Stimulation by Phosphatidylserine and Calmodulin of Calcium-dependent Phosphorylation of Endogenous Proteins from Cerebral Cortex*

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The Ca**-dependent phosphorylation of a number of proteins in the cytosol of the rat or guinea pig cerebral cortex was profoundly stimulated by phosphatidylserine; calmodulin, on the other hand, had only a minimal effect. The Ca**-dependent phosphorylation of different proteins from the total particulate fraction of the same tissue, in comparison, was specifically stimulated by either phosphatidylserine or calmodulin. The present findings, in line with the phospholipid-sensitive Ca**-dependent protein kinase recently recognized, suggest an involvement of phospholipid in regulating Ca**-dependent phosphorylation of endogenous substrate proteins. This new system presumably functions independently or in a complementary manner with the calmodulin-sensitive Ca**-dependent protein phosphorylation system previously reported by others.

Calcium ion has become recognized as an important intracellular mediator and shown to be involved in the regulation of a variety of biological processes (2, 3). The mode of its action has remained obscure, but recent evidence suggests that Ca**-dependent phosphorylation of endogenous substrate proteins may serve as a final pathway for this messenger (4). To date, all studies dealing with Ca**-dependent phosphorylation of proteins in tissues (5-8) have revolved around the multifunctional Ca**-binding protein, calmodulin, which confers a Ca** sensitivity upon specific protein kinases by serving as either an integral subunit or an obligatory cofactor for the enzymes (for reviews see Refs. 9 and 10).

Another species of Ca**-dependent protein kinase, requiring phospholipid as a cofactor, was recently described by Takai et al (11). Work in our laboratory has shown the widespread and differential distribution of this enzyme throughout the animal kingdom and in many mammalian tissues (12). Brain was found to have exceedingly high levels of this phospholipid-sensitive Ca**-dependent protein kinase (12). The present study was conducted to investigate the endogenous substrate proteins for Ca**-dependent phosphorylation systems in mammalian cerebral cortex and to compare the effects of phospholipid and calmodulin on this process.

EXPERIMENTAL PROCEDURES

Materials—Phosphatidylserine (bovine brain), lysine-rich histone (type III-S), and marker proteins for molecular weight determinations

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1 The abbreviation used is: SDS, sodium dodecyl sulfate.
Ca\textsuperscript{2+}-dependent Protein Phosphorylation

Calmodulin-deficient cyclic AMP phosphodiesterase was prepared by DEAE-cellulose chromatography as described earlier (12, 15). Cyclic AMP phosphodiesterase activity was assayed essentially as previously described (16). The reaction mixture (0.1 ml) contained: Tris/Cl (pH 7.5), 5 µmol; MgSO\textsubscript{4}, 1 µmol; CaCl\textsubscript{2}, 0.05 µmol; 5'-nucleotidase (snake venom), 20 µg; calmodulin-deficient cyclic AMP phosphodiesterase, 10 µg; cyclic [G-\textsuperscript{3}H]AMP, 0.033 pmol containing 34,000 cpm; and either pure calmodulin (0.5 to 6 µg) or the supernatant of the boiled cerebral cortex cytosol. The reaction was carried out for 1 to 5 min at 37°C.

Homogeneous calmodulin from the bovine brain was used either a gift from W. Y. Cheung of St. Jude Children's Research Hospital, Memphis, Tennessee, or was prepared by the fluphenazine affinity method described by Charbonneau and Cormier (17). Phospholipid-sensitive Ca\textsuperscript{2+}-dependent protein kinase was purified about 5000-fold from the bovine heart extract by the steps of ammonium sulfate precipitation and chromatographies on DEAE-cellulose, controlled-pored glass, and phosphatidylserine-Affigel 102. The enzyme was also purified about 100-fold from the rat brain extract by the steps of DEAE-cellulose and Sephadex G-200 chromatographies.\textsuperscript{2} The preparation was prepared by the method of Post and Sen (18). Protein was determined by the method of Lowry et al. (19).

RESULTS AND DISCUSSION

Ca\textsuperscript{2+}-dependent phosphorylation of cytosolic proteins from the rat cerebral cortex was found to be far more pronounced in the presence of phosphatidylserine (Fig. 1, comparing Lanes 1, 2, 3, 4, and 8) than in the presence of calmodulin (Fig. 1, comparing Lanes 1, 5, 6, 7, and 8). A wide variety of endogenous proteins, the phosphorylation of which was specifically stimulated by the combined presence of Ca\textsuperscript{2+} and phosphatidylserine, had molecular weights ranging from 28,000 to 95,000. A smaller protein, which migrated along with the tracking dye at the front of the 10% gel and was specific to stimulation by Ca\textsuperscript{2+} plus phospholipid (Fig. 1, Lanes 4 and 8), was determined to be a 15,000-dalton protein in separate experiments employing 12% gel (figure not shown). The phosphorylation of two proteins (41,000 and 85,000 daltons) was found to be stimulated by Ca\textsuperscript{2+} plus calmodulin (Fig. 1, comparing Lanes 1, 2, and 6). Their phosphorylation, however, was stimulated to a comparable degree by phosphatidylserine alone and to a much greater extent by Ca\textsuperscript{2+} plus phosphatidylserine. It appeared that phosphatidylserine was much more effective than calmodulin in supporting Ca\textsuperscript{2+}-dependent phosphorylation of brain cytosolic proteins under the present experimental conditions. The low magnitude of the effect observed for exogenously added calmodulin in the present studies probably was not due to the presence of endogenous calmodulin, since (a) the protein phosphorylation seen in the presence of added Ca\textsuperscript{2+} alone was very minimal (Fig. 1, Lane 2), and (b) the cytosol employed had been previously rendered deficient in calmodulin by chromatography on DEAE-cellulose (see "Methods"), as evidenced by the inability of its boiled supernatant to stimulate calmodulin-deficient cyclic AMP phosphodiesterase assayed in the presence of Ca\textsuperscript{2+} (Table I, Experiment 1). The relatively few substrate proteins seen for the calmodulin-sensitive system may be also an artifact due to the presence of excessive amounts of calmodulin-binding proteins in the cytosol preparation, thus depriving the phosphorylating enzyme(s) of free and active calmodulin. This possibility appeared to be excluded, since addition of calmodulin was found to stimulate phosphodiesterase added to calmodulin-deficient cytosol that was used for these phosphorylation experiments (Table I, Experiment 2). Results similar to those shown for the calmodulin-deficient cytosol (Fig. 1) were noted for the untreated cytosol (figure not shown), suggesting that the DEAE-cellulose treatment did not significantly remove the substrate proteins and their phosphorylating enzymes for the phospholipid- and calmodulin-sensitive systems. Results similar to those seen for the rat cerebral cortex cytosol (Fig. 1) were also noted for the guinea pig preparation (figure not shown).

Takai et al. (11) and we (1, 12) have previously shown the presence of a high level of phospholipid-sensitive Ca\textsuperscript{2+}-dependent protein kinase from the bovine heart or rat brain was also included in the incubation mixture. The separating gel used was 10% acrylamide containing 0.1% SDS.

\textsuperscript{2} B. C. Wise and J. F. Kuo, unpublished

\textsuperscript{3} 1. Effects of Ca\textsuperscript{2+}, phosphatidylserine, and calmodulin on phosphorylation of substrate proteins from the rat cerebral cortex cytosol depleted of endogenous calmodulin. Treatment of the cytosol, incubation conditions for phosphorylation, and subsequent SDS-polyacrylamide gel electrophoresis and autoradiography of the phosphorylated samples were as described under "Methods." Additions were made as indicated and consisted of CaCl\textsubscript{2} (0.1 µmol), phosphatidylserine (PS, 0.5 µg), and calmodulin (CDR, 2 µg). Phosphorylation of endogenous proteins was also carried out for shorter incubation times (0.5, 1, and 2 min). The results indicated that a 5-min incubation, as shown, was optimal. Essentially the same results were obtained when partially purified phospholipid-sensitive Ca\textsuperscript{2+}-dependent protein kinase (1 to 2 µg) from the bovine heart or rat brain was also included in the incubation mixture. The separating gel used was 10% acrylamide containing 0.1% SDS.

\textsuperscript{4} FIG. 1. Effects of Ca\textsuperscript{2+}, phosphatidylserine, and calmodulin on phosphorylation of substrate proteins from the rat cerebral cortex cytosol depleted of endogenous calmodulin. Treatment of the cytosol, incubation conditions for phosphorylation, and subsequent SDS-polyacrylamide gel electrophoresis and autoradiography of the phosphorylated samples were as described under "Methods." Additions were made as indicated and consisted of CaCl\textsubscript{2} (0.1 µmol), phosphatidylserine (PS, 0.5 µg), and calmodulin (CDR, 2 µg). Phosphorylation of endogenous proteins was also carried out for shorter incubation times (0.5, 1, and 2 min). The results indicated that a 5-min incubation, as shown, was optimal. Essentially the same results were obtained when partially purified phospholipid-sensitive Ca\textsuperscript{2+}-dependent protein kinase (1 to 2 µg) from the bovine heart or rat brain was also included in the incubation mixture. The separating gel used was 10% acrylamide containing 0.1% SDS.

\textsuperscript{5} Takai et al. (11) and we (1, 12) have previously shown the presence of a high level of phospholipid-sensitive Ca\textsuperscript{2+}-dependent protein kinase in the brain cytosol. The presence of a calmodulin-sensitive species of the enzyme, however, has not yet been demonstrated under the present assay conditions using lysine-rich histone as substrate (12). Since there is little evidence to indicate that experimental conditions employed are optimal, coupled with the possible presence of interfering substances in the enzyme and endogenous substrate protein preparations, our observation of relatively few substrates seen for the calmodulin-sensitive phosphorylation system (Fig. 1) does not necessarily suggest that the enzymes or substrates, or both, for this system are deficient in the cerebral cortex cytosol. Following the same reasoning, the present findings, while demonstrating the presence of an active phospholipid-sensitive phosphorylation system, do not necessarily imply the relative importance of phospholipid and calmodulin in regulating Ca\textsuperscript{2+}-dependent protein phosphorylation in the cerebral cortex and, perhaps, other tissues. It is not clear whether the presence of substrate proteins already in the phosphoryl-
ated form, or the presence of excessive amounts of phosphatases for the above phosphoproteins, may also account for the apparent scarcity of calmodulin-sensitive phosphorylation observed in the present studies. Yamauchi and Fujisawa (7) reported that the Ca\textsuperscript{2+}-dependent phosphorylation of proteins

\begin{table}[h]
\caption{Removal of calmodulin and calmodulin-binding proteins from the cerebral cortex cytosol with DEAE-cellulose treatment}

\begin{tabular}{|c|c|}
\hline
Addition & Cyclic AMP phosphodiesterase activity \\
& pmol hydrolyzed/min \\
\hline
Experiment 1 & \\
Calmodulin-deficient phosphodiesterase & 586 ± 17 \\
+ Calmodulin & 1982 ± 167 \\
+ Supernatant of boiled, untreated cytosol & 1979 ± 341 \\
+ Supernatant of boiled, DEAE-treated cytosol & 632 ± 75 \\
\hline
Experiment 2 & \\
Calmodulin-deficient phosphodiesterase & 409 \\
+ Calmodulin & 1021 \\
+ DEAE-treated cytosol (unboiled) & 547 \\
+ Calmodulin + DEAE-treated cytosol (unboiled) & 1087 \\
DEAE-treated cytosol (unboiled) + calmodulin & 180 \\
\hline
\end{tabular}
\end{table}

The effects of Ca\textsuperscript{2+}, phosphatidylserine, and calmodulin on phosphorylation of substrate proteins from the rat cerebral cortex cytosol chromatographed on DEAE-cellulose. Aliquots (0.03 ml) of Fraction 31 of Peak A (Fig. 2) were used as the source of endogenous substrate. The incubation conditions and the components added to the incubation as indicated were the same as described under “Methods” and Fig. 1. The resolving gel was 12% acrylamide containing 0.1% SDS. Essentially the same results were obtained when phospholipid-sensitive Ca\textsuperscript{2+}-dependent protein kinase (2 μg) from the bovine heart was also included in the incubation mixture.

Fig. 3. Effects of Ca\textsuperscript{2+}, phosphatidylserine, and calmodulin on phosphorylation of substrate proteins from the rat cerebral cortex cytosol chromatographed on DEAE-cellulose. Aliquots (0.03 ml) of Fraction 31 of Peak A (Fig. 2) were used as the source of endogenous substrate. The incubation conditions and the components added to the incubation as indicated were the same as described under “Methods” and Fig. 1. The resolving gel was 12% acrylamide containing 0.1% SDS. Essentially the same results were obtained when phospholipid-sensitive Ca\textsuperscript{2+}-dependent protein kinase (2 μg) from the bovine heart was also included in the incubation mixture.

In order to further define the endogenous protein phosphorylation, the rat cerebral cortex cytosol was chromatographed on DEAE-cellulose (Fig. 2). One major peak (A) and two minor peaks (B and C) of substrate proteins for phospholipid-sensitive Ca\textsuperscript{2+}-dependent protein kinase were obtained. The enzyme activity was eluted at the position coinciding with substrate Peak A (Fig. 2). However, no significant activity of calmodulin-sensitive Ca\textsuperscript{2+}-dependent enzyme, assayed using lysine-rich histone as substrate, was detected in any fraction. Calmodulin was eluted much later in the salt gradient, at a point corresponding to 400 to 450 mM KCl (data not shown). The autoradiograph of phosphorylated proteins from Fraction 31 of Peak A showed that the Ca\textsuperscript{2+}-dependent phosphorylation of four proteins of 95,000, 73,000, 50,000, and 15,000 daltons was stimulated by phospholipid, but not by calmodulin (Fig. 3). The four substrate proteins concentrated in Fraction 31 presumably were the same as those of similar molecular weight seen earlier for the cytosol (Fig. 1). Although studies have not yet been carried out for other fractions, it is likely that other substrate proteins may be selectively eluted in specific peaks.

The Ca\textsuperscript{2+}-dependent phosphorylation of the total (unfractionated) particulate of the cerebral cortex was found to be quite different from those of the cytosol shown above. In the

Fig. 2. DEAE-cellulose chromatography of the rat cerebral cortex cytosol analyzed for phospholipid-sensitive Ca\textsuperscript{2+}-dependent protein kinase and its substrate proteins. Cerebral cortex cytosol (8 ml, containing 150 mg of protein) was loaded onto a DEAE-cellulose column (1.5 × 15 cm) and proteins were eluted with a linear gradient of KCl (0 to 1 M; total volume, 300 ml). The fraction size was 2 ml. The enzyme activity and its substrate were assayed as described under “Methods.” Ca\textsuperscript{2+}-PK, Ca\textsuperscript{2+}-dependent protein kinase.

Fig. 4. Effects of Ca\textsuperscript{2+}, phosphatidylserine, and calmodulin on phosphorylation of substrate proteins from the rat cerebral cortex cytosol chromatographed on DEAE-cellulose. Aliquots (0.03 ml) of Fraction 31 of Peak A (Fig. 2) were used as the source of endogenous substrate. The incubation conditions and the components added to the incubation as indicated were the same as described under “Methods” and Fig. 1. The resolving gel was 12% acrylamide containing 0.1% SDS. Essentially the same results were obtained when phospholipid-sensitive Ca\textsuperscript{2+}-dependent protein kinase (2 μg) from the bovine heart was also included in the incubation mixture.
particulate from the guinea pig. Phosphorylation of a 21,000-dalton protein was specifically stimulated by Ca\(^{2+}\) plus phospholipid (Fig. 4, comparing Lanes 1, 2, 4, 6, and 8), whereas that of 58,000- and 66,000-dalton proteins were specifically stimulated by Ca\(^{2+}\) plus calmodulin (Fig. 4, comparing Lanes 1, 3, 4, 7, and 8). We noted previously that phospholipid-sensitive Ca\(^{2+}\)-dependent protein kinase distributes in both fractions of the brain (12). Qualitatively similar results, although less pronounced, were also noted for the particulate of the rat cerebral cortex (figure not shown). Schulman and Greengard have shown previously that Ca\(^{2+}\)-dependent phosphorylation of membrane proteins from the brain (5, 6) and other rat tissues (6) is stimulated by calmodulin. The same phenomenon was also reported by Landt and McDonald (8) for microsomal proteins from the rat adipocyte. In all cases, the major phosphoproteins have molecular weights of about 50,000 to 65,000, agreeing with those shown in Fig. 4. It is of interest to see whether the substrate proteins reported by these investigators (5, 6, 8) would be common substrates for the phospholipid-sensitive phosphorylating system, or if this system has other specific substrate proteins, as seen for the total particulate of the cerebral cortex shown in Fig. 4.

The present studies have clearly demonstrated the occurrence in cerebral cortex of a Ca\(^{2+}\)-dependent phosphorylation system for endogenous proteins stimulated specifically by phospholipid, which was distinguishable from the calmodulin-stimulated system reported earlier by others (5-8). We have also noted that trifluoperazine, an antipsychotic drug shown to inhibit the calmodulin-sensitive Ca\(^{2+}\)-dependent reactions (20), had some inhibitory effect on the observed phospholipid-sensitive Ca\(^{2+}\)-dependent phosphorylation of endogenous proteins such as presented in Figs. 1, 3, and 4 (data not shown). The presence of this new phosphorylating system is not unique to brain tissue, since we have noted a widespread occurrence of substrate proteins for this system in the cytosol of a variety of rat tissues. These findings are in line with the presence of phospholipid-sensitive Ca\(^{2+}\)-dependent protein kinase (21) reported for the brain (1, 11, 12) and many other tissues (1, 12). The profound stimulatory effects of phosphatidylserine on the Ca\(^{2+}\)-dependent phosphorylation of endogenous proteins (Fig. 1) suggest potential major roles for the phospholipid-sensitive Ca\(^{2+}\)-dependent phosphorylation system. The observation that phosphatidylserine and calmodulin stimulated Ca\(^{2+}\)-dependent phosphorylation of specific endogenous proteins (Fig. 4) also suggests independent roles for either system. The actions of the two systems, on the other hand, may be overlapping, since common substrate proteins for them were also noted (Fig. 1, comparing Lanes 4 and 6).

From the present studies (Figs. 1 and 4), and observations made with other tissues (such as heart), it appears that the phospholipid-sensitive system may be primarily cytosolic, whereas the calmodulin-sensitive one seems associated with subcellular particulate fractions, and probably the cytosol as well. Regarding the particulate fraction, since membranes, like purified phospholipids, are capable of activating phospholipid-sensitive Ca\(^{2+}\)-dependent protein kinase (21), it is conceivable that Ca\(^{2+}\) influx in response to certain stimuli could cause an enhanced phosphorylation of membrane proteins, or cytosolic proteins in contact or in close proximity with the membrane. It would thus seem possible that phosphorylation of membrane proteins stimulated by exogenous Ca\(^{2+}\) or conditions associated with increased Ca\(^{2+}\) influx, analogous to the case of neuronal membrane protein I (22), could be mediated through a phospholipid-sensitive system. In any case, it seems quite likely that both Ca\(^{2+}\)-dependent phosphorylation systems function in the cerebral cortex or other tissues, either independently or in a complementary manner. At this point, the functional significance of, and the interrelationship between, the two putative Ca\(^{2+}\)-dependent protein phosphorylation systems are not clear. Clarification of these problems may be facilitated by exploring phosphorylation of specific endogenous proteins in response to physiologic stimuli or pharmacologic agents in intact cell preparations, and by studying regulatory mechanisms of the two species of Ca\(^{2+}\)-dependent protein kinases.

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