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COMPARISON OF THE EFFECTS OF Ca^{2+} AND Mg^{2+} ON THE ADENYL CYCLASE OF BEEF BRAIN

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SUMMARY

The adenyl cyclase of beef cerebral cortex required both Mg^{2+} and Ca^{2+} for maximal activity. The requirement for Mg^{2+} was absolute, and it was demonstrated that this cation was necessary for the interaction of the substrate with the enzyme. Enzymic activity was dependent upon the ratio of Mg^{2+} to ATP, and maximal activity was obtained only when there was an excess of the divalent cation. Under these conditions, the apparent K_m for ATP was 1.2–1.3 mM and V was 45 nmoles/mg.

The requirement for Ca^{2+} was demonstrated by use of the chelating agent 1,2-bis-(2-dicarboxymethylaminoethoxy)ethane (EGTA) which specifically chelates Ca^{2+} in the presence of Mg^{2+}. Adenyl cyclase activity was inhibited non-competitively by this compound. The V and the initial velocity were reduced approx. 60% in the presence of 0.1 mM EGTA. There was no significant effect on the apparent K_m for ATP. The velocity of the reaction was returned to normal by the addition of Ca^{2+} in concentrations significantly lower than the concentration of the chelating agent.

INTRODUCTION

The activity of adenyl cyclase is affected by many factors, and among these are divalent cations. The addition of either Mg^{2+} or Mn^{2+} to adenyl cyclase preparations is required to elicit enzymic activity, and it is generally accepted that the Mg^{2+} is the natural co-factor[1]. The addition of Ca^{2+} in place of Mg^{2+} has no activating effect on the enzyme[2].

It has been demonstrated, however, that the adenyl cyclase of beef cerebral cortex depends upon both Ca^{2+} and Mg^{2+} for maximal activity[3]. Maximal activity in the presence of Mg^{2+} depends upon trace amounts of Ca^{2+} which appear to be bound to the particulate matter containing the adenyl cyclase. The chelation of these ions by the

Abbreviation: EGTA, 1,2-bis-(2-dicarboxymethylaminoethoxy)ethane.

relatively Ca^{2+}-specific chelator 1,2-bis-(2-dicarboxymethylaminoethoxy)ethane (EGTA) results in inhibition of the enzyme which is reversed by the addition of Ca^{2+}. A number of recent reports have provided evidence that Ca^{2+} may play a similar role in the control of the adenyl cyclase activity of other tissues as well[4–9]. In view of this evidence, the results presented here are of particular significance. A kinetic analysis of the effects of Ca^{2+} and Mg^{2+} on the adenyl cyclase activity of particulate fractions from beef cerebral cortex has been performed. The results show that both ions are required for activation of the enzyme, but the mechanisms by which they produce activation are quite dissimilar.

EXPERIMENTAL PROCEDURE

Materials

Theophylline was purchased from Nutritional Biochemicals, Inc.; EGTA from K and K Laboratories; and adenosine 3′,5′-monophosphate (cyclic AMP) and the disodium salt of ATP from Sigma Chemical Co. Ion-exchange resins used in the chromatographic steps were Bio-Rad preparations obtained from Calbiochem. All other chemicals were reagent grade preparations obtained from various commercial sources.

Methods

Particulate fractions containing adenyl cyclase were prepared from fresh beef cerebral cortex. The preparation of the 2000 × *g* fraction has been described elsewhere[3]. The 100 000 × *g* fraction was prepared by a procedure which incorporated techniques developed by Fitzpatrick *et al.*[10]. Cortical tissue was homogenized for 30 s in a Waring Blendor with 2 vol. of a solution composed of equal parts glycerol and 10 mM NaCl–KCl solution. The homogenate was centrifuged at 6000 × *g* for 15 min, and the pellet was washed with 10 mM NaCl–KCl solution. The washed pellet was homogenized in 1 vol. of 2 M sucrose using a motor-driven Teflon pestle. This homogenate was centrifuged at 13 300 × *g* for 10 min, and the residue was discarded. The supernatant suspension was diluted with 7 vol. of cold water, and the suspension was centrifuged at 11 900 × *g* for 10 min. The pellet was extracted with 1 vol. of 0.25 M sucrose, and the combined suspension was centrifuged at 100 000 × *g* for 30 min. The resulting pellet was resuspended in a small volume of 50% glycerol solution. The protein concentration of suspensions prepared in this manner ranged from 15–20 mg/ml. The enzyme was stored in this suspension at −70 °C. When incubated with 2 mM ATP and 5 mM $MgCl_2$ under the conditions described below, 1 mg of this particulate fraction catalyzed the formation of 25 nmoles of cyclic AMP in 30 min.

The adenyl cyclase activity of the 100 000 × *g* fraction was routinely assayed at 37 °C at a protein concentration of 1 mg/ml. The basic incubation solution contained 50 mM theophylline, 10 mM NaF, and 40 mM Tris (pH 7.5). ATP and $MgCl_2$ were added as described in individual experiments. The same incubation solution was used in assaying the 2000 × *g* fraction, but the protein concentration was 2.7 mg/ml and the temperature was 30 °C. Unless otherwise specified, the incubation time was 30 min. The reaction was terminated by adjusting the pH of the samples to 3.5 and heating to 100 °C. Protein was removed by centrifugation after readjustment of the pH to 7.5.

Quantitative analysis for cyclic AMP was performed by a modification of pro-

cedures already described[3,11]. In the modified version of this method, the preliminary purification of enzyme reaction mixtures was carried out on a 10-ml column of Dowex-2 resin (Bio-Rad AG 2-X, 200–400 mesh) in the formate form. The cyclic nucleotide was eluted from this column with 50 ml of 2 M formic acid (flow rate not exceeding 0.3 ml/min). An aliquot of this eluate was chromatographed on a cation exchange column (Bio-Rad AG 50-X8, 200–400 mesh) with the dimensions described in the original procedure. The cyclic nucleotide was eluted from this column with 0.05 M HCl and the fraction of eluate (75–215 ml) containing cyclic AMP was used for the measurement of absorbance[3]. The recovery of cyclic AMP from the columns was essentially quantitative (> 95%), and the results of assay of duplicate samples agreed within a few per cent.

For the determination of ATPase activity, the particulate fractions were incubated with 4 mM MgATP under conditions identical to those used in the adenyl cyclase assay. The inorganic phosphate released was determined by the method of Fiske and SubbaRow[12]. Protein was assayed by the method of Lowry *et al.*[13].

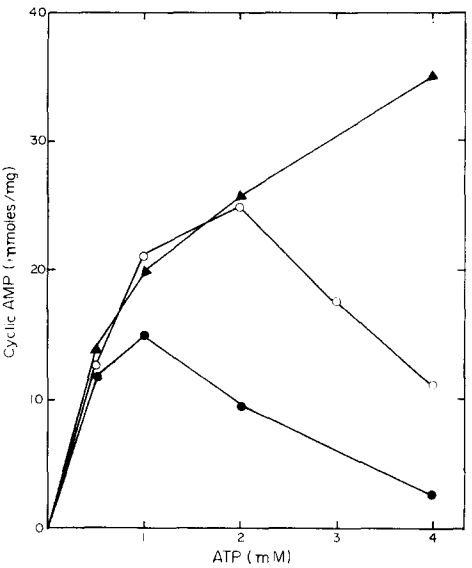

Fig. 1. Effect of variation of ATP concentration on the adenyl cyclase activity of the 100 000 × *g* fraction. In each experiment, the Mg^{2+} concentration was kept constant and the concentration of ATP was varied over the range indicated. ●—●, 1 mM Mg^{2+}; ○—○, 2 mM Mg^{2+}; ▲—▲, 5 mM Mg^{2+}.

RESULTS

Effect of variation of ATP and Mg^{2+} on adenyl cyclase activity

The activity of the adenyl cyclase of the 100 000 × *g* fraction was sensitive to variations in the relative concentrations of ATP and Mg^{2+}. As illustrated by the curves in Fig. 1, maximal activity was obtained only when the concentration of Mg^{2+} exceeded the concentration of ATP. Excess ATP inhibited the enzyme competitively.

The interrelationship between ATP and Mg^{2+} concentrations is further illus-

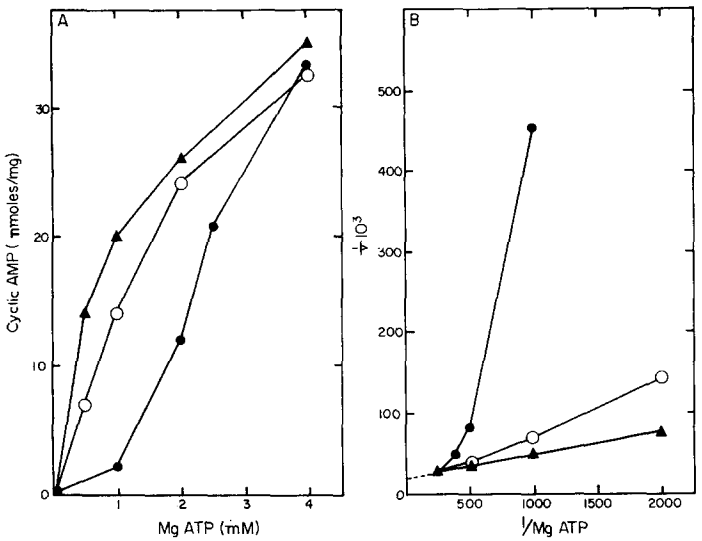

Fig. 2. Effect of variation of the relative concentrations of ATP and Mg^{2+} on the adenyl cyclase activity of the 100 000 × *g* fraction. (A) Enzyme activity obtained under the conditions listed below plotted as a function of MgATP concentration. ●—●, ATP maintained constant (4 mM) and Mg^{2+} concentration varied from 1 to 4 mM; ○—○, ATP and Mg^{2+} concentrations maintained equimolar over the concentration range; ▲—▲, Mg^{2+} concentration maintained constant (5 mM) and ATP concentration varied from 1 to 4 mM. (B) Reciprocal plots of the data of (A). The method of least squares was used to fit the bottom curve. The dotted line denotes extrapolation to the y-axis. Refer above for the definition of symbols.

trated by the experiments summarized in Fig. 2. These experiments were performed under conditions in which there was either an excess of ATP (bottom curve of Fig. 2A), an excess of Mg^{2+} (top curve of Fig. 2A), or equimolar concentrations of ATP and Mg^{2+} (middle curve of Fig. 2A). Approximately the same maximal velocity was approached under the three sets of conditions, but the initial velocities were controlled by the relative concentrations of ATP and Mg^{2+}. The reciprocal plots of these data (Fig. 2B) indicated that this was due to a progressive decrease in the K_m for the substrate (MgATP) as the concentration of Mg^{2+} increased in relation to the concentration of ATP. This plot was linear only when there was an excess of Mg^{2+}. The apparent K_m for MgATP calculated from this data was 1.27 mM.

Inhibition of adenyl cyclase activity by EGTA

The pattern of inhibition of the adenyl cyclase activity of the 100 000 × *g* fraction by EGTA was identical to that obtained earlier using the 2000 × *g* fraction as the source of enzyme[3]. As indicated by the data of Fig. 3, there was no effect of the chelating agent at concentrations of 0.0125 mM or less. Maximal inhibition (approx. 60%) was obtained at an EGTA concentration of 0.025 mM, and there was no further effect of increasing the concentration up to 0.1 mM. In all of the subsequent experiments, an EGTA concentration of 0.1 mM was used routinely.

The inhibition by EGTA was independent of substrate concentration. As shown by the data in Fig. 4A, the degree of inhibition remained the same when the concentration of ATP was varied over the range of 0.5 to 4 mM (in presence of 5 mM Mg^{2+}).

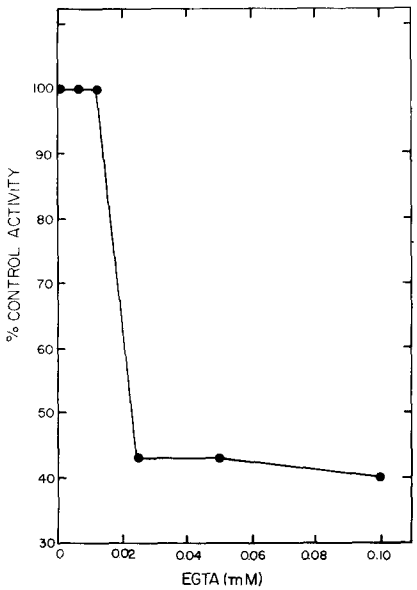

Fig. 3. Inhibition of adenyl cyclase activity by EGTA. The adenyl cyclase activity of the 100 000 $\times$ g fraction was determined in the presence of various concentrations of EGTA. The standard incubation mixture contained 2 mM ATP and 5 mM Mg^{2+}. Each point is the mean of the values obtained in three experiments.

The reciprocal plot of these data (Fig. 4B) indicated that this inhibition was non-competitive. The maximum velocity of the reaction was reduced by approx. 60%, but there was no significant effect on the apparent K_m for the substrate. The V for the reaction (as calculated by the method of least squares) in the presence of EGTA was

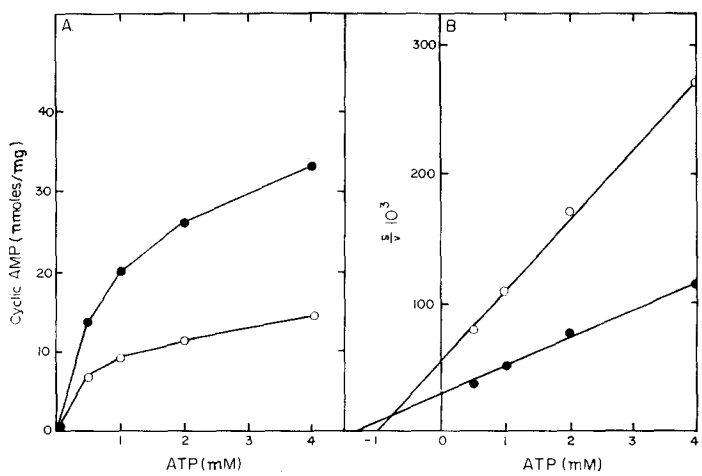

Fig. 4. Effect of variation of the substrate concentration on the inhibition of adenyl cyclase by EGTA. The 100 000 $\times$ g fraction was used as the source of enzyme. (A) The concentration of ATP was varied in the presence and in the absence of EGTA. The concentration of Mg^{2+} in all samples was 5 mM. (B) Reciprocal plots of the data of (A). The lines were fit to the points by the method of least squares. ●—●, Control, no EGTA; ○—○, 0.1 mM EGTA.

18.1 nmoles/mg, whereas in the control samples the value for this constant was 45.2 nmoles/mg. The apparent K_m values in the presence and in the absence of the chelator were calculated to be 0.94 and 1.27 mM, respectively.

Effect of Ca^{2+} on the inhibition by EGTA

Fig. 5 shows the effect of the inclusion of various concentrations of Ca^{2+} in reaction mixtures containing 0.1 mM EGTA. These curves have the appearance of titration curves with the enzymic activity as the end-point. It should be noted that there was significant restoration of activity at concentrations of Ca^{2+} lower than the concentration of EGTA. With both enzyme preparations, full activity was restored at 0.075 mM Ca^{2+}, whereas half the inhibited activity was restored at approx. 0.06 mM.

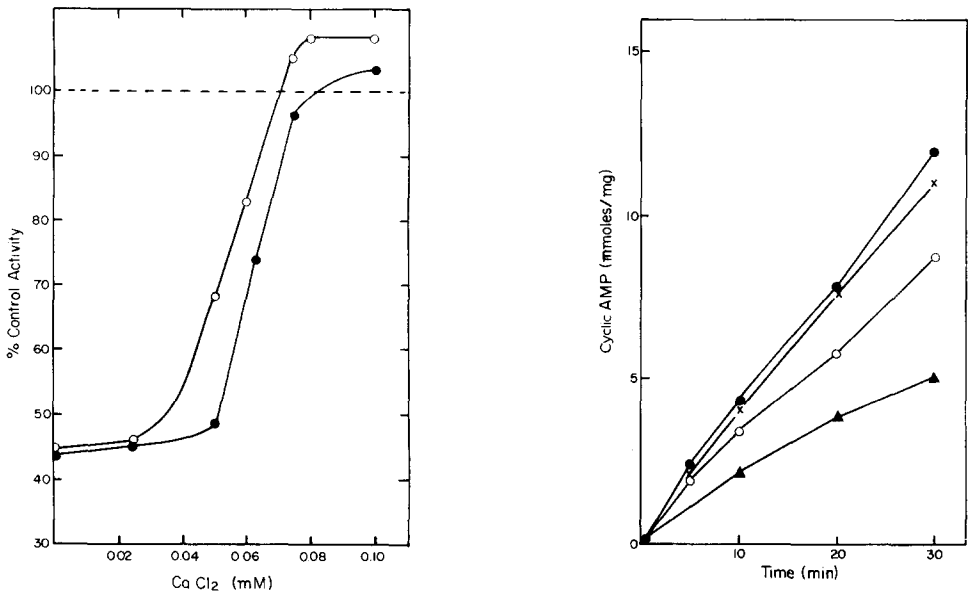

Fig. 5. Effect of inclusion of Ca^{2+} on the inhibition of adenyl cyclase by EGTA. Varying concentrations of $CaCl_2$ were added to samples of the 2000 × *g* fraction and the 100 000 × *g* fraction incubated with 0.1 mM EGTA. The concentration of Mg^{2+} in all samples was 5 mM. The concentration of ATP was either 2 mM (2000 × *g* fraction) or 4 mM (100 000 × *g* fraction). The dotted line represents the activity of similar samples incubated in the absence of EGTA. ●—●, 2000 × *g* fraction; ○—○, 100 000 × *g* fraction.

Fig. 6. Effect of EGTA and Ca^{2+} on the rate of formation of cyclic AMP. Samples of the 2000 × *g* fraction were incubated with EGTA and Ca^{2+} for various time intervals in the presence of 2 mM ATP and 5 mM Mg^{2+}. ×—×, control, no EGTA; ▲—▲, 0.1 mM EGTA, no Ca^{2+}; ○—○, 0.1 mM EGTA, 0.06 mM Ca^{2+}; ●—●, 0.1 mM EGTA, 0.08 mM Ca^{2+}.

The effect of these concentrations of Ca^{2+} on the rate of formation of cyclic AMP in the presence of the 2000 × *g* fraction, is illustrated by the data of Fig. 6. The concentrations of ATP and Mg^{2+} in these samples were 2 and 5 mM, respectively. In the control samples (no EGTA), cyclic AMP was formed at the rate of 383 pmoles/min per mg. The inclusion of 0.1 mM EGTA reduced this rate by 50% to 192 pmoles/min per mg. The rate was restored to normal (406 pmoles/min per mg) by 0.08 mM Ca^{2+},

whereas the inclusion of Ca^{2+} at a concentration of 0.06 mM resulted in a rate (308 pmoles/min per mg) which was approximately halfway between the maximally inhibited rate and the control rate.

The effect of Ca^{2+} on the reduction of maximal velocity by EGTA is illustrated by the data in Table I. The concentration of Mg^{2+} in these samples was 5 mM. The data show that the 60% reduction in V caused by EGTA was reversed by the inclusion of 0.075 mM Ca^{2+}. Again, there was no significant effect of either EGTA or Ca^{2+} on the apparent K_m for ATP.

TABLE I

EFFECT OF EGTA AND Ca^{2+} ON KINETIC PARAMETERS OF BRAIN ADENYL CYCLASE

Samples of the 100 000 × *g* fraction were incubated with EGTA and Ca^{2+} as designated in the presence of concentrations of ATP ranging from 0.5 to 4 mM. The concentration of Mg^{2+} in all samples was 5 mM. The values for K_m and V were calculated by the method of least squares from reciprocal plots of these data. Each value reported is the mean obtained from five experiments.

Expt	*EGTA (mM)*	*$CaCl_2$ (mM)*	*$K_m \times 10^3$ (± S.E.)*	*V (nmoles/mg ± S.E.)*
1	0	0	1.19 ± 0.14	45.8 ± 1.7
2	0.1	0	1.11 ± 0.2	18.1 ± 1.2
3	0.1	0.075	1.17 ± 0.01	42.9 ± 1.6

Effect of EGTA on cyclic nucleotide phosphodiesterase and ATPase activities

The particulate fractions used in these experiments contained very high activities of cyclic nucleotide phosphodiesterase and ATPase. The effect of EGTA on the rate of appearance of the cyclic nucleotide could be attributed to the stimulation of either or both of these enzymes. In order to eliminate this possibility, the effect of EGTA on the activity of these enzymes was determined.

The activity of ATPase in both enzyme preparations was inhibited 75–80% by 10 mM NaF, a routine component of the adenyl cyclase assay medium. In the absence of F^-, there was no effect of 0.1 mM EGTA on the ATPase activity of the 2000 × *g* fraction, and the only effect on the 100 000 × *g* fraction was a slight inhibition (10–20%). These results eliminated the possibility that stimulation of ATPase activity accounted for the decreased formation of cyclic AMP in the presence of EGTA.

The effect of EGTA on cyclic nucleotide phosphodiesterase activity was assayed by the measurement of the rate of disappearance of a known amount of cyclic AMP incubated with adenyl cyclase in the absence of ATP. In all other respects, the incubation conditions were identical to those used for the adenyl cyclase assay. As shown in Fig. 7, there was a loss of about 40% of the cyclic nucleotide, at an almost linear rate, during the 30-min incubation period. This loss was sustained in spite of the high concentration (50 mM) of theophylline in the reaction medium. Whether this loss actually occurred under the conditions of the adenyl cyclase assay is open to question, since Cheung[14] has shown that brain cyclic nucleotide phosphodiesterase is inhibited by ATP at concentrations comparable to those used in the adenyl cyclase assay. In any case, there was no significant effect of EGTA on the rate of loss of the cyclic nucleotide. The observed effect of EGTA on adenyl cyclase could not be ascribed, therefore, to the stimulation of phosphodiesterase activity.

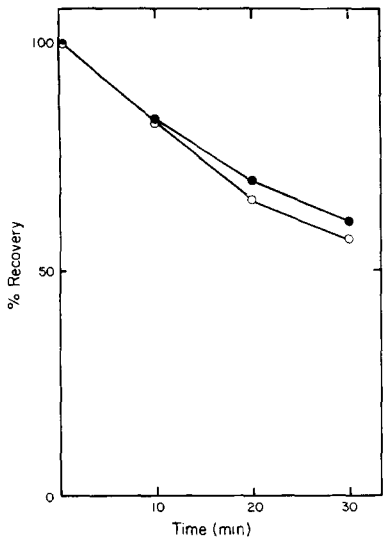

Fig. 7. Effect of EGTA on cyclic nucleotide phosphodiesterase activity. Cyclic AMP (0.5 mg) was incubated with samples of the 2000 × *g* fraction in the absence of ATP. All other conditions were identical to the usual adenyl cyclase assay. Samples were removed at timed intervals and analyzed for the remaining cyclic AMP. The points are the means of values obtained in three experiments. ○—○, control, no EGTA; ●—●, 0.1 mM EGTA.

DISCUSSION

The data presented here demonstrate that Ca^{2+} and Mg^{2+} activate the adenyl cyclase of beef cerebral cortex by distinct mechanisms. Both cations are necessary for maximal enzymic activity. Mg^{2+} must be added to the enzyme reaction mixtures, whereas the requirement for Ca^{2+} is demonstrated by inhibition of the adenyl cyclase activity with the relatively Ca^{2+} specific chelator EGTA. Since the early reports of this property of adenyl cyclase[4,15,16], EGTA has been shown to inhibit the adenyl cyclase prepared from a number of tissues. In most instances, EGTA inhibits only the hormonal response of the enzyme[5,7,8]. This effect has been attributed to interference with the binding of the hormone to its receptor[7]. Other reports indicate, however, that, like the adenyl cyclase of brain[3], the enzyme from fat cells[4] and liver[6] is inhibited by EGTA in the absence of hormones.

The activation by Mg^{2+} is undoubtedly due to its effect on the affinity of the enzyme for its substrate. The sensitivity of adenyl cyclase to variations in the relative concentrations of Mg^{2+} and ATP has been observed repeatedly[17–19], and it has been proposed that the substrate for the enzyme is the MgATP chelate[17]. Free ATP competes with this substrate for the catalytic site on the enzyme. The enhancement of activity by excess free Mg^{2+} has also been observed with adenyl cyclase preparations from fat cells[17] and cardiac tissue[18]. Birnbaumer *et al.*[17] have proposed that the binding of Mg^{2+} to a non-catalytic site influences activity at the catalytic site. It would appear that the same mechanism is operative in the brain enzyme (see Fig. 2), although the data do not permit a quantitation of the number of binding sites.

The results of the kinetic analysis of the inhibitory effect of EGTA on the brain

enzyme, and the reversal of this effect by Ca^{2+}, indicate that these ions control the velocity of the reaction without affecting the formation of the enzyme–substrate complex. The K_m for MgATP is not affected by the chelating agent, whereas the initial velocity and V are significantly reduced. With MgATP as the substrate, the enzyme is able to synthesize cyclic AMP in the absence of Ca^{2+} but does so at a reduced rate.

The degree of inhibition by EGTA is independent of the concentration of both MgATP and free Mg^{2+} (see Fig. 4). The effect of the chelating agent is observed in the presence of a large excess of Mg^{2+}. Since the stability constant of the MgATP chelate is higher than that of the CaATP chelate[20], it is most likely that Ca^{2+} rather than the chelate are involved in the activation. The binding of these Ca^{2+} to the particulate fractions containing the enzyme is apparently very stable The stability of this binding is demonstrated by the fact that the inhibition by EGTA is reversed by Ca^{2+} at concentrations significantly lower than the concentration of the chelating agent (see Fig 5). The data of Bar and Hechter[5] indicate a comparable binding of the Ca^{2+} involved in the activation of the adenyl cyclase of the fat cell membrane and adrenal microsomes.

It seems certain that Ca^{2+} plays a significant role in controlling the activity of adenyl cyclase in broken cell preparations isolated from various tissues. Whether these ions control the activity of the enzyme in intact cells has not been determined. As pointed out by Rasmussen[21], the activation of adenyl cyclase in intact cells does not require Ca^{2+} in the external medium. This does not preclude the possibility that intracellular Ca^{2+} or membrane-bound Ca^{2+} are involved in the control of adenyl cyclase activity in the intact cell.

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