EFFECT OF CALCIUM ON CYCLIC AMP-DEPENDENT AND CYCLIC GMP-DEPENDENT ENDOGENOUS PROTEIN PHOSPHORYLATION IN MOUSE BRAIN CYTOSOL

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In the presence of Ca^{++} (>1mM), cyclic AMP (10μM) inhibited, and cyclic GMP (10μM) stimulated, the phosphorylation of a protein in the brain cytosol fraction of A/Jax mice. Lower concentrations of cyclic AMP stimulated phosphorylation of this protein, while lower concentrations of cyclic GMP had no effect. This protein (called protein 49) co-migrated on SDS - polyacrylamide gels with the endogenously phosphorylated band of partially purified bovine heart protein kinase, and was estimated to have an apparent molecular weight of 49,000. The significance of this Ca^{++}-dependent effect of the two cyclic nucleotides is discussed.

Cyclic AMP (cAMP), cyclic GMP (cGMP), and Ca^{++} serve as second messengers in several biological systems (1-3). cAMP-dependent protein kinase enzymes are ubiquitously distributed in eukaryotes, and are believed to mediate the physiological effects of cAMP (4). These enzymes become activated following hormonal stimulation (5) and change the state of phosphorylation of endogenous protein substrates (6,7). Recent demonstration of the widespread occurrence of a cGMP-dependent protein kinase (8) suggests that cGMP may also function through a protein kinase step. Ca^{++} has been reported to inhibit cAMP-dependent protein kinase activity (9).

One of the substrates for protein kinase enzymes is the regulatory subunit of the protein kinase itself. This autophosphorylation reaction has been demonstrated using purified protein kinase preparations from bovine heart (10) and brain (11) tissues. Autophosphorylation apparently regulates the ability of cAMP to dissociate the protein kinase

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holoenzyme (12). Incubation of extracts from several vertebrate tissues with [^{32}P]-ATP, and subsequent separation of the endogenously phosphorylated protein substrates by sodium dodecyl sulfate (SDS)-gel electrophoresis, showed that the most heavily phosphorylated protein substrate in each tissue had an apparent molecular weight of 49,000 (13). This protein region on the gel, referred to as protein 49, co-migrated on SDS-gels with a protein which bound 8-azido-cAMP, a photo-affinity analog of cAMP, which reacts specifically with cAMP-binding sites. This finding, together with the estimated 49,000 molecular weight of the bovine heart (10) and brain (11) cAMP-binding subunits, suggested that protein 49 might be a protein kinase regulatory subunit (13). The extent of protein 49 phosphorylation was affected by cAMP, and the nature of this effect was determined by the divalent cation present (13). In the presence of Mg^{++}, cAMP stimulated protein 49 phosphorylation in cytosol fractions, but had no effect in particulate fractions. In the presence of Zn^{++}, cAMP decreased the phosphorylation of protein 49 in both cytosol and particulate fractions. This report describes the effects of cAMP and cGMP on protein 49 phosphorylation in the presence of Ca^{++}, using mouse brain cytosol.

METHODS:

Whole brains were removed from strain A/Jax-Minn mice (about 6 weeks of age, 20 gm body weight). Cytosol fractions were obtained by differential centrifugation as previously described (13). The endogenous protein phosphorylation assay contained (100µl, final volume): 40-100µg of protein, 50mM 2(N-morpholino) ethanesulfonic acid (MES) (pH 6.8), 5mM $CaCl_2$, 1.0-1.5µM [γ-^{32}P] ATP (5-20Ci/mmole), with or without 10µM cAMP or 10µM cGMP. The reaction was initiated by addition of [γ-^{32}] ATP; incubation was for 3 min at 30°C. The reaction was terminated by addition of 50µl of a "SDS stop solution" previously described (14), and the mixture was heated in a boiling water bath for 2 min to eliminate proteolytic activity. 75µl or 100µl aliquots were then subjected to electrophoresis on SDS - polyacrylamide slab gels (15). The gels were then stained with Coomassie blue, destained and dried, and autoradiographs made as described previously (14). Each autoradiograph was scanned using an Ortec 4300 microdensitometer and the area under the protein 49 peak was used as a quantitative measure of ^{32}P incorporation.

Protein kinase from bovine heart was purified through DEAE chromatography after the method of Miyamoto et al (16). [γ-^{32}P] ATP

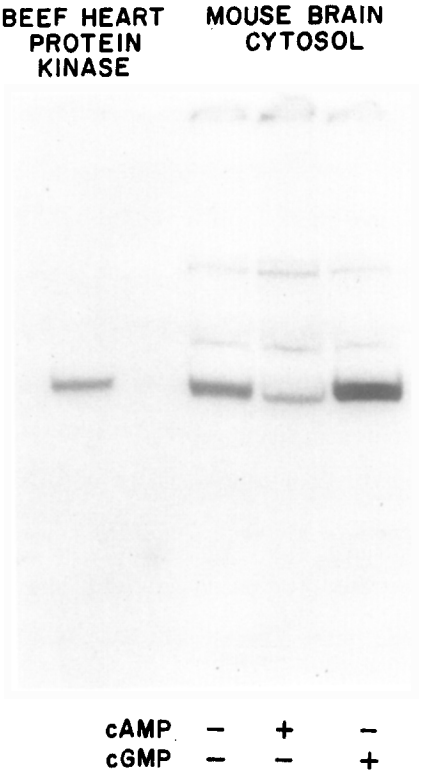

Fig. 1. Effects of 10μM cAMP and 10μM cGMP on endogenous phosphorylation of mouse brain cytosol in the presence of 5mM $CaCl_2$. Following incubation, samples were subjected to SDS-polyacrylamide gel electrophoresis and autoradiography as described in Methods. The autoradiograph is shown. A partially purified preparation of bovine heart protein kinase was incubated with [γ-^{32}P] ATP in the presence of 5mM $CaCl_2$ (in the absence of cyclic nucleotides), and subjected to electrophoresis on the same gel for comparison.

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was prepared according to the method of Post and Sen (17). Protein content was determined by the method of Lowry et al (18). cAMP and cGMP were purchased from Boehringer-Mannheim.

RESULTS:

Figure 1 is an autoradiograph showing the effects of 10μM cAMP and 10μM cGMP on endogenous phosphorylation of a brain cytosol fraction in the presence of 5mM Ca^{++}. Only a few proteins were phosphorylated, and the major phosphorylated band (protein 49) migrated at a position on the gel corresponding to a molecular weight of about 49,000, as determined using protein markers of known molecular weight. The

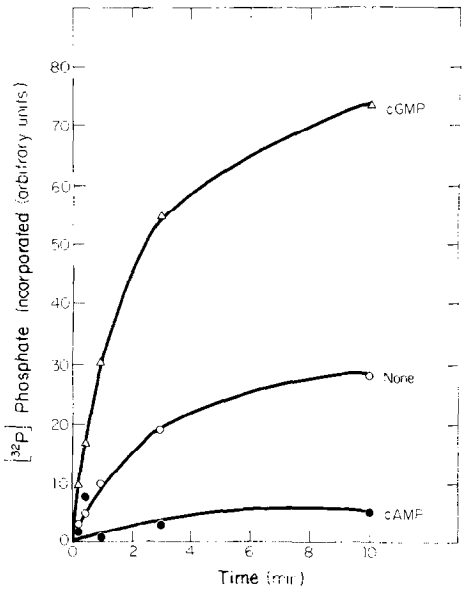

Fig. 2. Time course of the effects of 10μM cAMP and 10μM cGMP on the endogenous phosphorylation of protein 49 from mouse brain cytosol, in the presence of 5mM $CaCl_2$. Autoradiography was done as described in Methods, except for the variation in incubation time. The density of the protein 49 band was quantitated by microdensitometry, and expressed in arbitrary units.

extent of protein 49 phosphorylation in the presence of Ca^{++} was only about 16% of that phosphorylated in the presence of Mg^{++}. Treatment of the incubated samples with Pronase (Worthington; 0.2mg/ml) for 15 min at room temperature after the addition of the SDS-stop solution and prior to electrophoresis, abolished the radioactivity on the gel, indicating that the band at the 49,000 position was associated with protein. Incubation of a partially purified bovine heart protein kinase with 5mM Ca^{++} and [γ-^{32}P]-ATP resulted in a single band on the autoradiograph of the gel which co-migrated with protein 49. In the presence of Ca^{++}, cAMP inhibited the phosphorylation of protein 49, while cGMP stimulated protein 49 phosphorylation.

Figure 2 illustrates the time course of protein 49 phosphorylation with or without 10μM cAMP or 10μM cGMP, in the presence of 5mM Ca^{++}. The inhibitory effect of cAMP and the stimulatory effect of cGMP

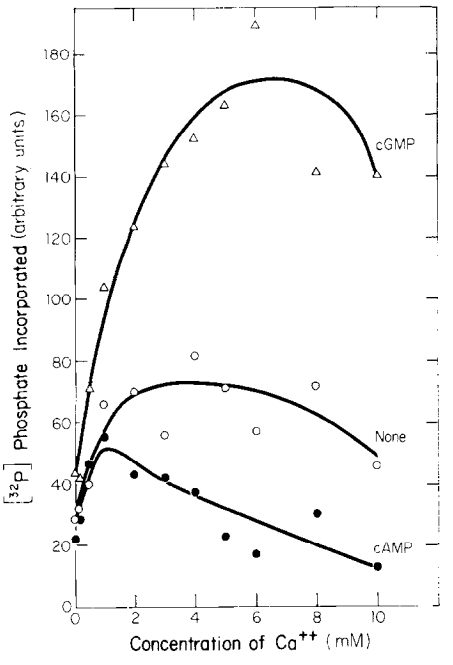

Fig. 3. Effects of different concentrations of $CaCl_2$ on the endogenous phosphorylation of protein 49. Samples were incubated with or without 10μM cAMP or 10μM cGMP. Incubation conditions were as described under Methods, except for the variation in Ca^{++} concentrations.

occurred at all incubation times from 15 sec to 10 min.

The effect of varying the Ca^{++} concentration on protein 49 phosphorylation is shown in Figure 3. Phosphorylation in the absence of added cyclic nucleotides was maximal between 1-8mM Ca^{++}. Protein 49 phosphorylation in the presence of 10μM cAMP was maximal at 1mM Ca^{++}, and then decreased at higher concentrations. 10μM cAMP inhibited protein 49 phosphorylation at all Ca^{++} concentrations above 1mM. 10μM cGMP stimulated protein 49 phosphorylation at all Ca^{++} concentrations tested. The optimal Ca^{++} level for cGMP stimulation was about 6mM.

A dose-response curve for the effects of cAMP and cGMP on protein 49 phosphorylation in the presence of 5mM Ca^{++} is shown in Figure 4. 0.5μM cAMP stimulated protein 49 phosphorylation while cAMP concentrations above 10μM inhibited this reaction. This concentration-depen-

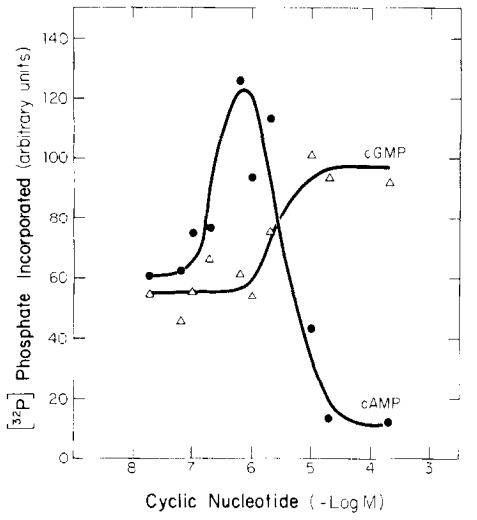

Fig. 4. Effects of varying cAMP or cGMP concentrations on the endogenous phosphorylation of protein 49. Incubation conditions were as described under Methods, except for the variation in cyclic nucleotide concentration.

dence of the direction of the cAMP effect on protein 49 phosphorylation was only observed in the presence of Ca^{++}. In the presence of 10mM Mg^{++}, cAMP stimulated protein 49 phosphorylation at all concentrations above 0.5μM (up to and including 0.2mM cAMP); while in the presence of 5mM Zn^{++}, cAMP inhibited this reaction at all concentrations above 1μM (data not shown). cGMP stimulated protein 49 phosphorylation at concentrations above 2μM in the presence of Ca^{++} (Fig 4). An inhibitory effect on protein 49 phosphorylation by cGMP, in the presence of any cation, has not been observed. Similar dose-dependent effects of cAMP and cGMP in the presence of Ca^{++} have been demonstrated using mouse lung and kidney tissues (data not shown).

DISCUSSION:

There is a heterogeneity of cyclic nucleotide-dependent protein kinase enzymes in eukaryotic tissues (18,19). It is possible that the cyclic nucleotide-divalent cation effects on protein 49 phosphorylation described in this report and previously (13) reflect this heterogeneity.

The 49,000 molecular weight region on a SDS-polyacrylamide gel may contain different protein kinase regulatory subunits, each responding to different cation-cyclic nucleotide conditions. For example, there was no effect of cAMP on protein 49 phosphorylation in the presence of Mg^{++} in particulate fractions of various tissues (13), and there was no effect of cAMP on the autophosphorylation of purified bovine brain protein kinase in the presence of Mg^{++} (11). In this latter preparation, cAMP inhibited autophosphorylation at all concentrations tested in the presence of Ca^{++} (11). Alternatively, there may be other proteins of 49,000 molecular weight -- not protein kinase regulatory subunits -- that are being phosphorylated. Separation of different protein kinase enzymes by various fractionation procedures, and examination of the effect of cAMP and cGMP on their activity in the presence of different cations, is in progress.

The biological effects of cAMP, cGMP, and Ca^{++} may be interrelated. For example, cAMP and cGMP have antagonistic effects on cell growth (20) and on the membrane potential of postsynaptic neurons (21). The effects of cAMP on cell growth can be modulated by Ca^{++} (22), and Ca^{++} seems to be required for some of the effects of cGMP (23). A Ca^{++}-dependent cGMP binding protein has been described (24). Regulation of the autophosphorylation of cyclic nucleotide-dependent protein kinases by cAMP, cGMP, and Ca^{++} (as well as the phosphorylation of other protein substrates) might be involved in some of the interdependent activities of these second messengers.

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