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Differential regulation of bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase isozymes by cyclic AMP-dependent protein kinase and calmodulin-dependent phosphatase

(cell calcium/protein phosphorylation)

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ABSTRACT **Purified bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase (3′,5′-cyclic-nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17) contains isozymes that are composed of two distinct subunits with molecular masses of 60,000 and 63,000 daltons. Analysis by $NaDodSO_4$ gel electrophoresis and autoradiography of a phosphodiesterase sample phosphorylated in the presence of [^{32}P]ATP and bovine heart cAMP-dependent protein kinase catalytic subunit revealed that only the 60-kDa subunit was phosphorylated. By using an isozyme preparation greatly enriched with the 60-kDa subunit, the following observations regarding the subunit phosphorylation were made. First, the phosphorylation resulted in the maximal incorporation of about 2 mol of phosphate per mol of subunit. Second, complete inhibition of 60-kDa subunit phosphorylation was approached at a saturating concentration of Ca^{2+} when a molar ratio of calmodulin to phosphodiesterase of 2:1 was used. No inhibition was observed in the presence of either Ca^{2+} or calmodulin alone. Third, the phosphorylation was accompanied by a decrease in the enzyme affinity for calmodulin; calmodulin concentrations required for 50% activation of nonphosphorylated and maximally phosphorylated phosphodiesterase isozyme samples were 0.51 and 9.3 nM, respectively. Fourth, the phosphodiesterase isozyme could be dephosphorylated by the calmodulin-dependent phosphatase (calcineurin) in the presence of Ni^{2+} or Mn^{2+}, the dephosphorylation being associated with an increase in the enzyme affinity for calmodulin. Fifth, peak II rabbit liver phosphoprotein phosphatase catalytic unit did not catalyze the dephosphorylation of the phosphodiesterase isozyme.**

Calmodulin-dependent cyclic nucleotide phosphodiesterase (3′,5′-cyclic-nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17) is one of the key enzymes involved in the complex interactions between the cyclic nucleotide and Ca^{2+} second messenger systems. The enzyme has been purified close to homogeneity from both bovine brain (1–4) and bovine heart (5) and extensively characterized. Recent studies (6, 7) showed that there are tissue-specific isozymes of calmodulin-dependent phosphodiesterase. Hansen and Beavo (6) demonstrated that bovine heart and brain phosphodiesterase have slightly different subunit molecular masses: 59,000 and 61,000 daltons, respectively. Using monoclonal antibodies, we showed that a bovine brain phosphodiesterase purified by a previously established procedure (2) contains two distinct subunits with molecular masses of 60,000 and 63,000 daltons, the two types of subunits exhibiting different peptide maps and antigenic properties (7). Since the enzyme is a dimeric protein (1–4), three isozymes—two homodimers and a heterodimer—could arise from the two subunits. Partial separation of the isozymes by using monoclonal antibodies indicates that the homodimers are major components of the enzyme preparation (7).

To understand the regulatory significance of the phosphodiesterase isozymes, we have begun to compare catalytic and regulatory properties of bovine brain isozymes. In previous studies (2, 8) it has been shown that bovine brain calmodulin-dependent phosphodiesterase can be phosphorylated in a cAMP-dependent protein kinase reaction. However, it is not clear which of the phosphodiesterase isozymes was present in these studies, and the effect of the phosphorylation on enzyme activity was not established.

In this study, we reexamined the possible regulation of calmodulin-dependent phosphodiesterase by cAMP-dependent protein kinase, using purified phosphodiesterase isozymes. We found that the isozymes are differently regulated by the protein kinase. Only the 60-kDa subunit-containing isozyme can be phosphorylated by cAMP-dependent protein kinase, resulting in a maximal incorporation of two phosphates per subunit. The phosphorylation is accompanied by a decrease to 1/20th in the affinity of the isozyme for calmodulin. Both the protein phosphorylation and the change in calmodulin affinity may be reversed by a calmodulin-dependent phosphatase (calcineurin).

EXPERIMENTAL PROCEDURES

Protein Preparation. Bovine brain calmodulin-dependent cyclic nucleotide phosphodiesterase, containing a mixture of isozymes, and calmodulin-dependent phosphatase of bovine brain were purified as described by Sharma *et al.* (9). Bovine brain calmodulin was purified by using a phenyl-Sepharose column as described by Gopalakrishna and Anderson (10). Bovine heart cAMP-dependent protein kinase catalytic subunit was purified to homogeneity as described by Demaille *et al.* (11). The catalytic unit of rabbit liver peak II phosphoprotein phosphatase (12) was a generous gift from R. Khandelwal (University of Saskatchewan). Heat-stable inhibitor protein of the protein kinase was purchased from Sigma. A monoclonal antibody C1, which is specific for the 60-kDa isozyme subunit, was produced and purified as described (7). The antibody-Sepharose 4B conjugate used for affinity chromatography purification of the 60-kDa phosphodiesterase isozyme was prepared as described (7).

METHODS

Purification of Phosphodiesterase Isozymes. Early steps for the isozyme purifications, including the calmodulin-Sepharose 4B chromatography stage, were as described for the purification of calmodulin-dependent phosphodiesterase (9). Fractions from a calmodulin-Sepharose 4B column containing calmodulin-dependent phosphodiesterase activity were pooled and concentrated by ultrafiltration with an Amicon PM-10 membrane. The concentrated sample was applied to a

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C1 antibody-Sepharose 4B column (1.5 × 8.0 cm), which had been preequilibrated with buffer A (20 mM Tris·HCl/1 mM imidazole/1 mM magnesium acetate, pH 7.0) containing 2 mM EDTA and 10 mM 2-mercaptoethanol. Fractions were eluted with 2.5 M $MgCl_2$, and those fractions containing phosphodiesterase activity were pooled and dialyzed against buffer A containing 0.1 mM EGTA, 10 mM 2-mercaptoethanol, and 10% sucrose to remove $MgCl_2$. The dialyzed sample was concentrated to at least 150 μg of protein per ml by Amicon ultrafiltration. This sample was greatly enriched with the 60-kDa subunit and usually contained <10% 63-kDa subunit. This preparation is referred to as the 60-kDa isozyme.

In order to further purify the 63-kDa isozyme, the breakthrough fraction and 150 ml of the initial column wash from the C1 antibody-Sepharose 4B column were pooled, concentrated, and further purified by Sephadex G-200 gel filtration as described for the purification of calmodulin-dependent phosphodiesterase (9).

Preparation of ^{32}P-Labeled 60-kDa Phosphodiesterase Isozyme. Phosphorylation of the 60-kDa isozyme (310 μg/ml) was carried out at 30°C in a 1-ml reaction mixture containing 30 μg of catalytic subunit of cAMP-dependent protein kinase per ml, 100 mM Tris·HCl (pH 7.0), 5 mM magnesium acetate, 5 mM 2-mercaptoethanol, and 0.2 mM [γ-^{32}P]ATP (400–500 cpm/pmol). After incubation for 60 min, an aliquot was taken for the determination of phosphate incorporation. The remaining sample was dialyzed overnight with several changes against buffer A containing 10 mM 2-mercaptoethanol and 10% sucrose to remove unreacted [γ-^{32}P]ATP. The dialyzed sample was used for 60-kDa phosphodiesterase isozyme dephosphorylation experiments.

Protein Phosphorylation, $NaDodSO_4$ Gel Electrophoresis, and Autoradiography. Phosphorylation of phosphodiesterase was carried out at 30°C in a reaction mixture containing 100 mM Tris·HCl (pH 7.0), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.2 mM [γ-^{32}P]ATP (100–200 cpm/pmol), and the catalytic subunit of cAMP-dependent protein kinase. Phosphorylation was monitored by the filter paper disc method (13) or by autoradiography following $NaDodSO_4$ gel electrophoresis. $NaDodSO_4$ gel electrophoresis was carried out as described (7) with 12% acrylamide gels. After electrophoresis the gel was stained in Coomassie brilliant blue, destained, and dried for autoradiography on Kodak Min-R x-ray film.

Other Methods. Assay of cyclic nucleotide phosphodiesterase activity was as described (2) except the assay mixture did not contain imidazole. The activation of calmodulin-dependent phosphatase by preincubation with activating metal ions was carried out as described (14).

RESULTS

Phosphorylation of 60-kDa Bovine Brain Calmodulin-Dependent Phosphodiesterase Isozyme. Purified bovine brain calmodulin-dependent phosphodiesterase contains three isozymes that are composed of two distinct subunits of 60 and 63 kDa. The two types of subunits are usually present in equal amounts (Fig. 1, lane 1) (7). Analysis by $NaDodSO_4$ gel electrophoresis and autoradiography of an isozymic mixture of phosphodiesterase, phosphorylated by the catalytic subunit of cAMP-dependent protein kinase in the presence of [γ-^{32}P]ATP, revealed that only the 60-kDa subunit of the phosphodiesterase was phosphorylated (Fig. 1, lane 5). Similarly, when purified isozyme preparations containing only 60-kDa or greatly enriched with 60-kDa subunit were used, only the 60-kDa subunit could be phosphorylated (Fig. 1, lane 7). The phosphorylation of the 60-kDa isozyme was essentially abolished when the heat-stable inhibitor of cAMP-dependent protein kinase was included in the reaction mixture, suggesting that the phosphorylation was catalyzed by

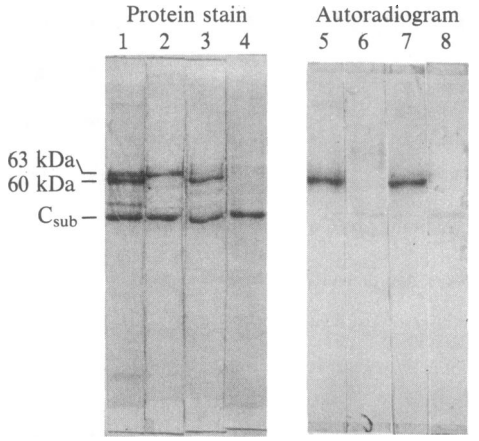

FIG. 1. Phosphorylation of calmodulin-dependent phosphodiesterase by the catalytic subunit of cAMP-dependent protein kinase (C_{sub}). Purified phosphodiesterase (containing a mixture of isozymes) (182.0 μg/ml), the 63-kDa isozyme (92.0 μg/ml), and the 60-kDa isozyme (92.0 μg/ml) were phosphorylated by 70 μg of C_{sub} per ml for 60 min as described. After phosphorylation, aliquots of the samples were subjected to $NaDodSO_4$ gel electrophoresis. All lanes contained about 5.0 μg of C_{sub}. Lanes: 1, purified phosphodiesterase (12.0 μg); 2, 63-kDa isozyme (6.0 μg); 3, 60-kDa isozyme (6.0 μg); 4, C_{sub} alone; 5–8, respective autoradiographs of lanes 1–4. The location of radioactive spots was determined by aligning the radioautographs on the gel.

cAMP-dependent protein kinase rather than by a contaminating kinase (results not shown). Using different concentrations of catalytic unit of the protein kinase (from 30 to 300 μg/ml) and prolonged reaction time (up to 60 min), we found maximal incorporation of phosphate to range from 1.8 to 2.3 mol of phosphate per mol of subunit. The initial rate of phosphorylation of the 60-kDa isozyme (5.0 μM) by the catalytic subunit of protein kinase was determined to be about 15% of that of histone H3 (20.0 μM) phosphorylation. In some preparations of the 60-kDa isozyme, a minor phosphorylated polypeptide of 58 kDa could be detected (Fig. 2 *Inset*). The identity of this phosphopeptide is not known.

Effect of Ca^{2+} and Calmodulin on the 60-kDa Subunit Phosphodiesterase Phosphorylation. The inclusion of both Ca^{2+} and calmodulin in the reaction mixture essentially eliminated the phosphorylation of the 60-kDa phosphodiesterase subunit by the protein kinase, whereas calmodulin or Ca^{2+} alone had little or no effect (Fig. 2 *Inset*). The inhibition by these effectors appears to be substrate-directed—i.e., due to the interaction of Ca^{2+}/calmodulin with the phosphodiesterase, since histone phosphorylation by cAMP-dependent protein kinase catalytic unit was not markedly affected by Ca^{2+} and calmodulin (results not shown).

To further substantiate the suggestion that Ca^{2+} and calmodulin affect the phosphorylation by binding to the protein substrate, the dependence of the protein phosphorylation on calmodulin concentration was examined. The inhibition was linearly dependent upon the calmodulin concentration at low concentrations of calmodulin and approached completion when a 2:1 molar ratio of calmodulin to phosphodiesterase was reached (Fig. 2). This is expected if the inhibition results from the interaction of calmodulin with the phosphodiesterase, since it is well established that one molecule of phosphodiesterase is capable of binding two molecules of calmodulin (15).

Relationship Between Phosphorylation and Activity of Phosphodiesterase Isozymes. Phosphorylation of the 60-kDa phosphodiesterase isozyme by the catalytic subunit of the protein kinase was accompanied by a decrease in enzyme activity when the enzyme assay was carried out in the presence of 3.4 nM calmodulin, a concentration giving rise to

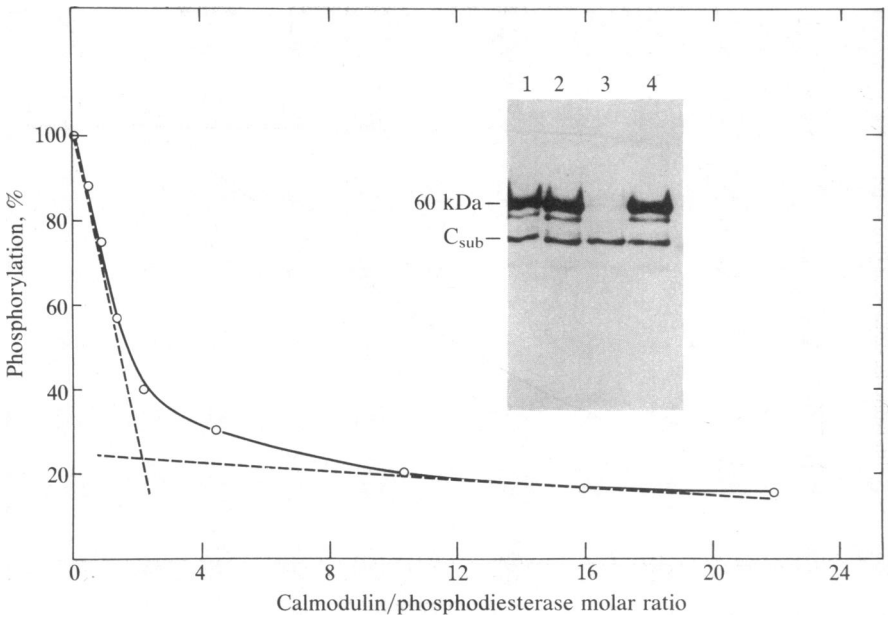

FIG. 2. Inhibition of phosphorylation of the 60-kDa isozyme by calmodulin in the presence of Ca^{2+}. The 60-kDa isozyme (0.148 nmol in 100 μl) was incubated in the presence of 0.1 mM Ca^{2+} with various amounts of calmodulin and 30 μg of catalytic subunit (C_{sub}) per ml under standard phosphorylation conditions as described. After incubation for 60 min, aliquots were removed and analyzed for phosphate incorporation. The amount of phosphate incorporated in the absence of calmodulin was 2.1 mol/mol of subunit and was taken as 100% phosphorylation. (*Inset*) Phosphorylation of the 60-kDa isozyme (0.148 nmol in 100 μl) was carried out with C_{sub} (30.0 μg/ml) in the presence of 0.1 mM Ca^{2+}, 0.1 mM EGTA, 0.1 mM Ca^{2+}/1.5 nmol of calmodulin per 100 μl, or 0.1 mM EGTA/1.5 nmol of calmodulin per ml. After a 60-min reaction time, aliquots were subjected to electrophoresis and autoradiography. Lanes: 1 and 2, samples phosphorylated in 0.1 mM Ca^{2+} and 0.1 mM EGTA, respectively; 3 and 4, samples phosphorylated in the presence of calmodulin in 0.1 mM Ca^{2+} and 0.1 mM EGTA, respectively.

about 80% maximal activation of the original enzyme (Fig. 3). After 60 min, 1.8 mol of phosphate was incorporated per mol of phosphodiesterase subunit, and the enzyme activity approached the level of basal enzyme activity. In contrast,

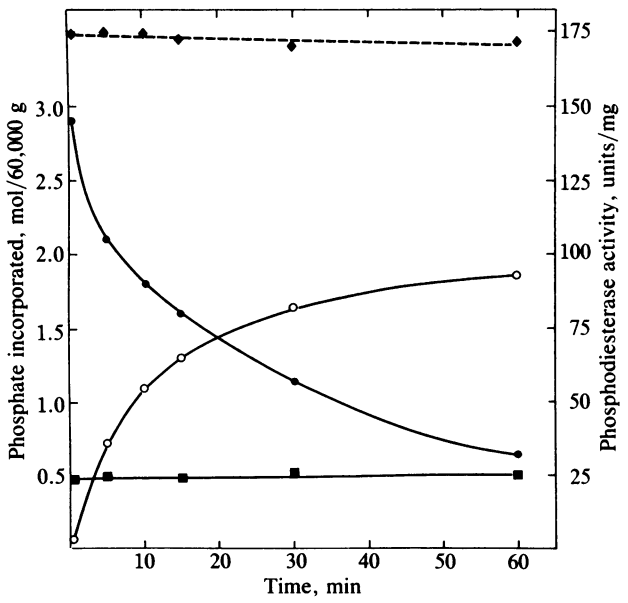

FIG. 3. Time courses of phosphorylation and activity modification of the 60-kDa isozyme by the catalytic subunit of protein kinase. Phosphodiesterase (310 μg/ml) was phosphorylated by the catalytic subunit of protein kinase (30 μg/ml) in a standard reaction mixture. At the time intervals indicated, separate aliquots were removed for analysis of phosphate incorporation (○) and phosphodiesterase activity in the presence of 3.4 nM (●) and 340.0 nM (◆) calmodulin with 0.1 mM Ca^{2+}. Basal activity was determined in the presence of 0.1 mM EGTA (■).

phosphorylation had little or no effect on the enzyme activity when the assay was carried out in the absence of calmodulin or in the presence of an excess amount of calmodulin (340.0 nM). The results suggest that phosphorylation lowers the enzyme affinity for calmodulin.

This suggestion was confirmed by comparing the dose-dependent activation of the nonphosphorylated phosphodiesterase with that of a phosphorylated isozyme sample containing 2.2 mol of phosphate per mol of subunit (Fig. 4). The concentration of calmodulin required for 50% activation of the phosphorylated phosphodiesterase was about 20-fold higher than that of the nonphosphorylated enzyme: 0.51 nM and 9.3 nM, respectively. In a previous study, we demonstrated that calmodulin-dependent bovine brain phosphodiesterase could be phosphorylated by cAMP-dependent protein kinase reactions but failed to detect any changes in the catalytic properties of this phosphodiesterase (2). The reason for this discrepancy is not clear.

Dephosphorylation of Phosphodiesterase Isozyme by Calcineurin. Two phosphoprotein phosphatases, a 35-kDa rabbit liver phosphoprotein phosphatase (peak II) (12) and a calmodulin-dependent phosphatase (calcineurin) were tested for their abilities to dephosphorylate the 60-kDa phosphophosphodiesterase isozyme. The phosphophosphodiesterase isozyme could be dephosphorylated by the calmodulin-dependent phosphatase when Ni^{2+} or Mn^{2+} was present (Fig. 5). The phosphatase activity was greatly enhanced by the inclusion of calmodulin (Fig. 5). When Ca^{2+} instead of Ni^{2+} or Mn^{2+} was used, little dephosphorylation of the enzyme occurred (results not shown). In contrast to the calmodulin-dependent phosphatase, peak II phosphoprotein phosphatase (0.5 unit/ml) possessed no detectable activity towards the 60-kDa phosphophosphodiesterase isozyme. One unit of phosphoprotein phosphatase was defined as that amount of enzyme that releases 1 nmol of phosphate from phosphorylated phosphorylase *b* per min at 37°C.

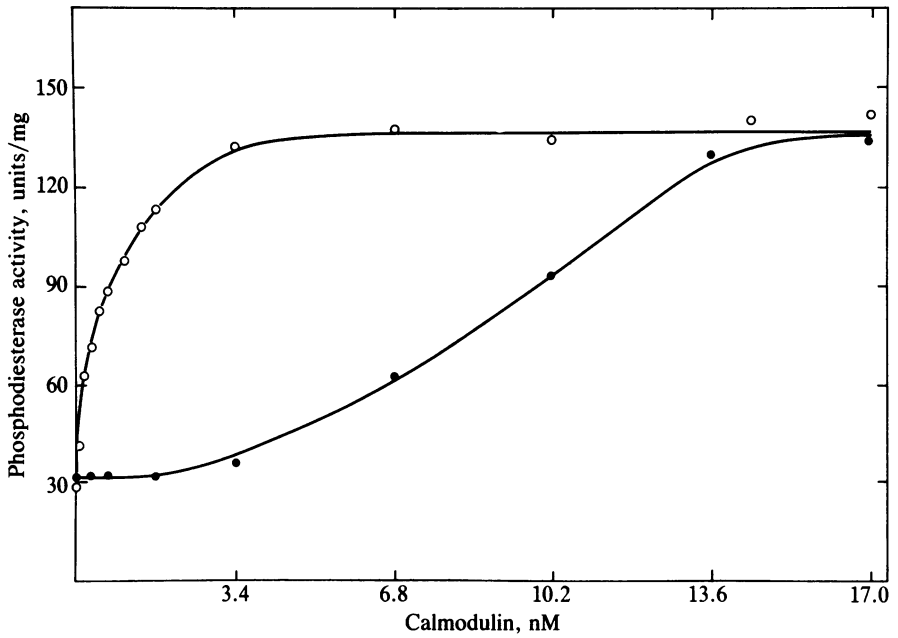

FIG. 4. Phosphodiesterase activation by calmodulin. A sample of the 60-kDa isozyme was phosphorylated for 60 min by the catalytic subunit of the protein kinase (as described in the legend for Fig. 3) to 1.9 mol of phosphate per mol subunit. Both the nonphosphorylated (○) and the phosphorylated (●) enzymes were assayed for phosphodiesterase activity with various concentrations of calmodulin as indicated.

Substrate dephosphorylation was accompanied by an increase in the phosphodiesterase affinity for calmodulin. When phosphorylated 60-kDa phosphodiesterase isozyme containing 2.2 mol of phosphate per mol of subunit was incubated at 30°C for 60 min with 30 μg of Ni^{2+}-activated calcineurin and 20 μg of calmodulin per ml, we observed the total dephosphorylation of the substrate. The dephosphorylated phosphodiesterase was then separated from calmodulin, phosphatase, and released phosphate by using a C1 antibody-Sepharose column. After dialysis to remove high concentrations of $MgCl_2$ resulting from the affinity chromatography, the enzyme was tested for calmodulin activation. Whereas the original phosphorylated isozyme required about 15 nM calmodulin for 50% activation, the dephosphorylated enzyme required only 0.8 nM calmodulin for 50% activation.

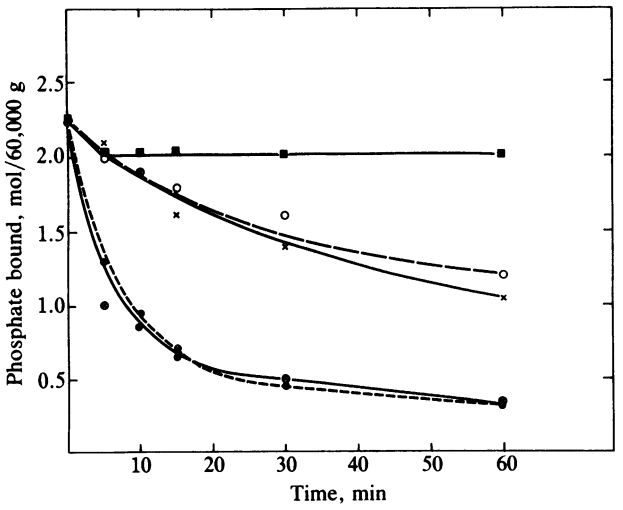

FIG. 5. Time course of dephosphorylation of the phosphorylated 60-kDa isozyme. Phosphorylated 60-kDa isozyme (150 μg/ml) was incubated at 30°C in a reaction mixture containing 100 mM Tris·HCl (pH 7.0), 5 mM magnesium acetate, and 5 mM 2-mercaptoethanol and the following additions: 0.05 mM Ni^{2+} and calmodulin (10 μg/ml) (■), 0.05 mM Ni^{2+} and Ni^{2+}-activated calcineurin (10 μg/ml) (○), 0.05 mM Ni^{2+} and Ni^{2+}-activated calcineurin (10 μg/ml) containing calmodulin (10 μg/ml) (●), 0.05 mM Mn^{2+} and Mn^{2+}-activated calcineurin (10 μg/ml) (×), and 0.05 mM Mn^{2+} and Mn^{2+}-activated calcineurin (10 μg/ml) containing calmodulin (10 μg/ml) (⊗). The phosphatase sample was incubated with 1 mM metal ion at room temperature for 1 hr prior to the initiation of the phosphatase reaction.

DISCUSSION

The observation that the 60-kDa subunit-containing isozyme but not the 63-kDa subunit-containing isozyme of the cyclic nucleotide phosphodiesterase can be phosphorylated by cAMP-dependent protein kinase and that the phosphorylation results in a marked decrease in the 60-kDa subunit affinity towards calmodulin supports the view that the isozymes of bovine brain phosphodiesterase possess distinct physiological and regulatory functions. Although the rate of phosphorylation of the phosphodiesterase isozyme is somewhat lower than those of many other protein substrates of cAMP-dependent protein kinase, the specificity of the phosphorylation and the marked change in regulatory properties of phosphodiesterase upon phosphorylation suggest that this phosphorylation is physiological. However, the suggestion has to be substantiated by further studies.

The phosphorylation of this phosphodiesterase isozyme and the concomitant decrease in the affinity of the enzyme toward calmodulin is reminiscent of the regulation of smooth muscle myosin light chain kinase by cAMP-dependent protein kinase, demonstrated by Adelstein and co-workers (16). In both cases, phosphorylation of the enzyme results in a drastic decrease in the enzyme affinity for calmodulin. The observation that phosphorylation of the phosphodiesterase isozyme is inhibited by Ca^{2+} and calmodulin suggests that the phosphorylation sites may reside in, or in proximity to, the calmodulin binding domain. This suggestion is in agreement with the finding that synthetic polypeptides that are substrates of cAMP-dependent protein kinase are capable of undergoing Ca^{2+}-dependent association with calmodulin (17).

Although phosphorylation of the 60-kDa subunit-containing isozyme drastically decreases the enzyme affinity for calmodulin, it does not totally abolish the Ca^{2+}-dependent

activation by calmodulin. In the presence of excess calmodulin, the activity of the 60-kDa subunit-containing isozyme is not affected by phosphorylation. Since the total calmodulin concentration in brain is >10 μM, it may be argued that phosphorylation of this phosphodiesterase isozyme by cAMP-dependent protein kinase does not bring about enzyme inhibition in brain cells. However, it is possible that a good portion of calmodulin in brain is protein bound (18) and, therefore, unavailable for phosphodiesterase activation. In addition, it has been shown that the effective Ca^{2+} concentration for the activation of cyclic nucleotide phosphodiesterase is dependent on the concentration of calmodulin. When calmodulin concentration is lowered, a higher concentration of Ca^{2+} is required for phosphodiesterase activation (19). Thus, it is expected that, even at a saturating concentration of calmodulin, a decrease in the enzyme affinity for calmodulin would result in a shift of the Ca^{2+}-activation curve to a higher Ca^{2+} concentration range. Since calmodulin regulates a multitude of proteins in a Ca^{2+}-dependent manner, the lowering of the calmodulin affinity of certain calmodulin-regulated proteins by phosphorylation may be a way to selectively eliminate or reduce the Ca^{2+} responses of specific processes.

One of the mechanisms by which Ca^{2+} can regulate cAMP concentrations in the cell is through the activation of cyclic nucleotide phosphodiesterase by Ca^{2+} and calmodulin. Results from the present study suggest that this regulatory mechanism can be fine tuned by various interactions of Ca^{2+} and cAMP. First, there are two major isozymes of the phosphodiesterase possessing different regulatory properties. Second, the 60-kDa subunit-containing isozyme can be regulated by at least three different mechanisms involving cAMP or Ca^{2+}: (*i*) cyclic AMP, through the action of the protein kinase, can lower the affinity of the phosphodiesterase isozyme for calmodulin; (*ii*) Ca^{2+} and calmodulin can prevent the decrease in affinity for calmodulin by inhibiting cAMP-dependent phosphorylation; and (*iii*) calmodulin, through activating a phosphatase (calcineurin), can reverse this cAMP effect. Although it is yet to be established that these regulatory mechanisms operate physiologically, it is not difficult to conceive of certain regulatory advantages of these mechanisms. Since a rise in cAMP concentration is often associated with the activation of adenylate cyclase, inhibition of the phosphodiesterase by cAMP-dependent phosphorylation represents a concerted enzyme regulation—i.e., inhibition of the degradative enzyme in concert with activation of the synthetic enzyme. It is conceivable that, for certain conditions of cell activation, a small and brief elevation in cAMP concentration may be required, and prevention of the inhibition of the phosphodiesterase isozyme by Ca^{2+} and calmodulin can be used by the cell to achieve such an objective. The stimulation of dephosphorylation of the phosphophosphodiesterase isozyme by calmodulin-stimulated phosphatase may be a mechanism for facilitating the return of the cell from an activated state to a resting state. Results from the present study provide a basis for the study of the mechanism of regulation of cAMP concentration under various physiological conditions. The interactions of cAMP and Ca^{2+}/calmodulin at the multiple levels of substrate, kinase, and phosphatase are probably determined by the physiological state and needs of the cell. Depending on these conditions, the extent of cellular responses can be controlled through these interactions.

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1. Morrill, M. E., Thompson, S. T. & Stellwagen, E. (1979) *J. Biol. Chem.* **254**, 4371–4374.
2. Sharma, R. K., Wang, T. H., Wirch, E. & Wang, J. H. (1980) *J. Biol. Chem.* **255**, 5916–5923.
3. Kincaid, R. L. & Vaughan, M. (1983) *Biochemistry* **22**, 826–830.
4. Kincaid, R. L., Manganiello, V. C., Odya, C. E., Osborne, J. C., Stith-Coleman, I. E., Danello, M. A. & Vaughan, M. (1984) *J. Biol. Chem.* **259**, 5158–5166.
5. LaPorte, D. C., Toscano, W. A. & Storm, D. R. (1979) *Biochemistry* **18**, 2820–2825.
6. Hansen, R. S. & Beavo, J. A. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 2788–2792.
7. Sharma, R. K., Adachi, A.-M., Adachi, K. & Wang, J. H. (1984) *J. Biol. Chem.* **259**, 9248–9254.
8. Eldik, L. J. V. & Watterson, D. M. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 789 (abstr.).
9. Sharma, R. K., Taylor, W. A. & Wang, J. H. (1983) *Methods Enzymol.* **102**, 210–219.
10. Gopalakrishna, R. & Anderson, W. B. (1982) *Biochem. Biophys. Res. Commun.* **104**, 830–836.
11. Demaille, J. G., Peters, K. A. & Fischer, E. H. (1977) *Biochemistry* **16**, 3080–3086.
12. Khandelwal, R. L., Vandenheede, J. R. & Krebs, E. G. (1976) *J. Biol. Chem.* **251**, 4850–4858.
13. Reimann, E. M., Walsh, D. A. & Krebs, E. G. (1971) *J. Biol. Chem.* **246**, 1986–1995.
14. Pallen, C. J. & Wang, J. H. (1984) *J. Biol. Chem.* **259**, 6134–6141.
15. Wang, J. H., Sharma, R. K., Huang, C. Y., Chau, V. & Chock, P. B. (1980) *Ann. N.Y. Acad. Sci.* **356**, 190–204.
16. Conti, M. A. & Adelstein, R. S. (1981) *J. Biol. Chem.* **256**, 3178–3181.
17. Malencik, D. A. & Anderson, S. R. (1983) *Biochemistry* **22**, 1995–2001.
18. Grand, R. J. A. & Perry, S. V. (1979) *Biochem. J.* **183**, 285–295.
19. Huang, C. Y., Chau, V., Chock, P. B., Wang, J. H. & Sharma, R. K. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 871–874.