Phosphorylation of Brain Cytosol Proteins
EFFECTS OF PHOSPHOLIPIDS AND CALMODULIN*

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Phospholipid-specific protein kinase has been demonstrated. A variety of effector-specific protein kinases have been identified in brain tissue and, most recently, a cyclic nucleotide-independent protein kinase has been the subject of intense interest (1-3). This particular protein kinase is stimulated by phospholipids in the presence of calcium (4, 5) and is inhibited by various phospholipid-interacting compounds (6-8), many of which are often used as CaM' antagonists (9-13). Furthermore, this phospholipid-sensitive kinase can apparently be distinguished from a CaM-activated protein kinase on the basis of substrate specificity (3). However, it has not been demonstrated with certainty that the phospholipid- and CaM-activated protein kinases are distinct enzymes, nor has a phospholipid-specific protein kinase been demonstrated.

The binding of calcium by CaM exposes hydrophobic regions on the CaM molecule (14), and it appears that such exposure of hydrophobic sites on CaM is the mechanism by which CaM activates, at least, myosin light chain kinase and phosphodiesterase (10, 15). In fact, certain CaM effects can be mimicked, in part, by hydrophobic agents such as SDS (15).

In the present study, we demonstrate that the activation of protein kinase by phospholipids can be mimicked by SDS as well as the hydrophobic probe ANS, indicating that the phospholipid effect on protein kinase represents a more general stimulation of enzyme activity than previously indicated (1-8). Furthermore, the phospholipid-dependent and CaM-dependent activations of protein kinase are distinct effects (by virtue of the endogenous substrates they phosphorylate), and it appears that the CaM-induced increase in phosphorylation is suppressed in the presence of certain hydrophobic probes.

EXPERIMENTAL PROCEDURES

Materials—Sucrose was obtained from Schwarz/Mann; Tris base from Bethesda Research Laboratories; MgCl2, CaCl2, and KCl from J. T. Baker Chemical Co.; ATP, ethylene glycol bis(β-aminoethyl ether)-N,N',N",N"'-tetraacetic acid, ANS, and phospholipids from Sigma; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 2-mercaptoethanol were obtained from Calbiochem. Acrylamide, N,N"'-methylene-bis-acrylamide, glycine, ammonium persulfate, N,N,N",N"'-tetramethylethylene-diamine, and Coomassie brilliant blue R-250 were all of electrophoretic grade quality and obtained from Bio-Rad. Ultrapure SDS was from BDH Chemicals, Ltd. XRP x-ray film used for autoradiography was obtained from Kodak. Sepharose 4B was purchased from Pharmacia, diethylaminoethyl cellulose (DEAE-52) from Whatman, and diglycidyl ether from Aldrich. Fluphenazine was generously provided by S. J. Lucania from the Squibb Institute of Medical Research.

Preparation of Cytosol—Male, Sprague-Dawley rats were decapitated, and the brains were rapidly removed. The cortex was homogenized in 1 volume of buffer containing 0.25 m sucrose and 20 mm Tris-HCl, pH 7.5. The homogenate was centrifuged at 100,000 g for 60 min, and the resulting supernatant, referred to as the cytosol, was used as the starting enzyme source.

CaM-deficient cytosol was prepared by two separate methods. In the first method, an aliquot of cytosol was loaded onto a DEAE column equilibrated with the homogenization buffer. The protein kinase was eluted along with certain substrate proteins with equilibrating buffer containing 250 mm KCl, essentially as described by Wrenn, et al. (3). The second method employed fluphenazine-Sepharose affinity chromatography (16). Fluphenazine-Sepharose was prepared by the method of Charbonneau and Cormier (17) with minor modifications (18). An aliquot of cytosol was adjusted to 1 mm CaCl2 and loaded onto a fluphenazine-Sepharose column (2.5 X 1.5 cm), previously equilibrated with homogenization buffer containing 1 mm CaCl2. The effluent from the column was used as the source of protein kinase and substrates. Ethylene glycol bis(β-aminoethyl ether)-N,N,N",N"'-tetraacetic acid was added to the effluent in excess of the CaCl2.

Protein Kinase Assay—The net incorporation of phosphate into cytosolic proteins (100 μg) was assayed at 30 °C in an assay mixture (200-μl final volume) containing 50 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.0; 10 mm MgCl2; 100 μm ethylene glycol bis(β-aminoethyl ether)-N,N,N",N"'-tetraacetic acid, and 5 μm ATP containing [γ-32P]ATP (1 X 106 dpm/nmol). Where indicated, CaCl2, calmodulin, phospholipids, and ANS were included in the assay at the concentrations listed. Reactions were initiated by addition of ATP and were terminated after 1 min by solubilizing the proteins with the addition of 100 μl of a solution containing 9% (w/v) SDS, 30 mm Tris-HCl, pH 8.0, 6% (v/v) 2-mercaptoethanol, and 27% (w/v) sucrose.

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The abbreviations used are: CaM, calmodulin; SDS, sodium dodecyl sulfate; ANS, 8-anilino-1-naphthalenesulfonate; PS, phosphatidylserine.

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Protein was determined by the method of Bradford (19), using bovine γ-globulin as a standard.

**Gel Electrophoresis and Autoradiography**—The solubilized proteins were resolved on SDS-polyacrylamide slab gels (60 μl/well). The acrylamide concentration was 6 and 10% in the stacking and running gels, respectively. Both stacking and running gels contained SDS at a final concentration of 0.1%. The running and gel buffers were as described by O’Farrell (30). Electrophoresis was performed under constant current. After electrophoresis, the gels were fixed and stained with 0.1% Coomassie brilliant blue R-250 in 50% methanol, 10% acetic acid. The gels were destained by diffusion in 30% methanol, 10% acetic acid and then dried under heat and partial vacuum. Autoradiography was carried out for 1–2 days with Kodak RP-5 x-ray film, using Cronex Lightning-Plus intensifying screens (DuPont). The molecular weights of the protein bands were determined from molecular weight standards that had been subjected to electrophoresis under identical conditions. Densitometric readings on selected autoradiographs were taken on an EC Apparatus Corp. densitometer.

**RESULTS**

Brain cytosol contains sufficient CaM to greatly enhance the phosphorylation of substrates acted upon by Ca2+-CaM-dependent protein kinase, when Ca2+ is added to the assay medium. Therefore, to study other Ca2+-dependent, but CaM-independent, effects on phosphorylation, it is necessary to remove the endogenous calmodulin prior to assay. Since any type of column chromatography could alter the substrate composition of the fraction assayed or alter the substrates themselves, we felt it was important to examine different methods of CaM removal. Fig. 1 shows the CaM and PS proteins in untreated cytosol in the absence of calcium, when Ca2+ is added to the assay. Since any type of column chromatography could alter the substrate composition of the fraction assayed or alter the substrates themselves, we felt it was important to examine different methods of CaM removal. Fig. 1 shows the CaM and PS stimulation of phosphate incorporation into endogenous proteins after chromatography on DEAE or fluphenazine-Sepharose. Lanes 1 and 2 show the pattern of phosphorylated proteins in untreated cytosol in the absence (lane 1) and presence of calcium (lane 2). Lanes 3–6 show the phosphorylation pattern of the DEAE eluate, and lanes 7–10 show the pattern in the fluphenazine-Sepharose effluent. It can be seen that both chromatographic procedures resulted in changes in the phosphorylation pattern. For example, it appears that basal phosphorylation (absence of calcium) is higher after either type of chromatography than before. Specifically, lanes 3 and 7 show the basal phosphorylation after chromatography of cytosol on DEAE and fluphenazine-Sepharose, respectively, and as compared to lane 1, there is an increased phosphorylation in several proteins, particularly with bands with Mr = 55,000, 50,000, and 44,000. Addition of calcium alone to the chromatographed cytosol results in an increased phosphorylation of several protein bands in the DEAE eluate (lane 4) and two protein bands (Mr = 55,000 and 77,000) in the fluphenazine-Sepharose effluent (lane 8). The calcium effect could be due to incomplete removal of CaM or some other effector, although this Ca2+-stimulation does not affect phosphorylation of all the proteins typically affected by CaM (see below). The CaM stimulation observed in the DEAE eluate is also quite different from that observed by Wrenn et al. (3) who obtained a very small stimulation of phosphorylation with CaM.

Since the effects of CaM on phosphorylation of endogenous cytosol proteins have been well-documented (18) and since the phosphorylation pattern seen in the fluphenazine-Sepharose effluent with addition of calcium and CaM (lane 9) more closely resembles the pattern seen in cytosol than does the DEAE eluate phosphorylation pattern (lane 5), we chose to use the fluphenazine-Sepharose effluent for the remaining experiments. The removal of endogenous CaM by fluphenazine-Sepharose when compared to DEAE chromatography also has the added advantages of selectivity, speed and ease of use, and reproducibility.

Fig. 2 shows the Ca2+-dependent effects of various phospholipids and CaM on the phosphorylation pattern of cytosol proteins. As previously described (18), CaM (lane 2) stimu-
lates phosphorylation of various proteins, with the major effect being on proteins with \( M_r = 50,000, 55,000, \) and 60,000. The effects of PS and phosphatidylinositol \( (5 \mu g/tube) \) are shown in lanes 3 and 4, respectively. These phospholipids primarily stimulated the phosphorylation of a protein with an approximate \( M_r \) of 47,000, while somewhat smaller effects were observed on two proteins with a higher \( M_r \) of 75,000 and 77,000. Phosphatidylcholine \( (lane 5) \) and phosphatic acid \( (lane 6) \) were less effective in stimulating protein phosphorylation. In general, the Ca\(^{2+}\)-dependent phospholipid effects were much smaller in magnitude than those observed by Wrenn, et al. (3). Fig. 2 also shows the effects of SDS \( (80 \mu M) \) on protein phosphorylation \( (lane 7) \). SDS has an effect similar to the phospholipids in that it predominately stimulates phosphorylation of proteins with \( M_r = 47,000 \) and 77,000. These effects of CaM, phospholipids, and SDS were Ca\(^{2+}\)-dependent (data not shown).

The effects of CaM, various phospholipids, and SDS were Ca\(^{2+}\)-dependent (data not shown). The effects of PS and phosphatidylinositol \( (5 \mu g/tube) \) are shown in lanes 3 and 4, respectively. These phospholipids primarily stimulated the phosphorylation of a protein with an approximate \( M_r \) of 47,000, while somewhat smaller effects were observed on two proteins with a higher \( M_r \) of 75,000 and 77,000. Phosphatidylcholine \( (lane 5) \) and phosphatic acid \( (lane 6) \) were less effective in stimulating protein phosphorylation. In general, the Ca\(^{2+}\)-dependent phospholipid effects were much smaller in magnitude than those observed by Wrenn, et al. (3). Fig. 2 also shows the effects of SDS \( (80 \mu M) \) on protein phosphorylation \( (lane 7) \). SDS has an effect similar to the phospholipids in that it predominately stimulates phosphorylation of proteins with \( M_r = 47,000 \) and 77,000. These effects of CaM, phospholipids, and SDS were Ca\(^{2+}\)-dependent (data not shown).

A more quantitative presentation of the CaM and phospholipid effects on phosphorylation of cytosol proteins is shown in Fig. 3. Densitometric scans of autoradiographs of gels similar to those presented in Fig. 2 demonstrate the overall effects of CaM, SDS, and phospholipids on protein phosphorylation and emphasize, in particular, the increase in phosphorylation of the \( M_r = 50,000, 55,000, \) and 60,000 bands produced by CaM, and the increase in phosphorylation of the \( M_r = 47,000 \) and 77,000 bands produced by phospholipids or SDS. Furthermore, the difference in the patterns of phosphorylation produced by the different effectors is clearly demonstrated.

The effects of addition of the hydrophobic probe ANS \( (200 \mu M) \) on protein phosphorylation are shown in Fig. 4. Lanes 1 and 2 show control and Ca\(^{2+}\)-CaM-stimulated phosphorylation, respectively. Lane 3 shows the effects of ANS in the presence of calcium and CaM, and it can be seen that ANS selectively blocks the calcium-CaM stimulation of protein phosphorylation but does not alter the stimulation produced by either PS \( (lane 6 \) compared to \( lane 5) \) or SDS \( (lane 8 \) compared to \( lane 7) \). In addition, the effects of phosphatidylinositol were not blocked by ANS (data not shown). Interestingly, ANS itself caused a Ca\(^{2+}\)-dependent stimulation of phosphorylation. Lane 10 in Fig. 4 demonstrates that ANS primarily enhanced the phosphorylation of protein bands with approximate \( M_r = 75,000 \) and 47,000, much like the effects produced by phospholipids and SDS (see above). PS and SDS were added to the assay mixture along with exogenous CaM to determine if the similarities with ANS extended to the inhibition of the CaM effect on protein phosphorylation. SDS \( (80 \mu M) \) blocked the CaM-stimulated increase in phosphorylation, while addition of PS \( (5 \) and 25 \( \mu g) \) resulted in an additive effect, i.e. increased phosphorylation of proteins affected by CaM and phospholipids (data not shown).

**DISCUSSION**

The study of Ca\(^{2+}\)-dependent phosphorylation in brain cytosol, other than that stimulated by calmodulin, is complicated by the presence of endogenous CaM. The phosphorylation of endogenous proteins in cortical cytosol has been
shown to be affected by phospholipids after chromatography on DEAE (3). However, the phosphorylation pattern produced by Ca"+-CaM in this preparation is not the same as that seen in untreated cytosol. In fact, the stimulation of phosphorylation by CaM appears to be quite variable after chromatography of cytosol on DEAE (3, 6). Thus, it appears that the method of removal of CaM may affect the phosphorylation pattern obtained with addition of CaM as well as phospholipids, as is shown in Fig. 1. This may reflect removal or alteration of some substrates as well as the rapid loss of CaM-stimulation but not phospholipid stimulation of protein kinase which is observed during purification of a CaM-dependent protein kinase. It is also possible that the increased phosphorylation seen after the selective removal of CaM by fluphenazine-Sepharose chromatography (see Fig. 1) could be explained by an inhibition of a CaM-dependent phosphatase (i.e. calcineurin (21)).

The effects of phospholipids and CaM on protein phosphorylation has been attributed specifically to the presence of Ca"+-dependent, phospholipid-sensitive, and CaM-sensitive protein kinases, respectively, in the central nervous system (1-8, 18). Evidence in support of this concept derives primarily from the observations that these different effectors stimulate the phosphorylation of different substrates (3, 6, 18, Fig. 2) and that the phospholipid-activated kinase is not responsive to CaM (4). However, the present data argue against the existence of specific (e.g. phosphatidylserine) phospholipid-activated kinases, indicating instead that the Ca"+-dependent stimulation of protein kinase in cytosol reflects the more general actions of the phospholipids as hydrophobic probes. The ability of SDS and ANS to mimic the phospholipid effects further substantiates this conclusion. Furthermore, we have observed that even short-term (within 24 h) purification of brain synaptosomal protein kinase results in the rapid loss of CaM-responsiveness (22). Thus, the inability of CaM to stimulate phospholipid-sensitive protein kinase may simply reflect the time-dependent loss of CaM-dependency in protein kinase, not the lack of CaM-dependency altogether.

The ability of ANS to block CaM-stimulated protein kinase in cortical cytosol indicates further that the Ca"+-exposed hydrophobic sites on CaM are essential for the activation of protein kinase. ANS also blocks the spectral changes in CaM induced by Ca"+ (14). However, in CaM-deficient cytosol, ANS itself stimulates the phosphorylation of proteins (M, 47,000 and 75,000, see Fig. 4) in a Ca"+-dependent manner and these same substrate proteins are also phosphorylated by the addition of either phospholipids or SDS (Fig. 2). The effects of ANS on protein kinase activity do not appear to be similar to the phenothiazine antipsychotics, which have been shown to block phospholipid-sensitive protein kinase activity (6-8) as well as CaM-dependent protein kinase activity (7, 18).

It is possible that different effectors could induce a single enzyme to act on different substrates. For example, a protein kinase in the cytosol could preferentially respond to CaM whereas translocation of the enzyme to the plasma membrane could promote contact with phospholipids, which stimulate the kinase to phosphorylate a different set of substrates. Of course, the possibility that CaM and phospholipids have differential effects on the substrates, rather than on enzymes, cannot be ruled out in the present results or others. In any case, it appears that phospholipids stimulate the phosphorylation of endogenous substrates in brain cytosol by acting largely as hydrophobic probes.

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