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Regulation of Nucleoside Cyclic 3′:5′-Monophosphate Phosphodiesterase Activity from Rat Brain by a Modulator and Ca^{2+}

(rat cerebral cortex/Ca^{2+}plus Mg^{2+}-dependent phosphodiesterase/cyclic AMP/cyclic GMP/modulator protein)

SHIRO KAKIUCHI, REIKO YAMAZAKI, YOSHIKO TESHIMA, AND KUNIHIRO UENISHI

The Research Division and Clinical Laboratory, Nakamiya Mental Hospital, Hirakata, Osaka, Japan

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ABSTRACT **Gel filtration of the 40,000 rpm supernatant fraction of a homogenate of rat cerebral cortex on a Sepharose 6B column yielded two fractions: fraction II with the "Ca^{2+} plus Mg^{2+}-dependent" phosphodiesterase activity and fraction III containing its modulator. The activity of fraction II was stimulated by micromolar concentrations of Ca^{2+} and the modulator when present together; the modulator stimulated the activity of fraction II only when the Ca^{2+} concentration was above a threshold value (about 2 μM with 0.4–1 μM substrate), and the stimulatory effect of Ca^{2+} was dependent upon the presence of the modulator. A possibility is discussed that the modulator may reversibly bind to the enzyme, which by itself is inactive, to form an active enzyme–modulator complex and that Ca^{2+} stimulates the activity of phosphodiesterase by shifting the equilibrium between these three species towards the formation of the active enzyme–modulator complex. Although fraction II hydrolyzed both cyclic AMP and cyclic GMP, hydrolysis of the latter was more significantly influenced by Ca^{2+} and the modulator than that of the former, and the "Ca^{2+} plus Mg^{2+}-dependent" phosphodiesterase is likely to be a cyclic GMP enzyme. This conclusion is based on the following evidence: (*a*) Ca^{2+} stimulated hydrolysis of cyclic GMP by fraction II more than that of cyclic AMP. (*b*) In the presence of Ca^{2+} and the modulator, fraction II hydrolyzed cyclic GMP about 8 times faster than cyclic AMP when incubated with 0.4 μM substrate. (*c*) Half-maximal stimulation of hydrolysis of cyclic GMP was attained at a lower concentration of Ca^{2+} (4 μM) than that of cAMP (8 μM). (*d*) Increase in the concentration of Ca^{2+} from 0.06 μM to 12 μM in the presence of the modulator caused a decrease in the K_m value of cyclic GMP hydrolysis by fraction II from 20 μM to 2 μM accompanied by 4-fold increase in the V_{max} value. Under similar conditions, there was only a slight decrease in the K_m value of cylic AMP hydrolysis (90 μM → 50 μM), although the V_{max} value increased 7-fold. The anomalous shape of the kinetic plot of cyclic GMP hydrolysis became linear when the Ca^{2+} concentration was increased in the presence of the modulator. The modulator seems to be a protein, but it is heat stable. It is probably identical to the protein activator of phosphodiesterase first described by Cheung.**

Adenosine cyclic 3′:5′-monophosphate (cAMP) and guanosine cyclic 3′:5′-monophosphate (cGMP) are hydrolyzed to the corresponding 5′-nucleotides by one or more nucleoside cyclic 3′:5′-monophosphate phosphodiesterases. Recent work has implicated phosphodiesterase as a regulatory enzyme (1–7). Cheung (1, 8) demonstrated that brain phosphodiesterase became relatively inactive upon purification, due to removal of a protein activator of the enzyme. The activator, isolated free of phosphodiesterase activity, effectively reconstituted the activity of the purified enzyme. This activator was found to be heat stable (1). A similar activator was also found in heart muscle (1, 4) and, more recently, it has been purified to apparent homogeneity (9). The mechanism and physiological significance of the regulation of phosphodiesterase activity by this activator are unclear. A recent report by Wang and his coworkers (6) indicated that cAMP slowly activated heart phosphodiesterase, possibly by enhancing the interaction between the enzyme and the activator.

Results from our laboratory (10–12) showed that the activity of brain phosphodiesterase was stimulated by micromolar concentrations of calcium ion in the presence of an optimum concentration (3 mM) of Mg^{2+} (the "Ca^{2+} plus Mg^{2+}-dependent" phosphodiesterase). During this work we found that a nondialyzable and thermostable factor present in brain extract was somehow involved in the mechanism of stimulation of the activity of brain phosphodiesterase by Ca^{2+} (11). In the presence of boiled supernatant fluid, as a source of this factor, the diluted crude enzyme (dialyzed supernatant fluid of a homogenate of rat brain) was stimulated by lower concentrations of Ca^{2+} than enzyme alone (11). The separation of this factor from enzyme activity by gel filtration and some of its properties were described in a preliminary note (13).

In the present work, the effects of this factor and of Ca^{2+} on the activity of brain phosphodiesterase were studied in conjunction, by use of a preparation of the modulator, fraction III, which was obtained under mild conditions, instead of using heat-treated preparations. Because of the physiological concentrations of cyclic nucleotides, assays were done at low substrate levels. As shown in the present paper, the actions of this factor and Ca^{2+} on the "Ca^{2+} plus Mg^{2+}-dependent" phosphodiesterase seem to be interrelated; the factor may or may not stimulate the enzyme activity, depending upon the concentration of free Ca^{2+} in the reaction mixture, and the effect of Ca^{2+} on the enzyme activity depends upon the presence of this factor in the medium. Therefore, this factor will be referred to here as the phosphodiesterase modulator. This paper also describes kinetic studies on brain phosphodiesterase.

METHODS

Dialyzed 40,000 rpm (100,000 × g) supernatant fluid was prepared from the cerebral cortexes of rats as described (11). Chromatography of the dialyzed supernatant fluid on a Sepharose 6B gel column separated the modulator (fraction

Abbreviation: EGTA, ethylene glycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid.

III) from the "Ca^{2+} plus Mg^{2+}-dependent" phosphodiesterase (fraction II), as illustrated in Fig. 1. Both fractions were concentrated in collodion bags at reduced pressure. Fraction III was rechromatographed in the same way to remove contaminating material from fraction II. The enzyme and modulator preparations, containing protein concentrations of 0.5–1 mg/ml, were divided into small portions, frozen quickly, and stored at −80° until use. Taking advantage of its thermal stability, the modulator was also prepared from hog-brain extract after heat treatment. The 40,000 rpm (100,000 × *g*) supernatant fluid from hog cerebral cortexes was heated for 5 min at 95° in a boiling water bath, and then chilled quickly and centrifuged for 60 min at 40,000 rpm (100,000 × *g*). The supernatant fraction was applied to a Sepharose 6B column. The modulator activity was eluted in the fractions corresponding to fraction III of Fig. 1. These fractions were collected and concentrated. The preparation of modulator obtained by this method was called the column fraction of boiled supernatant.

Activity of phosphodiesterase was determined by two different methods. Method 1 was a modification of the method of Butcher and Sutherland (14). The first-stage incubation was carried out for an appropriate time (15–30 min) at 30° with the following reaction components: 80 mM imidazole buffer (pH 6.9), 3 mM $MgCl_2$, and 0.3 mM dithiothreitol, with substrate, enzyme protein, and other additions as indicated, in a final volume of 0.75 ml. After incubation, the tubes were boiled for 3 min. Then they were incubated for another 20 min at 30° with 0.05–0.2 mg of *Crotalus atrox* venom (5′-nucleotidase) and 6 μmol of $MnCl_2$ (the second-stage incubation). The reaction was stopped by addition of perchloric acid to a final concentration of 3 percent. Pi in the reaction mixture was determined colorimetrically. Method 2 was a modification of the method of Beavo *et al.* (15) and essentially the same as method 1, except that tritiated substrate (2×10^5 dpm) was used. After the incubation, the reaction mixture was applied to a Dowex 1-X8 column containing 0.75 ml of 200–400 mesh resin. The column was then eluted first with 20 ml of 0.002 N HCl, then with 20 ml of 0.002 N HCl, and finally with 30 ml of 0.1 N HCl. Tritiated adenosine or guanosine was recovered quantitatively in the first, 20-ml fraction, and remaining substrate in the third, 30-ml fraction. Those were determined by a liquid scintillation spectrometer. Paper chromatographic analysis showed that the results obtained by methods 1 and 2 corresponded well with the amount of cyclic AMP or cyclic GMP hydrolyzed. Ca^{2+}-EGTA buffer was prepared essentially as described by Weber and Winicur (17). To calculate the concentration of free Ca^{2+}, the apparent binding constant of Ca^{2+}-EGTA of 8×10^5 M^{-1} was used (18). Assay of the modulator was based on the ability of the modulator to enhance the activity of diluted 40,000 rpm supernatant fluid of brain homogenate in the presence 2.5 μM Ca^{2+}. The amount of modulator that doubled the enzyme activity in the standard system was defined as 10 units. Protein was determined by a modification of the method of Lowry *et al.* (16).

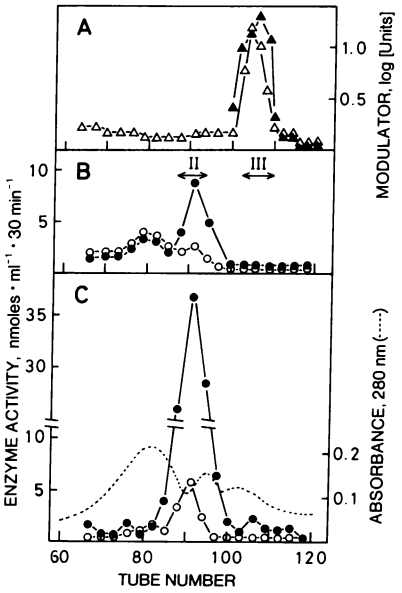


FIG. 1. Fractionation of the supernatnat fluid on a Sepharose 6B column. The column (5 × 84 cm) was eluted with 10 mM Tris·HCl (pH 7.5)–1 mM $MgCl_2$–0.1 mM EGTA, at a flow rate of 13.4 ml/hr, and fractions of 10.2 ml were collected. Modulator activity (*A*) was assayed before (▲——▲) and after (△——△) samples were heated for 3 min in a boiling-water bath. Phosphodiesterase activity was assayed by method 2 with 1 μM cAMP (*B*) or 0.4 μM cGMP (C), in the presence of the modulator (the column fraction of heated supernatant) and either 0.06 μM (○——○) or 12 μM (●——●) Ca^{2+}.

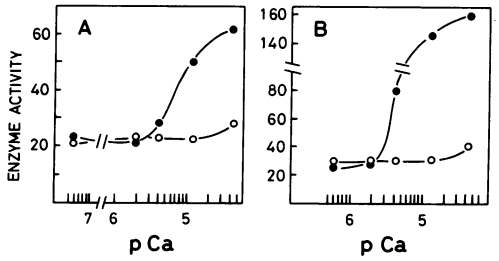


FIG. 2. Effect of addition of the modulator (fraction III) on hydrolysis of 1 μM cAMP (*A*) and 0.4 μM cGMP (*B*) by the "Ca^{2+} plus Mg^{2+}-dependent" phosphodiesterase (fraction II) at various concentrations of Ca^{2+}. Assay was performed by method 2 in the presence (●——●) or absence (○——○) of the modulator. Ca^{2+}-EGTA buffer was used.

RESULTS

The modulator (fraction III) was separated from the "Ca^{2+} plus Mg^{2+}-dependent" phosphodiesterase (fraction II) by gel filtration of the supernatant fluid (Fig. 1). Fig. 1 also indicates that the activity for cGMP hydrolysis coincided with that for cAMP hydrolysis, the former being greater than the latter. Both required the modulator and Ca^{2+}. The rate of hydrolysis of 1 μM cAMP or 0.4 μM cGMP by fraction II was titrated with Ca^{2+} with or without added modulator (Fig. 2). Ca^{2+} stimulated the activity of fraction II only in the presence of the modulator, and the effect of the modulator on the activity of fraction II was seen only when the concentration of Ca^{2+} was above a threshold value. This threshold value was about 2 μM in the presence of 1 μM cAMP or 0.4 μM cGMP as substrate (Fig. 2). With the saturating concentration (1 mM) of substrate, the threshold Ca^{2+} concentration was about 0.7 μM (for cAMP hydrolysis) or 0.2 μM (for cGMP hydrolysis). When the concentration of Ca^{2+} was above the threshold value, the modulator stimulated hydrolysis of cAMP by fraction II in a dose-related fashion (Fig. 3). With 2.5 μM

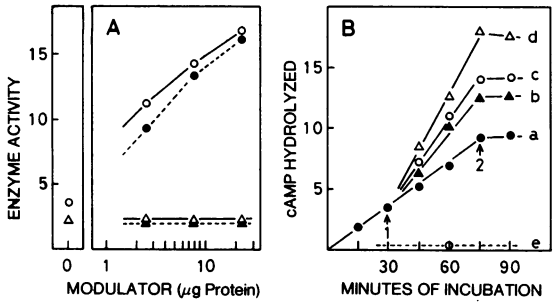


FIG. 3. Hydrolysis of cAMP by the "Ca^{2+} plus Mg^{2+}-dependent" phosphodiesterase (fraction II) in the presence of various concentrations of modulator. Assay was by method 1 with 1.2 mM cAMP. (*A*) Fraction III was added to the reaction mixture before (○——○, △——△) or after (●- - -●, ▲- - -▲) fraction III was heated for 4 min in a boiling water bath. Ca^{2+} concentration was either 0.06 μM (△——△, ▲- - -▲) or 2.5 μM (○——○, ●- - -●) (Ca^{2+}-EGTA buffer). (*B*) The reaction mixture, which contained 2 μM Ca^{2+}, received 0 μg (*a*), 7.5 μg (*b*), 15 μg (*c*), or 150 μg (*d*) of protein of the column fraction of boiled supernatant (the modulator) per ml at *arrow 1*. At *arrow 2*, EGTA was added at a final concentration of 4 mM. Tube *e* represents a control assay with heat-inactivated enzyme. Results are expressed in μmol·mg of protein^{-1}·30 min^{-1} (*A*) or μmol·mg of protein^{-1} (*B*).

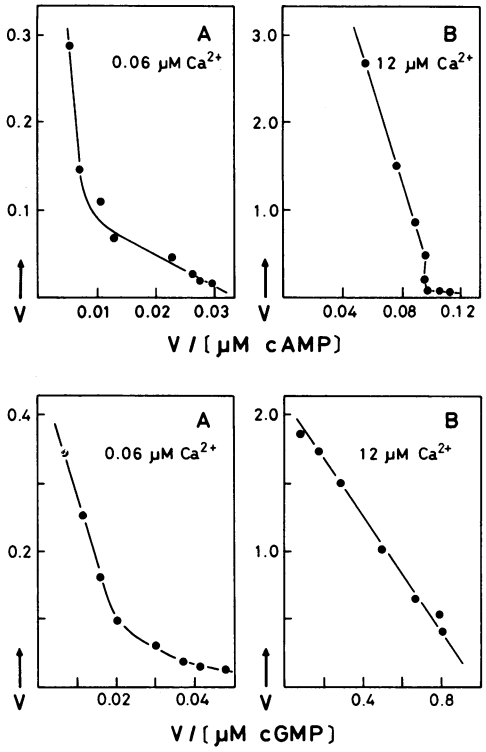
Ca^{2+}, increase in enzyme activity was nearly proportional to the logarithm of the concentration of modulator in the medium, whereas with 0.08 μM Ca^{2+}, addition of increasing concentrations of modulator had no effect on enzyme activity (Fig. 3*A*). Thus, the phosphodiesterase activity of fraction II depended upon both the modulator and Ca^{2+}. Fig. 3*A* also indicates that boiling fraction III for 3 min had no appreciable effect on its stimulatory effect on fraction II (see also Fig. 1*A*)

The modulator seems to be a protein since its activity was destroyed by treatment of the preparation with trypsin or *Bacillus subtilis* protease (Nagarse), but not by treatment with lecithinase C or pancreatic ribonuclease. Bovine-plasma albumin, albumin fraction V, egg albumin, gamma-globulin, trypsin, ribonuclease, myoglobin, lysozyme, ribonucleic acid, and deoxyribonucleic acid were tested for possible modulator activity, but results indicated that none of these substances possessed such activity. The stimulatory effect of the modulator on brain phosphodiesterase was not a catalytic process

TABLE 1. *Comparison of the rates of hydrolysis of cAMP and cGMP by fraction II*

Substrate	Activity of fraction II: 0.06 μM Ca^{2+} (nmol·mg of protein^{-1}·30 min^{-1})	Activity of fraction II: 12 μM Ca^{2+} (nmol·mg of protein^{-1}·30 min^{-1})
Cyclic AMP, 1.2 mM	1520	9910
Cyclic GMP, 1.2 mM	1880	5500
Cyclic AMP, 0.4 μM	10.2	46.4
Cyclic GMP, 0.4 μM	36.6	350.6

The rates of hydrolysis of 1.2 and 0.4 μM cyclic nucleotides by fraction II were determined by assay methods 1 and 2, respectively, with the modulator (the column fraction of boiled supernatant).



FIGS. 4 (*top*) and 5 (*bottom*). Hofstee plots of hydrolysis of cAMP (Fig. 4, *top*) and cGMP (Fig. 5, *bottom*) by the "Ca^{2+} plus Mg^{2+}-dependent" phosphodiesterase in fraction II in the presence of the modulator (the column fraction of boiled supernatant) and either 0.06 μM (*A*) or 12 μM Ca^{2+} (*B*). Velocity is expressed in nmol·mg of protein^{-1}·30 min^{-1}.

that requires Ca^{2+}, since the effect was dose-related, not time-dependent, as shown in Fig. 3*B*.

Comparison of Figs. 2*A* and *B* indicates that, in the presence of the modulator, the rate of cGMP hydrolysis was stimulated by lower concentrations of Ca^{2+} than that of cAMP hydrolysis; half-maximal stimulation of the rates of their hydrolysis were observed at about 4 and 8 μM Ca^{2+}, respectively. Moreover, the magnitude of the increase in the rate of cGMP hydrolysis on addition of the modulator and Ca^{2+} was greater than that of cAMP hydrolysis. As shown in Table 1 (see also Fig. 1), with 0.4 μM substrate, Ca^{2+} and the modulator stimulated the rate of cAMP hydrolysis and that of cGMP hydrolysis 4.5 and 9.6-fold, respectively. Therefore, on saturation with Ca^{2+} and the modulator, fraction II hydrolyzed cGMP about eight times faster than cAMP. At the saturating substrate level (1.2 mM), however, the reverse was found, i.e., the modulator and Ca^{2+} exerted less effect on the rate of cGMP hydrolysis than on the rate of cAMP hydrolysis and, in the presence of the modulator and 12 μM Ca^{2+}, fraction II hydrolyzed cGMP slower than cAMP (Table 1).

The kinetic parameters of cAMP and cGMP hydrolysis by fraction II were determined in the presence of the modulator. In accord with the reports by Appleman and his colleagues (5, 19), plots of the data generally showed biphasic kinetics and did not follow classical Michaelis–Menten kinetics. This anomalous kinetic behavior of the phosphodiesterase reaction has been interpreted by these authors (5) and others (3) as indicative of negative cooperativity of the interaction between

the enzyme and substrate. Fig. 4 illustrates Hofstee plots (20) of hydrolysis of cAMP by fraction II. With 0.06 μM Ca^{2+} (Fig. 4*A*), apparent K_m values of 4 and 90 μM were estimated by extrapolation of the linear portions, with V_{max} values of 0.13 and 0.8 μmol·mg of protein^{-1}·30 min^{-1}, respectively. When the concentration of Ca^{2+} was increased to 12 μM (Fig. 4*B*), the higher K_m decreased to 50 μM with about 7-fold increase in the V_{max}. However, no essential change was observed in the lower K_m with increase in the Ca^{2+} concentration, although the V_{max} corresponding to the lower K_m increased more than 3-fold. For cGMP hydrolysis, in the presence of 0.06 μM Ca^{2+}, an apparent K_m of 20 μM and a V_{max} of 0.5 μmol·mg of protein^{-1}·30 min^{-1} were obtained from the linear portion corresponding to higher substrate levels (Fig. 5*A*). When the concentration of Ca^{2+} was increased to 12 μM, the Hofstee plots became linear and the K_m decreased to 2 μM with increase in the V_{max} to 2.2 μmol·mg of protein^{-1}·30 min^{-1} (Fig. 5*B*).

DISCUSSION

This work provides conclusive evidence that the modulator is involved in stimulation of the activity of brain phosphodiesterase by Ca^{2+}, and results support those obtained previously (11) using diluted crude enzyme (the dialyzed supernatant fluid) and the boiled supernatant fluid as preparations of the modulator. A preparation of brain phosphodiesterase (fraction II) that was devoid of the modulator did not respond to Ca^{2+}. A preparation of modulator (fraction III) isolated from brain extract under mild conditions, which was free of enzyme activity, effectively reconstituted the responsiveness of fraction II to stimulation by Ca^{2+}. Stimulation of enzyme activity by Ca^{2+} required the presence of the modulator, and the effect of the modulator on enzyme activity was dependent upon the concentration of Ca^{2+} in the medium. Thus, the actions of the modulator and Ca^{2+} on the enzyme are interrelated. The protein factor described by Cheung was defined as an activator of the enzyme, while the modulator is concerned with the effect of Ca^{2+} on phosphodiesterase activity. However, the two factors are very similar, and their possible identity requires investigation.

The effect of the modulator on brain phosphodiesterase seems to be specific. Its effect is to increase the initial velocity of the enzyme reaction in the presence of Ca^{2+}. The mechanisms of the action of the modulator and Ca^{2+} on phosphodiesterase are still unknown. One possible explanation of their effects is that Ca^{2+} acts as an activator of the enzyme, exerting its effect through the modulator. In this case, the modulator, possibly binding to enzyme, would function as a mediator of the action of Ca^{2+} since, in the absence of the modulator, Ca^{2+} did not affect enzyme activity. An alternative possibility is that the modulator may reversibly bind to the enzyme, and that the enzyme alone and the enzyme–modulator complex may represent inactive and active forms of phosphodiesterase, respectively. The observations that the modulator (fraction III) was easily separated from enzyme (fraction II) by gel filtration and that simple dilution of the mixture of enzyme and the modulator in the phosphodiesterase assay system greatly reduced the effect of modulator (data not shown) suggest that enzyme and the modulator could reversibly associate and dissociate, supporting the latter possibility. Recent reports by Wang and his coworkers (6, 9) on heart phosphodiesterase suggested that the protein activator and the enzyme may exist in equilibrium among their respective free forms and the active protein complex and this equilibrium may be modulated by cAMP. The effect of Ca^{2+} on brain phosphodiesterase shown in the present paper can be explained by assuming that the equilibrium between the enzyme, the modulator, and the enzyme–modulator complex is determined by the concentration of Ca^{2+}; increase in the Ca^{2+} concentration shifts the equilibrium towards formation of the enzyme–modulator complex, thereby increasing the activity of the mixture. This reaction is shown in the following equation:

$$[\text{Enzyme}]_{\text{inactive}} + \text{Modulator} \overset{Ca^{2+}}{\rightleftharpoons} [\text{Enzyme–Modulator}]_{\text{active}}$$

The concentration of Ca^{2+} that regulates the activity of brain phosphodiesterase in the presence of the modulator was about 1 μM, which seems to be within the physiological concentration range of intracellular calcium ion, as discussed previously (21). Thus, it is possible that, *in vivo*, the above equilibrium and, therefore, the activity of brain phosphodiesterase may change continuously as the concentration of the intracellular calcium ion changes under varying cellular conditions.

The concentrations of cyclic nucleotides in tissues are quite low (22) and phosphodiesterase would never be saturated with substrate *in vivo*. In this regard, results obtained with low concentrations of substrate seem more significant than those obtained with the saturating concentration of substrate, and so is the change in the K_m than the change in V_{max}. Although Ca^{2+} and the modulator affected both cAMP hydrolysis and cGMP hydrolysis, the latter may be more significantly influenced by Ca^{2+} and the modulator *in vivo*, and the "Ca^{2+} plus Mg^{2+}-dependent" phosphodiesterase is likely to be a cGMP enzyme. This conclusion is based on the following observations. (*a*) At low concentrations of substrate, Ca^{2+} and the modulator increased the rate of cGMP hydrolysis by fraction II more than that of cAMP hydrolysis. (*b*) Hydrolysis of cGMP by fraction II was responsive to lower concentrations of Ca^{2+} than that of cAMP. (*c*) Increase in the concentration of Ca^{2+} from 0.06 to 12 μM in the presence of the modulator caused much more decrease in the K_m of cGMP hydrolysis (20 μM to 2 μM) than that of cAMP hydrolysis (90 μM to 50 μM), although increase in V_{max} was greater with cAMP hydrolysis (7-fold) than with cGMP hydrolysis (4-fold). It is of interest to note that the anomalous shape of the kinetic plot of cGMP hydrolysis became linear when the concentration of Ca^{2+} was increased in the assay. The presence of cGMP in brain was reported recently (23–26), and phosphodiesterases that are more specific for cGMP than for cAMP have been found in a variety of tissues, including brain (5, 19). Although the functional role of cGMP in the nervous system is still unclear, the physiological significance of this nucleotide in the nervous system is strongly suggested by the observation that the concentration of cGMP in the tissue was shown to increase in response to exogenous acetylcholine (25, 27, 28).

NOTE ADDED IN PROOF

After this paper was submitted for publication, a report by T. S. Teo and J. H. Wang (1973) *J. Biol. Chem.* **248,** 5950–5955 was published, which shows that the activation of bovine-heart cAMP phosphodiesterase requires the simultaneous presence of Ca^{2+} and a heat-stable protein activator.

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