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Biochemistry

Ca^{2+}-dependent protein phosphorylation system in membranes from various tissues, and its activation by "calcium-dependent regulator"

(calcium/protein kinase)

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ABSTRACT **Analysis of membranes from a variety of tissues has revealed a widespread distribution of a protein phosphorylation system dependent on the presence of both Ca^{2+} and "calcium-dependent regulator" (CDR). This protein phosphorylation system has been studied in some detail in nervous tissue. Neuronal membranes contain a protein phosphorylation system that requires Ca^{2+} and a soluble heat-stable protein [Schulman, H. & Greengard, P. (1978) *Nature (London)* 271, 478–479]. This protein has been purified to homogeneity from bovine cerebral cortex, with use of an assay based on its ability to stimulate Ca^{2+}-dependent protein phosphorylation in membranes. This protein kinase activator appears to be identical to CDR of cyclic nucleotide phosphodiesterase. Throughout its purification, this single entity was found to activate both Ca^{2+}-dependent protein kinase and cyclic nucleotide phosphodiesterase. The kinase activator purified here and authentic CDR were equally effective in their ability to activate Ca^{2+}-dependent protein kinase.**

Previous studies from this laboratory indicated that brain membranes contain a Ca^{2+}-dependent protein phosphorylation system (1) in addition to the well-characterized cyclic AMP (cAMP)-dependent protein phosphorylation system. The Ca^{2+}-dependent protein kinase required a heat-stable protein factor as well as Ca^{2+}. Similarly, a heat-stable protein (calcium-dependent regulator; CDR) and Ca^{2+} have been shown to activate cyclic nucleotide phosphodiesterase (2, 3). CDR is an intracellular calcium receptor with multiple functions. In addition to regulating phosphodiesterase, it has been shown to activate a detergent-solubilized preparation of brain adenylate cyclase (4, 5) and a Ca^{2+}, Mg^{2+}-ATPase in plasma membranes from human erythrocytes (6, 7), and it appears to be a component of myosin light chain kinase in chicken gizzard (8) and rabbit skeletal muscle (9, 10). Our earlier studies (1) showed that Ca^{2+}-stimulated phosphorylation in brain was lost upon preparation of cytosol-free membranes and could be restored by reconstitution with either brain cytosol or purified CDR (1). In the present study, the endogenous activator present in brain cytosol was purified and compared with authentic CDR. In addition, cytosol-free membranes from a variety of non-neuronal tissues were examined for the occurrence of protein phosphorylation that was dependent on Ca^{2+} and CDR.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP (200 Ci/mmol) (1 Ci = 3.7×10^{10} becquerels) was purchased from ICN as the triethylammonium salt. [^{3}H]cAMP was purchased from New England Nuclear Corp., Boston, MA.

CDR was prepared from bovine cerebral cortex by the method of Teo *et al.* (11). CDR-depleted cyclic nucleotide phosphodiesterase was prepared from bovine cerebral cortex by removing endogenous CDR on a DEAE-Sephadex A-50 column as described by Watterson *et al.* (12). The concentrations of pure CDR and protein kinase activator were standardized by amino acid analysis and by measurement of absorbance at 280 nm using $A_{1\ mg/ml} = 0.18$. Nondenaturing polyacrylamide gel electrophoresis was performed by the method of Davis (13).

Assay of Protein Kinase Activator. Protein kinase activator was assayed for its ability to stimulate endogenous protein phosphorylation of synaptic membranes. Activator-deficient membranes were prepared from rat cerebral cortex as described previously (1). The reaction mixture (final volume, 100 μl) contained: 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes) buffer (pH 7.0), 10 mM $MgCl_2$, 0.1 mM dithioerythritol, 0.2 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (minus calcium) or 0.2 mM EGTA + 0.5 mM $CaCl_2$ (plus calcium), 5 μM [γ-^{32}P]ATP ($3.0–7.0 \times 10^4$ cpm/pmol), 34 μg of membrane protein, and variable amounts of column fractions containing the activator. After preincubation for 30 sec at 30°C, the reaction was initiated by addition of the [γ-^{32}P]ATP. Incubation was carried out for 30 sec, the reaction was terminated by addition of 50 μl of a "sodium dodecyl sulfate ($NaDodSO_4$) stop solution," and a 75-μl aliquot of the sample was analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and autoradiography as described previously (14, 15). The radioactive band at 51,000 daltons (see arrow, Fig. 1) was localized by autoradiography and cut out of the dried gel, and the radioactivity was quantitated by liquid scintillation spectrometry. Assay of kinase activator based on stimulation of ^{32}P incorporation into several other protein bands gave similar results.

Assay of Cyclic Nucleotide Phosphodiesterase. Cyclic nucleotide phosphodiesterase activity and the ability of CDR and protein kinase activator to stimulate cyclic nucleotide phosphodiesterase were assayed by a modification of the procedure of Thompson and Appleman (16). The reaction mixture (final volume, 100 μl) contained: 20 mM Tris·HCl (pH 8.0), 1 mM $MnCl_2$, 0.1 mM $CaCl_2$, 20 μM [^{3}H]cAMP (25 μCi/μmol), an appropriate amount of enzyme, and CDR or protein kinase activator. Incubation was carried out for 10 min. The reaction mixture was then treated with an excess of alkaline phosphatase (Worthington) and the [^{3}H]adenosine produced was measured after its batch separation with an anion-exchange resin (Bio-Rad AG1-X8).

Purification of Protein Kinase Activator. Kinase activator was purified by using the same sequence of chromatographic steps used by Watterson *et al.* (12) for purification of CDR with

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Abbreviations: CDR, "calcium-dependent regulator"; cAMP, cyclic AMP; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

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only minor modifications. Bovine cerebral cortex (360 g) was the source of the cytosol. The first DEAE-Sephadex step was carried out at pH 6.7 rather than pH 7.2 and the second DEAE-Sephadex step was carried out at pH 6.9 rather than pH 7.5. The last ammonium sulfate precipitation step was omitted.

Preparation of Activator-Deficient Membrane Fractions from Various Tissues. Total membrane fraction was prepared from 1 g of each of the rat tissues studied. The tissue was first minced with a McIlwain tissue chopper. It was then homogenized in 15 vol of 5 mM Tris·HCl (pH 7.0)/0.5 mM EDTA/1 mM 2-mercaptoethanol by using 12 up-and-down strokes with a Teflon–glass homogenizer at 1000 rpm. Cellular debris, nuclei, and connective tissue were eliminated by centrifugation at 900 × *g* for 10 min. The remaining material was centrifuged at 150,000 × *g* for 40 min, and pellets were resuspended in the original volume of homogenization buffer and centrifuged again to reduce the amount of activator. Pellets were resuspended in the homogenization buffer to give appropriate protein concentrations.

RESULTS

Purification of the Endogenous Activator of Ca^{2+}-Dependent Protein Kinase. The heat stability of the protein kinase activator (1) and the involvement of Ca^{2+} in the endogenous phosphorylation of membrane proteins suggested that a Ca^{2+}-binding protein similar to that which modulates the activities of cyclic nucleotide phosphodiesterase and adenylate cyclase might be involved. Indeed, it was demonstrated that the Ca^{2+}-binding protein (CDR) isolated from bovine brain could stimulate protein phosphorylation in activator-depleted membranes (1). Purification of the endogenous kinase activator was therefore undertaken in order to determine its relationship to CDR. Bovine cerebral cortex, which manifests Ca^{2+}-dependent protein phosphorylation similar to that seen with rat cerebral cortex (data not shown), was used as the source of kinase activator. Kinase activator was assayed by its ability to stimulate endogenous protein phosphorylation in activator-depleted membranes prepared from rat cerebral cortex.

The results of the procedure used for purification of the protein kinase activator are outlined in Table 1. Throughout the purification, only a single peak of protein kinase activator was found, suggesting that only one protein in the cytosol is able to reconstitute Ca^{2+}-dependent protein kinase activity. When CDR was monitored, it was found to comigrate with the kinase activator throughout the purification. The yields of the two activities were nearly identical at each step of the purification (Table 1). In addition, when column fractions were monitored by a third method, namely protein staining patterns obtained upon $NaDodSO_4$/polyacrylamide gel electrophoresis, both activities copurified with an 18,000-dalton polypeptide. The

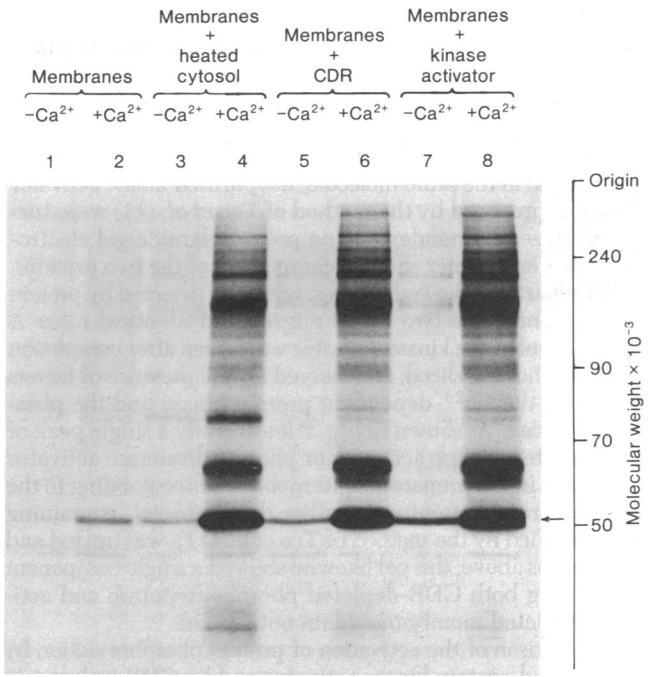

FIG. 1. Autoradiograph illustrating endogenous Ca^{2+}-dependent protein phosphorylation of brain membranes, and activation by heated cytosol, purified CDR, or purified kinase activator. Cytosol-free membranes (34 μg of protein) were incubated in the standard reaction mixture containing 0.2 mM EGTA (minus calcium) or 0.2 mM EGTA + 0.5 mM $CaCl_2$ (plus calcium), 5 μM [γ-^{32}P]ATP (5 × 10^4 cpm/pmol), and, where indicated, heated cytosol (14 μg of protein), purified CDR (0.25 μg of protein), or purified kinase activator (0.25 μg of protein). The arrow indicates the 51,000-dalton phosphoprotein used for quantitative measurements of stimulation of ^{32}P incorporation into membrane protein.

kinase activator (and CDR) comprised about 1% of the soluble protein in bovine brain cytosol (Table 1), as also indicated for CDR by Watterson *et al.* (12).

Stimulation of Endogenous Protein Phosphorylation by CDR and Kinase Activator. As reported earlier (1), membranes depleted of kinase activator do not show significant Ca^{2+}-dependent phosphorylation (Fig. 1, lanes 1 and 2). Upon addition of untreated cytosol (data not shown), or heat-treated cytosol (Fig. 1, lanes 3 and 4), the Ca^{2+}-dependent phosphorylation was recovered. The same phosphorylation pattern could be obtained by substitution of authentic CDR, prepared from bovine cerebral cortex by the method of Teo *et al.* (11), for the heat-treated cytosol (Fig. 1, lanes 5 and 6). Minor differences in the two patterns are apparently due to soluble substrates that are present in the cytosol but absent in the purified CDR preparation. The kinase activator, purified as de-

Table 1. Purification of protein kinase activator from bovine cerebral cortex

Purification step	Total volume, ml	Total protein, mg	Protein kinase activator: Specific activity, units/mg	Recovery, %	Purification, fold	Phosphodiesterase activator: Specific activity, units/mg	Recovery, %	Purification, fold
Homogenization	830	6225	620	100	1.0	500	100	1.0
CM-Sephadex C-50	1380	5208	660	89	1.1	550	92	1.1
DEAE-Sephadex A-25	144	111	13,400	38	22	12,500	44	25
DEAE-Sephadex A-50	79	26.5	38,600	26	62	35,000	30	69
Sephadex G-100	40	15.5	43,600	18	70	38,500	19	76

One unit of activator is defined as the amount necessary to produce half-maximal stimulation either of ^{32}P incorporation into activator-deficient membranes or of cAMP hydrolysis by activator-deficient phosphodiesterase in 1 min under standard assay conditions.

scribed in the preceding section, was qualitatively and quantitatively similar to CDR in its stimulation of protein phosphorylation (Fig. 1, compare lanes 6 and 8).

Identification of the Endogenous Ca^{2+}-Dependent Protein Kinase Activator as CDR. To examine more closely whether the activator for the protein kinase and for the phosphodiesterase reside in the same molecule, the purified kinase activator and CDR prepared by the method of Teo *et al.* (11) were further analyzed by nondenaturing polyacrylamide gel electrophoresis. Fig. 2 *upper* shows a comparison of the two proteins. Each preparation showed only a single band detected by protein staining, and these two bands migrated at identical rates. A duplicate gel of the kinase activator was frozen after completion of electrophoresis, sliced, and assayed for the presence of factors activating the Ca^{2+}-dependent protein kinase and the phosphodiesterase. As shown in Fig. 2 *lower*, only a single peak of either protein kinase activator or phosphodiesterase activator was found in the preparation, its mobility corresponding to the band of protein staining. Another duplicate gel, containing CDR purified by the method of Teo *et al.* (11), was treated and analyzed as above; this gel likewise showed a single component stimulating both CDR-depleted phosphodiesterase and activator-depleted membranes (data not shown).

Comparison of the activation of protein phosphorylation by the purified protein kinase activator and by CDR is shown in Fig. 3. Addition of either protein kinase activator or CDR caused a dose-dependent increase in the rate of ^{32}P incorporation into a representative membrane protein (see arrow, Fig. 1). The two factors were equally effective in stimulating protein kinase activity. Each caused about a 6-fold stimulation, with half-maximal stimulation at about 0.25 μg of protein. Phosphate incorporation into several other proteins was measured quantitatively in the same way and showed a similar response to the two factors. The two proteins were also indistinguishable in their

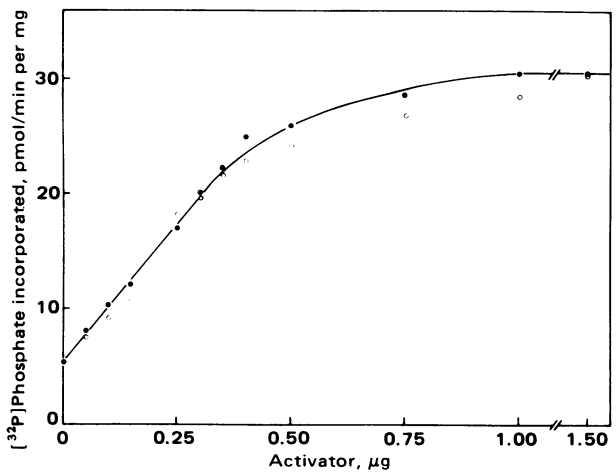

FIG. 3. Activation of Ca^{2+}-dependent protein phosphorylation by purified kinase activator and by authentic CDR. Cytosol-depleted membranes were activated by various amounts of purified kinase activator (●) or CDR (○) in the presence of Ca^{2+} under standard conditions.

ability to stimulate a partially purified CDR-depleted phosphodiesterase preparation (data not shown).

CDR confers an extreme Ca^{2+} sensitivity upon cyclic nucleotide phosphodiesterase (17). It was therefore of interest to determine the dependence of protein phosphorylation on Ca^{2+} in the presence of the kinase activator or CDR. The effect of various concentrations of Ca^{2+} on protein phosphorylation is shown in Fig. 4. With either activator, half-maximal stimulation occurred at a free Ca^{2+} concentration of about 0.3 μM. This is consistent with the findings that under various conditions half-maximal activation of CDR-dependent phosphodiesterase

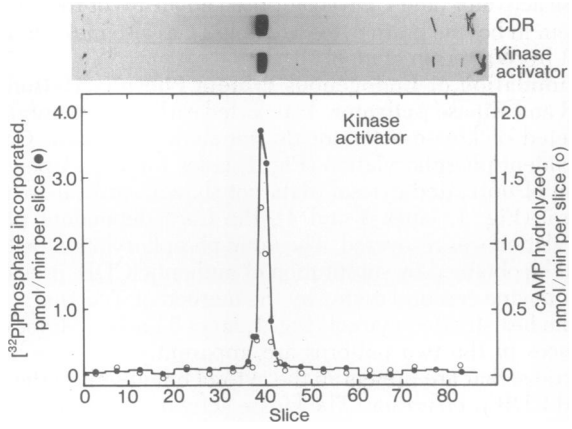

FIG. 2. Comparison of authentic CDR and purified kinase activator by nondenaturing polyacrylamide gel electrophoresis. (*Upper*) Samples containing 30 μg of purified CDR (upper gel) or 20 μg of purified kinase activator (lower gel) were subjected to nondenaturing polyacrylamide gel electrophoresis in long gels (9.8 cm) containing 15% acrylamide, pH 8.9. (*Lower*) A duplicate sample of kinase activator (10 μg) was electrophoresed simultaneously with the above samples, frozen, and sliced into 1.1-mm segments. Protein was eluted from each slice by incubation at 4°C for 48 hr in 200 μl of 50 mM Pipes buffer, pH 7.0. Aliquots (8 μl) of each fraction, or a pool of five consecutive fractions (40 μl total), were assayed for activation of Ca^{2+}-dependent protein phosphorylation (●). Aliquots (2 μl) of each fraction, or a pool of five consecutive fractions (10 μl total) were also assayed for activation of CDR-depleted phosphodiesterase (○). Distances along the two gels are directly related to distances in the profiles of activity. The solid line is drawn to indicate the profile of protein kinase activator.

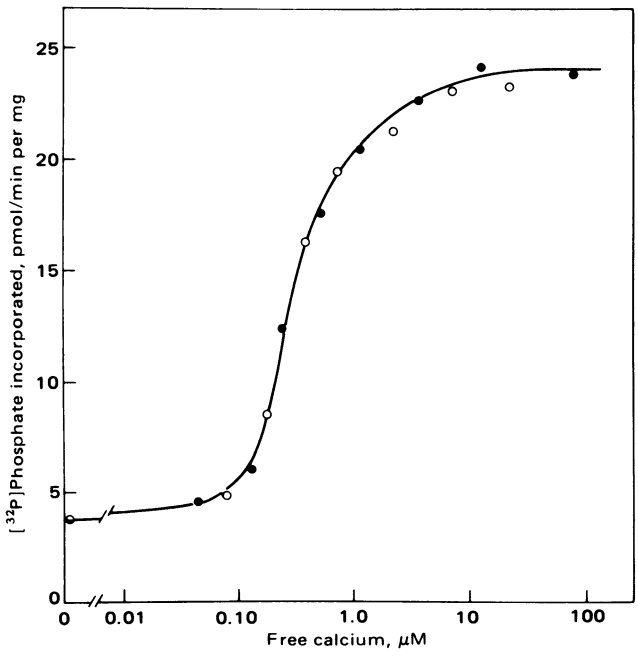

FIG. 4. Activation by Ca^{2+} of protein phosphorylation, with either purified kinase activator or authentic CDR as the source of activator. Activator-depleted membranes were incubated with either 1 μg of kinase activator (●) or 1 μg of CDR (○) under standard conditions at pH 7.1. Free Ca^{2+} concentration was varied by use of a Ca^{2+}/EGTA buffer containing 0.4 mM EGTA. Free Ca^{2+} concentration was determined by using an apparent binding constant for Ca^{2+}-EGTA of 7.61×10^6 M^{-1} (18).

Table 2. Stoichiometric interaction of protein kinase activator with endogenous Ca^{2+}-dependent protein phosphorylation system

Amount of cytosol added, μg protein	Preincubation, sec	Increase in [^{32}P]phosphate incorporation, pmol/mg per min	
		$+Ca^{2+}$	$-Ca^{2+}$
5.6	5	4.4 ± 0.2	—
5.6	300	4.7 ± 0.3	0.4 ± 0.2
10.5	5	8.4 ± 1.1	—
10.5	300	8.6 ± 0.3	1.3 ± 0.1

Activator-deficient membranes from rat cerebral cortex were preincubated with 5.6 or 10.5 μg of cytosol and 100 μM free Ca^{2+}. The time of preincubation was either 5 sec or 300 sec at 30°C. Incubation was then initiated by the addition of [γ-^{32}P]ATP. Where indicated (minus calcium), 1 mM EGTA was added after the preincubation period and prior to addition of [γ-^{32}P]ATP to determine whether activation was reversible.

and binding of Ca^{2+} to CDR occurs in the range of 0.2 μM to 3 μM free Ca^{2+} (17, 19).

Nature of the Interaction of the Kinase Activator with the Membrane. Nishizuka and his colleagues (20) have recently described a protein kinase in liver and brain that can be activated by Ca^{2+} and a protein factor. However, the mechanism of that activation appears to be quite different from the one studied in the present investigation. Nishizuka and associates have purified the protein factor and shown it to be a Ca^{2+}-dependent protease that converts an inactive protein kinase to an active one. This Ca^{2+}-dependent stimulation of protein phosphorylation is an irreversible process that is dependent on the time of preincubation of the protease with the kinase proenzyme. The Ca^{2+}-dependent protein phosphorylation that we have described does not result from proteolytic activation of a protein kinase.

As shown in Table 2, activation of endogenous protein phosphorylation by the kinase activator appears to result from a reversible and stoichiometric interaction between the activator and protein kinase. Activation of endogenous protein phosphorylation by the kinase activator was assayed in a two-step process. First, activator-depleted membranes were preincubated for different lengths of time with different amounts of cytosol in medium lacking [γ-^{32}P]ATP. The effect of preincubation time or of amount of cytosol on protein kinase activity was then assayed by addition of [γ^{32}P]ATP and measurement of ^{32}P incorporation into a representative phosphopeptide. Submaximal activation of the protein kinase, achieved by addition of low levels of kinase activator, was not changed by increasing the preincubation period. In contrast, the degree of stimulation was controlled by the amount of kinase activator added. In addition, if EGTA was added after the preincubation of membranes and cytosol, but prior to addition of [γ-^{32}P]ATP, stimulation by the kinase activator was prevented (Table 2). Thus, activation of the membrane-bound kinase by the kinase activator is distinct from the irreversible, Ca^{2+}-dependent, activation of protein kinase characterized by Nishizuka and associates (20).

Occurrence of CDR- and Ca^{2+}-Dependent Protein Phosphorylation in Non-Neuronal Tissues. Calcium ion has important functions in the physiology of numerous tissues (21). As for brain, little is known about the biochemical basis of the action of Ca^{2+} in these tissues. In light of the finding of CDR- and Ca^{2+}-dependent protein phosphorylation in brain membranes, it was of interest to determine whether the action of Ca^{2+} on membrane function in other tissues might also be mediated by such a mechanism. Analysis of endogenous protein phosphorylation in total membrane fractions from six rat tissues (lung, spleen, skeletal muscle, vas deferens, heart, and adrenal) is shown in Fig. 5. A total membrane fraction, washed free of cytosol and CDR, was prepared from each of the tissues and then assayed for endogenous Ca^{2+}-dependent phosphorylation in either the absence or the presence of kinase activator purified from bovine brain. It can be seen that membranes from each of the six tissues contained a CDR- and Ca^{2+}-dependent protein phosphorylation system. For example, in membranes from vas deferens, neither Ca^{2+} alone nor protein kinase activator alone was effective, whereas addition of both Ca^{2+} and protein kinase activator stimulated protein phosphorylation (Fig. 5 *right*, compare lanes 1–4). Each tissue displayed a different pattern of Ca^{2+}-stimulated protein phosphorylation that was distinct from the cAMP-dependent phosphorylation seen in the same tissue (data not shown). One of the low molecular weight polypeptides (20,000 daltons) seen in the particulate fraction from vas deferens may be myosin light chain, which has recently been shown to be phosphorylated by myosin light chain

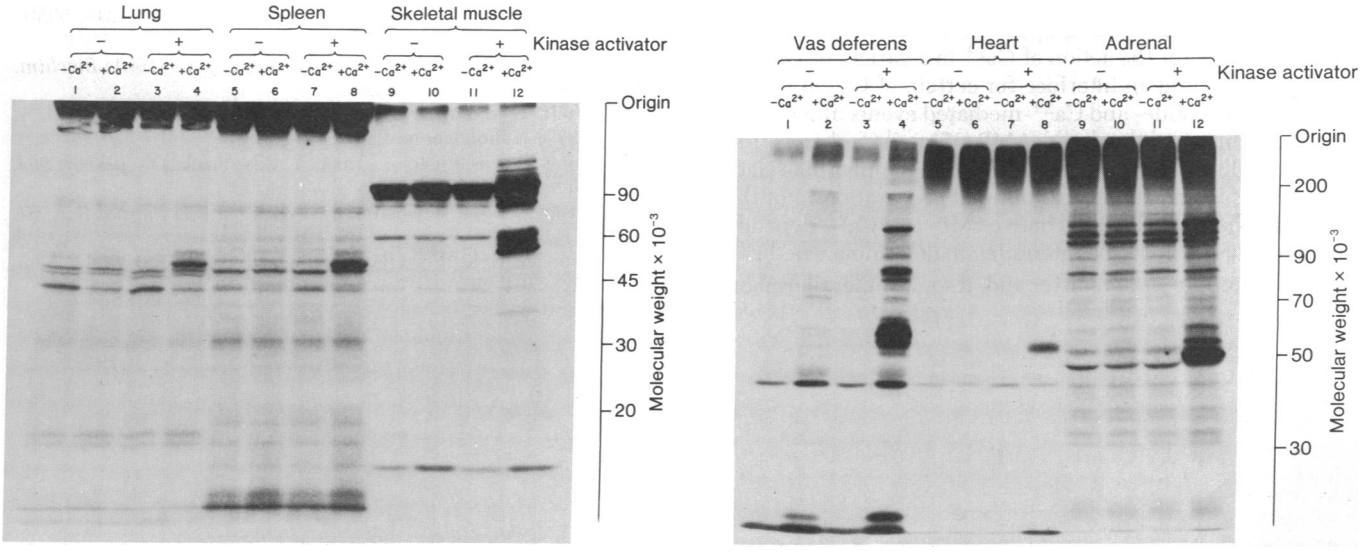

FIG. 5. Autoradiograph illustrating endogenous CDR- and Ca^{2+}-dependent phosphorylation of membrane proteins from various rat tissues. Total membrane fractions, washed free of CDR, were assayed for endogenous Ca^{2+}-dependent phosphorylation in either the absence or the presence of 1 μg of CDR (kinase activator purified from bovine brain). The tissues studied, and the amounts of membrane protein applied to each lane were: lung, 7.7 μg; spleen, 11.4 μg; skeletal muscle, 5.0 μg; vas deferens, 5.4 μg; heart, 5.3 μg; and adrenal, 6.8 μg.

kinase, an enzyme requiring both CDR and Ca^{2+} for activity (8). The conditions used to assay Ca^{2+}-dependent protein phosphorylation in various tissues in the present study were those that had been standardized for brain membranes and were not optimized for any other tissue. It is therefore possible that substrates for the Ca^{2+}-dependent kinase, in addition to those detected here, exist in these membranes. The analysis does demonstrate, however, a widespread distribution of CDR- and Ca^{2+}-dependent protein phosphorylation in membranes from various tissues.

DISCUSSION

In the present study, the endogenous activator of the Ca^{2+}-dependent protein phosphorylation system of brain membranes was purified from bovine brain cytosol by a procedure that omitted the heating step normally used in purifying CDR. This procedure was chosen to ensure that no activators were destroyed or produced by the boiling step. The results presented here demonstrate that the protein kinase activator is indistinguishable from CDR in its chromatographic and electrophoretic properties, its ability to activate Ca^{2+}-dependent protein phosphorylation, and its ability to confer Ca^{2+} sensitivity on the protein kinase. In addition, preliminary experiments indicate that the two proteins are identical on the basis of amino acid composition, UV spectrum, and peptide mapping (unpublished data).

Earlier studies of protein phosphorylation in intact synaptosomes indicated that Ca^{2+} influx enhanced the incorporation of radioactive phosphate into several polypeptides (22). The CDR- and Ca^{2+}-dependent protein phosphorylation system examined here may be the agent mediating these changes. This CDR-dependent protein phosphorylation system is extremely sensitive to low concentrations of free Ca^{2+} and would therefore be responsive to physiological changes in Ca^{2+} ion concentration. It may mediate some of the actions of Ca^{2+} in the presynaptic terminal.

The finding of CDR- and Ca^{2+}-dependent protein phosphorylation in membranes from a variety of tissues suggests that such a phosphorylation system may be of general importance as a mechanism by which some of the actions of Ca^{2+} are mediated. In addition to the CDR- and Ca^{2+}-dependent protein kinase, membranes from these tissues also contain a tissue-specific array of endogenous substrates for the kinase. This finding offers a promising approach for examining the biochemical basis for the action of Ca^{2+} in a variety of tissues, as well as suggesting an interface for certain of the interactions of cyclic nucleotide- and Ca^{2+}-mediated events. It has, for instance, been found that both cAMP (15) and Ca^{2+} (refs. 1 and 22; unpublished experiments) stimulate the phosphorylation of proteins Ia and Ib in synaptosomal preparations. For those proteins phosphorylated by both cAMP- and Ca^{2+}-dependent protein kinases, it will be interesting to determine whether the sites of phosphorylation differ and, if so, whether phosphorylation by the two kinase systems elicits different changes in the activity of the substrate protein.

The active form of Ca^{2+} for a variety of reactions appears to be the CDR·Ca^{2+} complex. Increases in intracellular calcium are extremely transient because of the rapid uptake of Ca^{2+} by mitochondria and sarcoplasmic reticulum. Binding of Ca^{2+} to CDR, which is present in many tissues at very high concentrations, may prolong the effectiveness of Ca^{2+}, and thereby facilitate its regulation of various biochemical reactions.

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