Sphingosine activates casein kinase II in the presence of endogenous substrates as well as a synthetic peptide substrate. The activation response occurred between 12 and 25 μg/ml sphingosine and exhibited positive cooperativity with a Hill coefficient of 3.0. Sphingosine not only increased the V_{max} of casein kinase II but decreased the K_{m(app)} for the peptide substrate from 0.5 to 0.06 mM. In contrast, the K_{m(app)} for MgCl\(_2\) was increased from 0.12 to 0.7 mM. Consequently, sphingosine altered significantly several parameters which determine casein kinase II activity. The effect of sphingosine was relatively specific, inasmuch as related lipids were less potent activators or largely ineffective in stimulating casein kinase II. On the other hand, the effect of sphingosine itself could be potentiated or inhibited by other lipids. Ceramide and sphingosylphosphorylcholine augmented the sphingosine effect. Phospholipids alone did not alter the activity of casein kinase II significantly, but abolished enzyme activation by sphingosine with different potencies (phosphatidylserine > phosphatidylethanolamine > phosphatidylinositol > sphingosylphosphorylcholine). Moreover, the sphingosine effect could be abrogated by KCl and NaCl, which alone are known to induce enzyme activation and dissociation of aggregated casein kinase II protein; LiCl and NH\(_4\)Cl also inhibited the sphingosine effect. Polyamines, known activators of casein kinase II, partially mimicked the effect of sphingosine on endogenous polyphosphate phosphorylation but failed to do so with the peptide substrate. These observations demonstrate that sphingosine is a potent activator of casein kinase II. The potential pharmacological and physiological modulation of casein kinase II by sphingoid bases is discussed.

Sphingolipids are membrane lipids with important roles in cell growth, differentiation, and oncogenesis (1, 2). The discovery that sphingosine and related lysosphingolipids inhibit protein kinase C activity in vitro (3, 4) and in cell systems (5–7) established important links between sphingolipids and signal transduction mechanisms. Sphingosine also modulates the activity of other biochemical targets in vitro; it inhibits phosphatidate phosphohydrolase (8), calmodulin-dependent enzymes (9), binding of factor VII to tissue factor (10), and binding of thyrotropin releasing hormone to its receptor (11). Sphingosine has also been shown to activate the epidermal growth factor receptor kinase (12) and to activate phospholipase D (13, 14). At the cellular level, sphingosine appears to inhibit most, if not all, protein kinase C-dependent processes (1, 15). It also, however, appears to influence activities not related to protein kinase C. Most notably, sphingosine appears to be mitogenic to Swiss 3T3 cell fibroblasts at low concentrations (16) and to inhibit tissue factor activity in mononuclear cells (10).

Casein kinase II (CK-II) is a tetrameric protein kinase (17, 18) found in cytoplasm and nucleus (19). It is distinguished by its specificity for serine residues within clusters of acidic amino acids (20), its ability to use either GTP or ATP as nucleotide substrates (17), and its inhibition by low concentrations of heparin (21). Although CK-II is activated in response to growth factors (22, 23) and during different phases of the cell cycle (24, 25), the actual biochemical steps involved in the regulation of CK-II activity are poorly understood. The enzyme is also activated by high concentrations of polyamines (26) although the significance of this regulation is unknown.

During the characterization of a rat brain sphingosine-activated protein kinase (27), it was noted that this enzyme had chromatographic and catalytic properties, suggesting that it may be identical to CK-II. This observation prompted the present study aimed at investigating the effects of sphingosine on purified CK-II.

**MATERIALS AND METHODS**

\([\gamma^{32}P]ATP (~30 Ci/mm)\) was from Du Pont-New England Nuclear. Spermine, spermidine, N-erythrospingosine, N-erythropysan-gine, octadecylamine, psychosine, sphingosylphosphorylcholine, and all the phospholipids were from Sigma. C\(_2\)-ceramide was synthesized by acetylation of sphingosine as described (25). Biosynthesis Inc. supplied the synthetic peptide substrate RRREEETEEE. Phospholipid suspensions were prepared by drying the organic solvent under \(N_2\) followed by sonication in 25 mM Tris-HCl, pH 7.7. The other lipids were dissolved as concentrated (5–10 mg/ml) solutions in dimethyl sulfoxide or ethanol/H\(_2\)O.

CK-II was purified from adult male Sprague-Dawley rat brains. Fifteen brains were homogenized in 6 volumes of a buffer containing 25 mM Tris-HCl, pH 7.7, 1 mM EDTA, 2 mM dihydrothreitol, 100 μg/ml phenylmethysulfonyl fluoride, and 10 μg/ml leupeptin. The 250,000 x g supernatant was successively chromatographed on DEAE-cellulose, octyl-Sepharose, heparin-Sepharose, and casein-Sepharose columns; the identity of the purified CK-II was ascertained by its α/β-subunit composition, α-subunit autophosphorylation, utilization of GTP as a substrate, inhibition by low concentrations of heparin, and immunoreactivity with antiserum against calf thymus and hog spleen CK-II (gifts from Drs. M. E. Dahmus (University of California, Davis, CA) and O.-G. Issinger (University of Saarland, Homburg, Federal Republic of Germany), respectively). The purity

\[1\] The abbreviations used are: CK, casein kinase; PS, phosphatidylserine; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine.

---

*This work was supported in part by National Institutes of Health Grant GM-43825. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Pew scholar.

**To whom correspondence should be addressed: Div. of Cell Biology, Burroughs Wellcome Co., Research Triangle Park, NC 27709. Tel.: 919-245-4184.
of the final enzyme preparations ranged from ~30 to 70% and had a specific activity of 0.1–0.2 μmol/min/mg protein. The endogenous M, 42,000 substrate was partially purified from rat brain homogenates by extraction at pH 11, heat denaturation at 70 °C, ammonium sulfate precipitation, and desalting on a Sephadex G-50 column (27), yielding a relatively crude substrate preparation which contained multiple polypeptides but was devoid of detectable protein kinase activity. Endogenous polypeptide phosphorylation was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (29) followed by autoradiography. Phosphate incorporation into the synthetic peptide was measured by the P-81 filter method (30). Assays were performed for 10 min at 30 °C in a 30-μl reaction mixture containing 25 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 50 μM ATP, 2 μCi of [γ-32P]ATP, 1 mM peptide substrate, and ~20 ng of enzyme protein. Any changes in these parameters are specified where appropriate. Linear conditions for the assay of peptide phosphorylation were initially established prior to the detailed examination of the regulation of enzyme activity. Assays were performed in triplicate, and each experiment was repeated a minimum of three times. Proteins were determined by the Bradford method (31).

RESULTS AND DISCUSSION

Sphingosine stimulated the phosphorylation of selected endogenous brain polypeptides by CK-II (Fig. 1) including a M, 42,000 component whose phosphorylation was also stimulated by higher concentrations of polyamines (Fig. 1). These observations are consistent with the ability of sphingosine to stimulate the phosphorylation of endogenous synaptosomal polypeptides (27). Whether any of these polypeptides is a physiologic substrate for CK-II has not been verified.

In order to characterize quantitatively the activation of CK-II by sphingosine, we resorted to the use of the synthetic peptide RRREEETEEE, previously designed as a selective CK-II substrate (32). Sphingosine activation was evident at concentrations above 12 μg/ml, reaching a maximum at 25 μg/ml (Fig. 2). This relatively narrow concentration range reflects the sigmoidal nature of the dependence of CK-II activation on the concentration of sphingosine. In fact, a Hill plot of the data (Fig. 2, inset) yielded a Hill coefficient of 3.0, confirming the positive cooperativity of the sphingosine response. Whether this cooperative response stems solely from the lipid-enzyme interaction or from lipid-lipid association has not been determined. A possible interaction between sphingosine and CK-II substrates has not been ruled out either. In contrast to sphingosine, polyamines failed to activate CK-II phosphorylation of the synthetic substrate (data not shown). Combined, Figs. 1 and 2 show that sphingosine stimulates CK-II phosphorylation of endogenous polypeptides as well as synthetic peptides.

The sphingosine effect was further investigated by varying individually the concentrations of ATP, peptide substrate, or MgCl₂ at fixed levels of the other two variables. Sphingosine increased the Vmax of CK-II 2.3-fold when the enzyme was assayed at different ATP concentrations (Fig. 3A); however, the Kcatapp for ATP (7.5 μM) remained constant. Sphingosine also increased the Vmax values, which were obtained by changing the concentration of peptide substrate or MgCl₂ at fixed ATP levels. The Kcatapp for MgCl₂ was increased from 0.12 to 0.7 mM (Fig. 3B). More importantly, sphingosine caused a significant decrease in the Kcatapp value for the peptide from 0.5 to 0.08 mM (Fig. 3C). Thus a major effect of sphingosine on CK-II appears to be an enhancement of affinity to peptide/protein substrates. This mechanism appears to be shared by several second messengers which activate protein kinases by relieving the inhibitory effect of intrinsic pseudosubstrate domains (33). The effects of sphingosine on CK-II may result from a direct enzyme-sphingosine association which exerts an allosteric effect on the interaction of the CK-II with its substrate and with MgCl₂.

Activation of CK-II by sphingosine displayed significant structural selectivity. Octadecylamine and psychosine were less effective activators of the kinase, behaving as partial agonists. Ceramide and sphingosylphosphorylcholine were without significant effect (Table I). Sphinganine (dihydrosphingosine) could not be evaluated because of high filter background. These results indicate that a free NH₂ is required for activity and that a C1 hydroxyl may increase the potency. Substitutions at the C1 carbon significantly decrease (psychosine) or eliminate (sphingosylphosphorylcholine) activity, suggesting steric hindrance.

Although ceramide and sphingosylphosphorylcholine did not alter CK-II activity when used alone, they were able to modify the response of the kinase to sphingosine itself. Accordingly, these two compounds (at 30 μg/ml) were able to enhance the sphingosine effect by 60 and 90%, respectively (data not shown).
Activation of Casein Kinase II by Sphingosine

The four major phospholipids, PC, PE, PI, and PS, were also examined for a possible direct effect on CK-II activity or on its response to sphingosine. When the phospholipids were added individually, they had no apparent influence on CK-II activity; however, they were able to modify the stimulation of the protein kinase by sphingosine (Fig. 4). PS abolished the sphingosine response fully at the lowest concentration tested (5 µg/ml) (data not shown). PE and PI behaved similarly, although complete inhibition of the sphingosine effect required 20 µg/ml of either phospholipid. PI appeared to display a biphasic effect insofar as it potentiated the sphingosine response by 30% at 5 µg/ml phospholipid. This biphasic response was even more accentuated in the case of PC, which potentiated the sphingosine response by 50% at 10 µg/ml phospholipid while inhibiting the response by 40% at 80 µg/ml phospholipid. The mechanism by which various lipids antagonize the activation of CK-II by sphingosine has not been addressed, although it may entail enzyme-phospholipid binding and/or phospholipid-sphingosine association.

NaCl and KCl are known to stimulate CK-II activity (34, 35) and appear to control the state of aggregation of casein kinase II by preventing the formation of elongated enzyme "polymers," which are observed in the absence of salt (36). These effects of KCl and NaCl on the activity of CK-II and its molecular state prompted us to examine the influence of KCl on phosphorylation of the synthetic substrate by CK-II and on the sphingosine response of the protein kinase. In the absence of sphingosine, KCl exerted a modest biphasic effect on the phosphorylation of the synthetic peptide by casein kinase II with a maximum of about 40% activation at 100 mM KCl (Fig. 5). In the presence of sphingosine, a small stimu-
the recruitment of protein kinase C to cell membranes by potentiated by low concentrations of phosphatidylcholine, with other targets of sphingosine.

the phosphorylation of a threonine residue by a mechanism.

cytoplasm and not in membranes. Moreover, regulation of CK-II by diacylglycerol is inhibited in uitro.

The present study demonstrates that sphingosine can serve as an activator as well as inhibitor of different protein kinases. Thus, sphingosine inhibits protein kinase C and calmodulin-dependent kinases but can activate CK-II. The dual action of sphingosine is well illustrated by its effects on epidermal growth factor receptor phosphorylation (12, 38). As expected, sphingosine inhibited the phorbol ester-induced phosphorylation of the receptor (12); however, sphingosine alone induced the phosphorylation of a threonine residue by a mechanism that appears to be independent of protein kinase C. Sphingosine is a potent activator of CK-I1 in uitro. However, sphingosine alone induced a purely inhibitory influence at concentrations above 150 mM LiCl, NaCl, NH4Cl, and KCl, respectively. The effect of salt on sphingosine-CK-I1 interaction may not be duplicated in intact cells.

Determining of intracellular levels of sphingosine in response such as Myb GAP-43, and topoisomerases I and II activation by lipids may not be duplicated in intact cells.

CoKKing P. R., Patton, K. L., Hannun, Y. A., Greenberg, C. S., and Weinberg, J. B. (1989) J. Biol. Chem. 264, 18440–18444.

References

1. Hannun, Y. A., and Bell, R. M. (1989) Science 243, 500–507
2. Hakomori, S. (1981) Annu. Rev. Biochem. 50, 733–764
3. Hannun, Y. A., Loomis, C. R., Merrill, A. H., Jr., and Bell, R. M. (1986) J. Biol. Chem. 261, 12604–12609
4. Hannun, Y. A., and Bell, R. M. (1987) Science 235, 670–674
5. Hannun, Y. A., Greenberg, C. S., and Bell, R. M. (1987) J. Biol. Chem. 262, 13620–13626
6. Merrill, A. H., Jr., Sereni, A. M., Stevens, V. L., Hannun, Y. A., Bell, R. M., and Kinkade, J. M. Jr. (1986) J. Biol. Chem. 261, 12610–12615
7. Wilson, E., Oicott, M. C., Bell, R. M., Merrill, A. H., Jr., and Lambeth, J. D. (1986) J. Biol. Chem. 261, 12616–12623
8. Mieth, T. J., Ariga, N., Egan, R. W., and Billah, M. M. (1991) J. Biol. Chem. 266, 2013–2016
9. Jefferson, A. B., and Schulman, H. (1988) J. Biol. Chem. 263, 15241–15244
10. Conkling, P. R., Patton, K. L., Hannun, Y. A., Greenberg, C. S., and Weinberg, J. B. (1989) J. Biol. Chem. 264, 18440–18444.

11. Wilson, L., Cory, R. N., and Gerashenkov, M. C. (1990) Endocrinology 126, 1668–1672
12. Faucher, M., Girones, N., Hannun, Y. A., Bell, R. M., and Davis, R. (1988) J. Biol. Chem. 263, 5319–5327
13. Merrill, A. H., Jr., and Stevens, V. L. (1989) Biochim. Biophys. Acta 1010, 131–139
14. Kiss, Z., and Anderson, W. B. (1990) J. Biol. Chem. 265, 7345–7350
15. Lavie, Y., and Lascovich, M. (1990) J. Biol. Chem. 265, 3868–3872
16. Zhang, H., Buckley, N. E., Gibson, K., and Spiegel, S. (1990) J. Biol. Chem. 265, 78–81
17. Hatthew, G. M., and Traugh, J. A. (1982) Curr. Top. Cell. Regul. 21, 101–127
18. Hatthew, G. M., Zoller, M. J., and Traugh, J. A. (1981) J. Biol. Chem. 256, 14420–14444
19. Rose, K. M., Bell, L. E., Siefen, D. A., and Jacob, S. T. (1981) J. Biol. Chem. 256, 7468–7477
20. Kuenzel, E. A., Mulligan, J. A., Sommernick, J., and Krebs, E. G. (1987) J. Biol. Chem. 262, 9136–9140
21. Hatthew, G. M., Lubben, T. H., and Traugh, J. A. (1980) J. Biol. Chem. 255, 8088–8041
22. Ackermann, P., and Osheroff, N. (1989) J. Biol. Chem. 264, 11968–11971
23. Sommernick, J., Mulligan, J. A., Lozenza, F. J., and Krebs, E. G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 8834–8838
24. Muller-Lorillon, O., Marot, J., Cayla, A., Pouhle, R., and Belle, R. (1988) Eur. J. Biochem. 171, 107–117
25. Takio, K., Kuenzel, E. A. Walsh, K. A., and Krebs, E. G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 4851–4855
26. Hatthew, G. M., and Traugh, J. A. (1984) J. Biol. Chem. 259, 7011–7015
27. Sahyoun, N. E., McDonald, O. B., and Misra, U. K. (1989) J. Biol. Chem. 264, 1062–1067
28. Okazaki, T., Bielawska, A., Bell, R. M., and Hannun, Y. A. (1990) J. Biol. Chem. 265, 15922–15926
29. Laemmli, U. K. (1970) Nature 227, 680–685
30. Roskoski, R., Jr. (1983) Methods Enzymol. 99, 3–6
31. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
32. Kuenzel, E. A., and Krebs, E. G. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 737–741
33. Soderling, T. R. (1990) J. Biol. Chem. 265, 1823–1826
34. Hatthew, G. M., and Traugh, G. A. (1983) Methods Enzymol. 99, 317–331
35. Dahms, M. E., and Natzle, J. (1977) Biochemistry 16, 1901–1909
36. Glover, C. V. C. (1986) J. Biol. Chem. 261, 14349–14354
37. Hannun, Y. A., and Bell, R. M. (1990) J. Biol. Chem. 265, 2962–2972
38. Davis, R. J., Girone, N., and Faucher, M. (1988) J. Biol. Chem. 263, 5373–5379
39. Khan, W. A., Macarella, S. W., Lewin, A. H., Wyrick, C. D., Carroll, F. T., and Hannun, Y. A. (1991) Biochem. J., in press
40. Luscher, C., Kuenzel, E. A., Krebs, E. G., and Eisenman, R. N. (1989) EMBO J. 8, 1111–1119
41. Luscher, C., Christenson, E., Litchfield, D. W., Krebs, E. G., and Eisenman, R. N. (1990) Nature 344, 517–522
42. Durban, E., Goodenough, M., Mills, J., and Busch, H. (1985) EMBO J. 4, 2921–2926
43. Ackermann, P., Glover, C. V. C., and Osheroff, N. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3164–3168
44. Apel, E. D., Litchfield, D. W., Clark, R. H., Krebs, E. G., and Storm, D. R. (1991) J. Biol. Chem. 266, 10544–10551
45. Karlund, J. K., and Czech, M. P. (1988) J. Biol. Chem. 263, 15872–15875