelles, suggesting that sphingosine functioned by simple neutralization of the charge of the lipid component.

When histone was used as the substrate, both PKC-phospholipid and histone-phospholipid interactions are essential for phosphorylation by PKC (3). The effect of sphingosine on histone-PS interaction was examined by light scattering intensity measurements. In the absence of sphingosine, large changes in the light scattering intensity occurred upon the addition of histone to micelles containing 8 mole % PS (Fig. 2, trace #1). This showed that histone interacted with, and aggregated PS-Triton mixed micelles (3). However, micelles containing 8 mole % PS plus 8 mole % sphingosine did not interact with histone as indicated by the lack of significant change in light scattering intensity (Fig. 2, trace #2). Inhibition of PKC activity by sphingosine may therefore be due to inhibition of substrate-phospholipid interaction.

The addition of 5 mole % phosphatidylinositol (PI) to micelles containing 8 mole % PS and 8 mole % sphingosine restored the micelles ability to bind histone and undergo aggregation (Fig. 3,

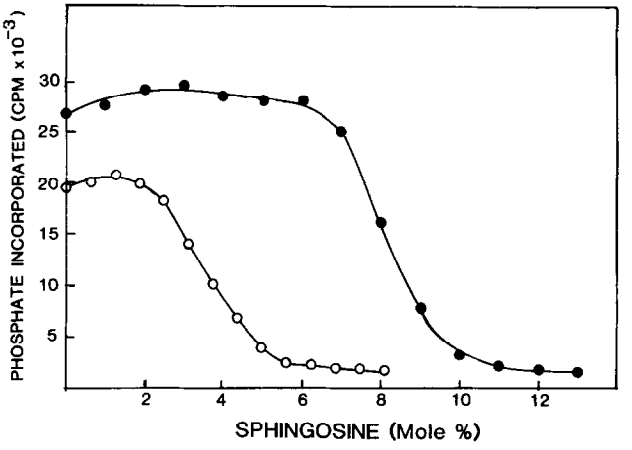

FIGURE 1. Inhibition of PKC by sphingosine. The activity of PKC was measured as described in Material and Methods. The results show the inhibition of PKC by sphingosine in the presence of PS-Triton mixed micelles containing either 8 mole % PS (●), or 5 mole % PS (O).

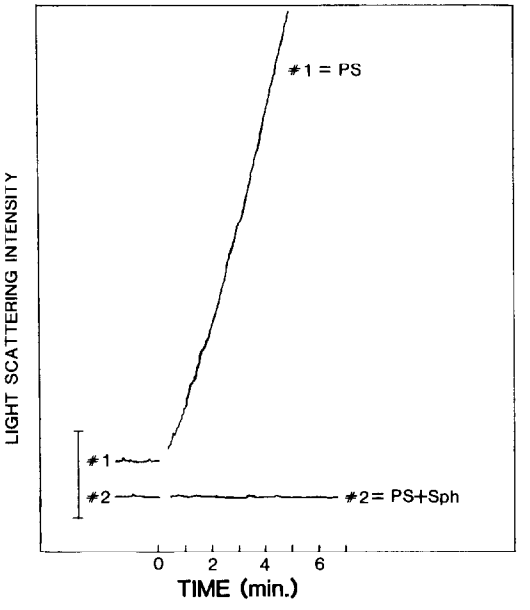

FIGURE 2. Effect of sphingosine on interaction of histone with PS-Triton mixed micelles. Changes in the light scattering intensity of micelles containing 8 mole % PS (Trace #1), or 8 mole % PS plus 8 mole % sphingosine (trace #2) upon addition of histone were monitored as function of time. The light scattering intensity of the micelles were recored first, then 100 μg histone were added (designated by time 0 in the figure). The two traces were offset for clarity, but the light scattering intensities of the micelles were essentially the same and were equal to the bar at the left of the figure. The measurements were performed with 1.6 ml buffer containing 20 mM HEPES, pH 7.5, 5 mM Mg^{2+}, 0.2 mM Ca^{2+}, and 20 μM ATP. The final concentration of Triton was 0.3%. The temperature was maintained at 25° C. The abbreviation Sph. in the figure used to indicate the presence of 8 mole % sphingosine.

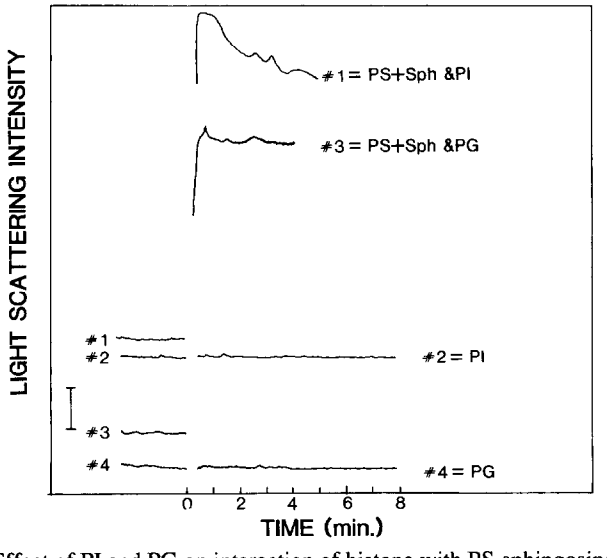

FIGURE 3. Effect of PI and PG on interaction of histone with PS-sphingosine-Triton mixed micelles. Triton-PS-sphingosine micelles (8 mole % PS and 8 mole % sphingosine; traces #1 and #3) , or Triton micelles (traces #2 and #4) were used. Aqueous dispersions of either PI (traces #1 and #2) or PG (traces #2 and #4) were added to give a final concentration of 5 mole %. After incubation for at least 5 minutes, 100 μg histone were added and the changes in the light scattering intensity were monitored. The various traces were offset for clarity. Other experimental conditions were the same as in Figure 2.

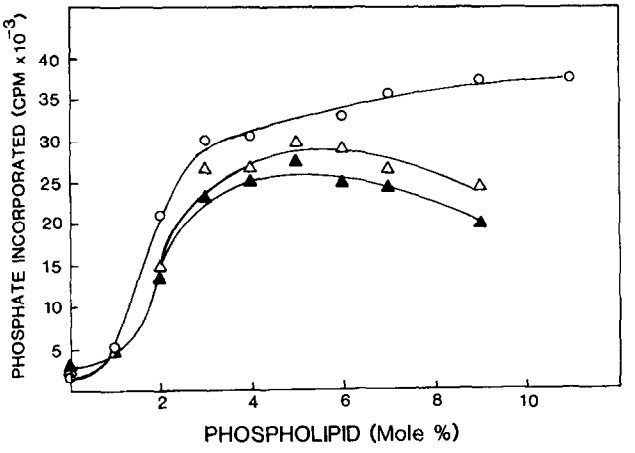

FIGURE 4. Reversibilty of sphingosine inhibition by PS, PI, and PG. Phospholipid vesicles of either PS (O), PI (Δ), or PG (▲) were added to Triton mixed micelles containing 5 mole % PS and 7 mole % sphingosine to give the indicated additional concentration of phospholipid. After equilibrating the resultant micelles for 5 minutes, they were used for assay of PKC activity as described (8).

trace #1). Micelles containing only 5 mole % PI did not interact with histone (e.g. Fig. 3, trace #2). In fact, micelles containing up to 15 mole % PI did not interact with histone (unpublished data). Similarly, the addition of phosphatidylglycerol (PG) to PS-sphingosine-Triton micelles also allowed interaction with histone (Fig 3, trace #3). Again, micelles containing only PG were not able to interact with histone (Fig. 3, trace #4). These results suggested that sphingosine could interact with either PG, PI, or PS. Addition of PI or PG served to free PS for interaction with histone.

The results given above together with the proposed mechanism for sphingosine inhibition by simple charge neuteralization of PS suggested that PI and PG would reverse PKC inhibition by sphingosine. Triton mixed micelles containing only PI or PG do not interact with substrate (above) and do not support the activity of PKC to a significant level (8). Figure 4 shows that, in the absence of added phospholipids, micelles containing 5 mole % PS and 7 mole % sphingosine were not effective in supporting the activity of PKC. However, addition of either PS, PI, or PG reversed the sphingosine inhibition. Greater stimulation by PS was expected since 5 mole % PS provided less than maximum PKC activity (7,8). However, maximum enhancement by either PI or PG coincided with the activity of micelles containing 5 mole % PS in the absence of sphingosine (25,000 ± 2000 cpm under the experimental conditions shown in Figure 4). The results were therefore consistent with the interpretation that PI and PG functioned by binding sphingosine molecules thereby rendering PS available for interaction with histone and PKC.

Hannun et al. (4) concluded that sphingosine did not function by inhibition of the catalytic domain of PKC or by inhibition of PKC-membrane binding, but by inhibition of PKC-phorbol ester binding. Inhibition of phorbol ester binding was also proposed to be the mechanism for PKC inhibition by lysosphingolipids (5). However, the binding of phorbol ester to PKC , when assayed with PS-Triton mixed micelles, is strongly dependent on the PS composition of the micelles (9). Under our proposed mechanism of sphingosine function, inhibition of phorbol ester

binding to PKC by sphingosine or lysosphingolipis could also be a consequence of the limited availability of PS, rather than direct interference with the phorbol ester binding domain of PKC.

These results suggested that sphingosine interacted with PS and thereby limited the availability of this essential phospholipid component. Such a mechanism could also account for inhibition by other compounds bearing both positively charged amine groups and hydophobic regions (4,5). The hydrophobic chain may assure the partition of these molecules into the lipid so that the amino group could interact with negatively charged PS. It is important to emphasize that this proposed effect is limited to in vitro circumstances. Extending the same mechanism to in vivo conditions would require that high concentrations of sphingosine or lysosphingolipids, adequate to at least partially neutralize the charge of biological membranes, be produced. If this actually occurred, many changes in membranes and membrane-associated proteins would be expected. Sphingosine has been reported to inhibit PKC in platelets (4). However, inhibition required high levels of sphingosine and it is possible that charge neutralization occurred in that case as well. The mechanism of charge neutralization is neither a very specific nor a very sensitive manner of regulation. Whether any in vivo situations actually provide such effects is not known.

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