Activation of 3′,5′-Cyclic Adenosine Monophosphate Phosphodiesterase by Calcium Ion and a Protein Activator†

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ABSTRACT: 3′,5′-cAMP phosphodiesterase was partially purified from bovine cerebral cortex. A heat-stable activating factor was separated from the enzyme by chromatography on DEAE-cellulose. The enzyme in crude ammonium sulfate fractions was stimulated by 5 mM $CaCl_2$. This stimulation was reversed by the calcium chelator EGTA. The main phosphodiesterase peak obtained by DEAE-cellulose chromatography was not stimulated by Ca^{2+}. Upon addition of column effluent containing a heat stable factor, Ca^{2+} activation was restored. Protein activator was inactive when endogenous contaminating Ca^{2+} was complexed with EGTA. It was concluded that activation of phosphodiesterase requires the presence of both protein activator and Ca^{2+}. From an analysis of activation of cGMP hydrolysis a kinetic model for the interaction of Ca^{2+} and protein activator with the phosphodiesterase was developed. Heterotropic cooperativity between the binding of Ca^{2+} and protein activator to the phosphodiesterase was observed, *i.e.*, Ca^{2+} decreased the apparent dissociation constant for protein activator and protein activator decreased the apparent dissociation constant for Ca^{2+}.

Recently considerable attention has been directed toward elucidating the regulatory properties of 3′,5′-cAMP phosphodiesterase (EC 3.1.4.17) (phosphodiesterase[1]). In a number of tissues this enzyme exists in multiple molecular forms having different catalytic properties (Brooker *et al.*, 1968; Jard and Bernard, 1970; Thompson and Appleman, 1971; Uzunov and Weiss, 1972). A heat-stable protein was found to be capable of activating phosphodiesterase from bovine brain (Cheung, 1970). This factor could be separated from phosphodiesterase by chromatography on DEAE-cellulose (Cheung, 1971). A similar protein activator has been purified from bovine heart (Teo *et al.*, 1973). Kakiuchi and Yamazaki (1970a,b) have shown that phosphodiesterase present in crude fractions of rat brain was stimulated by minute amounts of Ca^{2+} when Mg^{2+} was present in the assay. They found that Ca^{2+} stimulation was enhanced by a nondialyzable factor present in the extract (Kakiuchi *et al.*, 1970c). The factor was heat stable and resembled the protein activating factor described by Cheung (1971). While the present work was in progress, the activation of phosphodiesterase has been shown to require the presence of both the protein activating factor and calcium ions simultaneously (Teo and Wang, 1973; Teshima and Kakiuchi, 1974). In this paper experiments are presented which concur with the finding that activation of phosphodiesterase has this dual requirement. Kinetic data presented reveal a possible heterotropic cooperativity between Ca^{2+} and protein activating factor in the activation of the cAMP-cGMP phosphodiesterase from bovine cerebral cortex.

Materials and Methods

[^{3}H]cAMP (24 Ci/mmol) and [^{3}H]cGMP (4.3 Ci/mmol) were purchased from New England Nuclear. Ethanol was evaporated under reduced pressure before solutions were prepared for use.

Phosphodiesterase Assay. Each incubation tube contained 50 mM imidazole (pH 7.0), 5 mM $MgSO_4$, 30 μM cAMP or 3 μM cGMP (5×10^5 dpm/assay), enzyme fraction, and water or other addition as required to a final volume of 200 μl. The reaction was started by the addition of substrate. After incubation at 30° for 10 min when cGMP was the substrate, and 30 min when cAMP was used, 25 μl of glacial acetic acid was added to stop the reaction; an aliquot (200 μl) was spotted along with appropriate carriers (0.05 μmol of 3′,5′-NMP, 5′-NMP, and nucleoside) on Whatman 3MM paper and chromatographed (descending) for 16–18 hr using 1 M ammonium acetate–95% ethanol (3:7 v/v). After drying the papers, areas containing 5′-NMP, 3′,5′-NMP, and nucleoside were cut out and the radioactivity was determined by scintillation spectrometry. Reaction velocity was calculated from the per cent conversion of 3′,5′-NMP to 5′-NMP and nucleoside.

When determining kinetic parameters, protein concentration in the assay was held constant. This was necessary since endogenous activator could be present in the enzyme

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[1] Abbreviations used are: phosphodiesterase, 3′,5′cAMP phosphodiesterase; cAMP, adenosine 3′,5′-cyclic monophosphate; cGMP, guanosine 3′,5′-cyclic monophosphate; cNMP, nucleoside 3′,5′-cyclic monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate; S, substrate; P, product; E, enzyme; A, activator; Ap, protein activator; Ca, Ca^{2+}; ES, enzyme-substrate complex; EAp, enzyme-protein activator complex; ECa, enzyme-Ca^{2+} complex; ApCa, protein activator-Ca^{2+} complex; EApCa, enzyme-protein activator-Ca^{2+} complex; EApCaS, enzyme-protein activator-Ca^{2+}-substrate complex; K_a, activator constant; K_S, dissociation constant of S from ES (=[E][S]/[ES]); K_{Ap+Ca}, dissociation constant of Ca from ApCa (=[Ap][Ca]/[ApCa]); K_{ApCa}, dissociation constant of ApCa from EApCa (=[E][ApCa]/[EApCa]); K_{Ap}, dissociation constant of Ap from EAp (=[E][Ap]/[EAp]); K_{Ca}, dissociation constant of Ca from ECa (=[E][Ca]/[ECa]); K_{Ap}', dissociation constant of Ap from EApCa (=[ECa][Ap]/[EApCa]); K_{Ca}', dissociation constant of Ca from EApCa (=[EAp][Ca]/[EApCa]); V_1, maximal velocity in absence of either or both activators; V_2, maximal velocity in the presence of both activators.

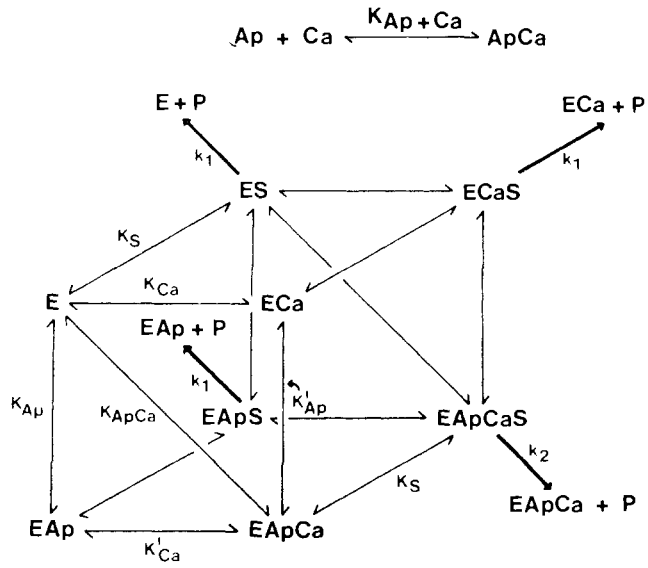

FIGURE 1: Activation model for phosphodiesterase. Each double headed line indicates a rapid equilibrium step with the dissociation constant indicated. Each heavy single headed arrow indicates an irreversible rate-limiting step with the rate constant indicated alongside.

preparation. Variation of protein concentration would alter the amount of activating factor (as well as endogenous Ca^{2+}) which could alter the reaction velocity in a nonlinear manner. Due to the fixed level of phosphodiesterase used, up to 35% hydrolysis of substrate occurred in some assay tubes. In order to compensate for this somewhat high level of conversion, the average substrate present during the assay was determined and this value was used in the kinetic analysis (Lee and Wilson, 1971). All lines for v *vs.* $v/[S]$ and v *vs.* $(v - v_0)/[A]$ plots were determined by least-squares fitting on a duplex IBM 360/67 computer. A standard routine for the analyses was obtained from the University of British Columbia Computing Center general library.

Enzyme Preparation. Phosphodiesterase was partially purified from bovine cerebral cortex, the starting material being an acetone powder prepared and stored at −20°. The dried powder was homogenized in 20 volumes of 5 mM Tris-HCl (pH 7.5). Following centrifugation at 37,000*g* for 30 min, the residue was suspended in 10 volumes of buffer and centrifuged as above. The combined supernatant fluid was brought to 40% saturation by the addition of solid ammonium sulfate, the pH being maintained at 7 by the dropwise addition of 2 N KOH. After 1 hr at 4°, the suspension was centrifuged at 37,000*g* for 30 min, and the pellet was dissolved in 5 mM Tris-HCl (pH 7.5) and exhaustively dialyzed against this buffer. An aliquot (800 mg of protein) was applied to a DEAE-cellulose column (2 × 30 cm) previously equilibrated with 5 mM Tris-HCl (pH 7.5) and the column was washed with this buffer until a washout protein peak had been removed. Protein was then eluted using a linear gradient of 0–500 mM potassium phosphate (pH 7.0) (1 l. of each); 10-ml fractions were collected. Tubes containing phosphodiesterase activity were combined, dialyzed against 5 mM Tris-HCl (pH 7.5), concentrated using an Amicon PM-10 ultrafilter, and stored at −20°. The enzyme hydrolyzed both cAMP (K_m, 30 μM) and cGMP (K_m, 3 μM) with each nucleotide inhibiting the hydrolysis of the other. In the experiments to be described, cGMP was used as the substrate. Similar results were obtained using cAMP. Protein was determined by the method of Lowry *et al.* (1951).

Determination of Activator Dissociation Constants. In Figure 1, a model for the activation of an enzyme with basal activity by two activating factors is depicted. Rate equations for this model, and two additional ones, were developed using the method of Cha (1968). The derivation of rate equations for the other models is similar to that shown for the model in Figure 1. Since this model was later found to fit the data, only the rate equations describing it are presented. The other two models are discussed further in Results. Inherent in the derivation by the method of Cha (1968) is the assumption of either a rapid equilibrium between the various enzyme species or a combined steady state and rapid equilibrium system. Two other simplifying assumptions were made both of which are confirmed in the Results section: (1) the K_m of the enzyme for cGMP does not change in the presence of protein activator and/or Ca^{2+}. The maximal velocity, however, increases in the presence of both protein activator and Ca^{2+}; (2) the apparent activator constant for protein activator does not change as the substrate concentration is varied.

In the model presented, four enzyme–activator species (E, EAp, ECa, and EApCa) are capable of existing. There are three simultaneous pathways to arrive at EApCa from E + Ap + Ca^{2+}. These are E → ECa → ECaAp, E → EAp → EApCa, and E → EApCa. Rate equations for all three pathways were derived by the method of Cha (1968) and found to be identical. Equation 1 represents the pathway E → ECa → ECaAp, while eq 2 represents the pathway E → EAp → EApCa. The equation representing the third pathway (E → EApCa) is not presented but as mentioned, produced equations identical with 1 or 2. In order to determine

$$v = \left(\frac{[S]}{K_S + [S]}\right) \frac{V_1 K'_{Ca}(K_{Ap}K_{Ca} + K_{Ca}[Ap] + K_{Ap}[Ca]) + V_2 K_{Ca}[Ap][Ca]}{K'_{Ap}(K_{Ap}K_{Ca} + K_{Ca}[Ap] + K_{Ap}[Ca]) + K_{Ca}[Ap][Ca]} \quad (1)$$

$$v = \left(\frac{[S]}{K_S + [S]}\right) \frac{V_1 K'_{Ap}(K_{Ap}K_{Ca} + K_{Ca}[Ap] + K_{Ap}[Ca]) + V_2 K_{Ap}[Ap][Ca]}{K'_{Ap}(K_{Ap}K_{Ca} + K_{Ca}[Ap] + K_{Ap}[Ca]) + K_{Ap}[Ap][Ca]} \quad (2)$$

the K_S for substrate, eq 1 can be rearranged into a form analogous to the Eadie formulation of the Michaelis–Menten expression:

$$v = \frac{V_1 K'_{Ca}(K_{Ap}K_{Ca} + K_{Ca}[Ap] + K_{Ap}[Ca]) + V_2 K_{Ca}[Ap][Ca]}{K'_{Ca}(K_{Ap}K_{Ca} + K_{Ca}[Ap] + K_{Ap}[Ca]) + K_{Ca}[Ap][Ca]} - \frac{v}{[S]} K_S \quad (3)$$

For analysis of plots in which the activators were varied, a velocity (v_0) is defined as the velocity at a given substrate concentration in the absence of one or both of the activators (eq 4). Equation 1 can then be rearranged into the form

$$v_0 = V_1[S]/(K_S + [S]) \quad (4)$$

$$v = \frac{v_0 K'_{Ca} + V_2 v_0 [Ca]/V_1}{K'_{Ca} + [Ca]} - \left(\frac{v - v_0}{[Ap]}\right) \frac{K_{Ap}K'_{Ca}(K_{Ca} + [Ca])}{K_{Ca}(K'_{Ca} + [Ca])} \quad (5)$$

In a similar manner eq 2 can be rearranged into the form

$$v = \frac{v_0 K'_{Ap} + V_2 v_0 [Ap]/V_1}{K'_{Ap} + [Ap]} - \left(\frac{v - v_0}{[Ca]}\right) \frac{K_{Ca}K'_{Ap}(K_{Ap} + [Ap])}{K_{Ap}(K'_{Ap} + [Ap])} \quad (6)$$

Table I: Effect on Reaction Velocity of Combining Protein from Ca^{2+} Sensitive Peak of DEAE Column with Main Peak.[a]

Assay	Enzyme Source	EGTA (μM)	V_0	$V_{Ca^{2+}}$	Ap Source	Ap (μg)	V_{Ap}	$V_{Ap+Ca^{2+}}$
1	Tube 40	100	3.75	3.7	Tube 80	3.0	7.0	47.5
2	Tube 44	100	4.90	4.8	Tube 80	3.0	7.6	42.4
3	Tube 48	100	4.80	4.7	Tube 80	3.0	7.5	39.0
4	Peak I	0	20.8	21.2	Boiled peak II	35.0	22.5	118
5	Peak I	250	22.2	22.0	Boiled peak II	35.0	105	119

[a] Tube numbers refer to fractions of the DEAE column shown in Figure 2; peak I refers to an aliquot (3.8 μg of protein) of the concentrated main phosphodiesterase peak as described in the text. Peak II refers to the concentrated minor Ca^{2+} sensitive peak of Figure 2. EGTA was present, at the concentrations indicated. Substrate was 3.0 μM CGMP. Abbreviations used are: V_0, velocity determined with no further additions; $V_{Ca^{2+}}$, velocity with 5 mM $CaCl_2$ present; V_{Ap}, velocity when Ap was present as indicated; $V_{Ap+Ca^{2+}}$, velocity in presence of Ap and 5 mM $CaCl_2$. Velocities are expressed as pmol/min.

It should be noted that in the absence of one or both activators eq 1, 2, and 3 reduce to the simple Michaelis-Menten formulation of reaction velocity

$$v = V_1[S]/(K_S + [S])$$

and

$$V = V_1 - (V/[S])K_S$$

Equations 5 and 6 contain combinations of constants in the components of the equations representing the intercepts and slopes. Individual dissociation constants can be derived from replots of the slopes and intercepts of graphs obtained by varying one activator at several fixed concentrations of the other. By rearrangements, it can be shown that the intercept (a_1) of eq 5 is represented by the expression

$$a_1 = \frac{V_2 v_0}{V_1} - \frac{a_1 - v_1}{[Ca]} K'_{Ca} \quad (7)$$

A similar equation can be obtained for the values of the intercept (a_2) of eq 6

$$a_2 = \frac{V_2 v_0}{V_1} - \frac{a_2 - v_0}{[Ap]} K'_{Ap} \quad (8)$$

It is apparent that a replot of the intercepts of eq 5 or 6 can be used to obtain the values of K_{Ca}' and K_{Ap}', respectively. The use of the slopes to obtain K_{Ap} and K_{Ca} requires a somewhat different rearrangement. The slope of eq 6 (b_2) is given by the expression

$$b_2 = -\frac{K_{Ca} K'_{Ca}(K_{Ap} + [Ap])}{K_{Ap} K'_{Ca} + K_{Ca}[Ap])}$$

which can be rearranged to the form

$$\frac{-1}{b_2} = \frac{1}{K_{Ca}} + \frac{[Ap]}{K_{Ap}}\left(\frac{1}{b_2} + \frac{1}{K'_{Ca}}\right) \quad (9)$$

A similar rearrangement can be performed on the slope expression of eq 5 (b_1), and results in the equation

$$\frac{-1}{b_1} = \frac{1}{K_{Ap}} + \frac{[Ca]}{K_{Ca}}\left(\frac{1}{b_1} + \frac{1}{K'_{Ca}}\right) \quad (10)$$

These equations require that the value of the constant K_{Ca}' be known. This constant can be obtained from eq 7 following a suitable analysis.

Results

In accord with previous findings (Kakiuchi and Yamazaki, 1970b), phosphodiesterase was inhibited by the calcium chelator EGTA; enzyme activity in the ammonium sulfate fraction was inhibited 50% when 0.5 mM EGTA was present in the assay. When 5 mM $CaCl_2$ was present, activity was increased sixfold above the EGTA inhibited state. The ammonium sulfate fraction was subjected to chromatography on DEAE-cellulose; selected fractions were dialyzed against 5 mM Tris-HCl (pH 7.5) and assayed in the presence and absence of 5 mM $CaCl_2$. When assays were performed in the absence of Ca^{2+}, a single peak of phosphodiesterase activity (peak I) with a tailing edge was evident (Figure 2). When Ca^{2+} was present in the assay, enzyme activity in the main peak was not increased; however, a second peak of activity (peak II) now appeared being eluted between tubes 75 and 90 (Figure 2). Assays were performed in which aliquots from peak II (tube 80) were added to assays from peak I (tubes 40, 44, and 48). The results are shown in Table I (assays 1-3). When 5 mM $CaCl_2$ was present activity in tubes from peak I was not increased (compare V_0 and $V_{Ca^{2+}}$). When similar assays were supplemented with small amounts (3 μg) of protein from peak II (tube 80, $CaCl_2$ absent) there was only a modest increase in activity (V_{Ap}) possibly caused by residual phosphodiesterase in this fraction. When both $CaCl_2$ and an aliquot from peak II were present, activity in tubes from peak I was increased tenfold (compare V_0 and $V_{Ap+Ca^{2+}}$). When a portion of the solution from peak II was placed in a boiling

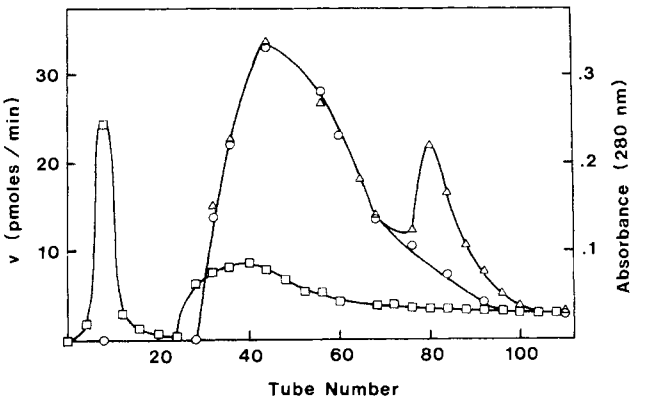

FIGURE 2: DEAE-cellulose column chromatography of ammonium sulfate fraction of bovine cerebral cortex. Conditions are described in the text. Phosphodiesterase was measured in the absence (○) and presence (△) of 5 mM $CaCl_2$; (□) OD 280 nm. The substrate was 3 μM cGMP.

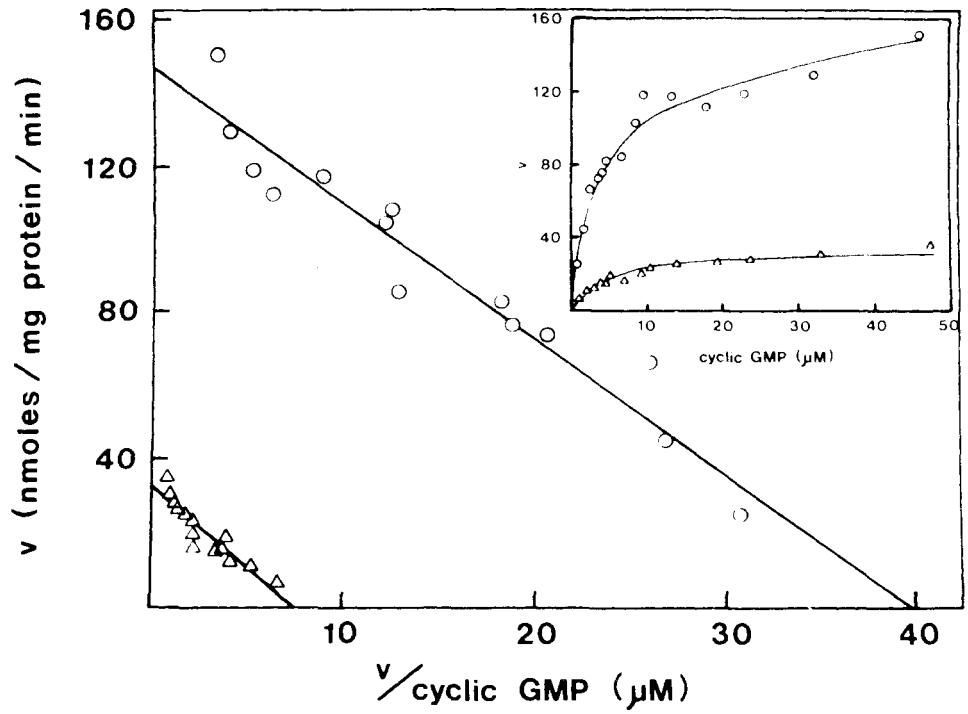

FIGURE 3: Effect of protein activator and Ca^{2+} on the K_m and V_{max} for cGMP. Phosphodiesterase (3.8 μg of protein) was assayed in the absence (△) and presence (○) of protein activator (1.4 μg of protein) plus $CaCl_2$ (5 mM).

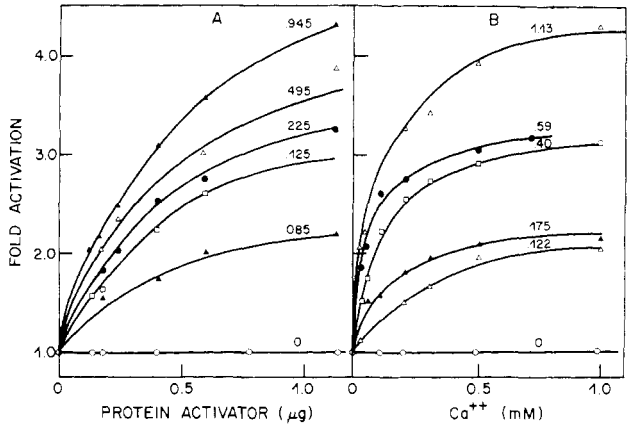

FIGURE 4: Activation of phosphodiesterase by protein activator and Ca^{2+}. Phosphodiesterase and protein activator were purified as in Figure 3. cGMP (3 μM) was the substrate. (A) Protein activator was present at varying concentrations and assay tubes were supplemented with $CaCl_2$ at concentrations (in mM) indicated by the numbers on each curve. (B) $CaCl_2$ was varied in the presence of fixed concentrations of protein activator (in μg). Control tubes (no added activator (A) and no added Ca^{2+} in (B)) contained 100 μM EGTA.

water bath for 5 min and a similar aliquot of the heated supernatant added to the assay of tubes from peak I, the small increase in activity in the absence of added Ca^{2+} was no longer present. In the presence of both Ca^{2+} and an aliquot of heated supernatant from peak II, a sixfold stimulation of activity in peak I tubes was still in evidence. Thus, it seemed that the second peak which appeared to be Ca^{2+}-sensitive phosphodiesterase was, in fact, due to the presence of the heat-stable protein activator (which had been largely resolved from phosphodiesterase) acting on phosphodiesterase tailing through the final column fractions. Phosphodiesterase in peak II was destroyed by boiling, leaving the heat-stable factor intact. The data suggested that neither Ca^{2+} nor the protein activator was capable of stimulating phosphodiesterase alone, but together were highly effective. Cheung (1970, 1971) has not described a Ca^{2+} requirement for the protein activator. In his studies he employed somewhat larger amounts of protein activator (20–50 μg) than were present in the experiments just described. Experiments were then performed using higher concentrations of activator protein obtained from peak II of a DEAE-cellulose column fractionation. The peak II tubes were combined and concentrated in an Amicon PM-10 ultrafilter assembly; the solution was placed in a boiling water bath for 5 min (to destroy phosphodiesterase) then stored at −20°. When phosphodiesterase (DEAE-cellulose column eluate) was assayed in the presence of 35 μg of protein activator in the absence of added $CaCl_2$, a strong activation occurred (Table I, assay 4, compare V_0 with V_{Ap}) which was almost equivalent to that produced by activator protein plus 5 mM $CaCl_2$ ($V_{Ap+Ca^{2+}}$). When 250 μM EGTA was present in the assay, this amount of protein activator caused no stimulation (Table I, assay 5, compare V_0 with V_{Ap}). This suggested that stimulation by protein activator in the first instance was due to contaminating Ca^{2+}. Indeed, the concentration of this cation in the assay mixture as determined by atomic absorption spectrophotometry was found to be 5 μM. This is sufficient (see later results) to greatly increase phosphodiesterase activity when activator protein is present.

In order to clarify the mechanism of interaction of activating factor and Ca^{2+} with the phosphodiesterase, a kinetic analysis of cGMP hydrolysis was undertaken. In Figure 3 the kinetics of cGMP hydrolysis by the DEAE-cellulose purified enzyme is presented. Protein activator (1.4 μg) in the presence of $CaCl_2$ (5 mM) was found to increase the V_{max} of the reaction while leaving the Michaelis constant unaffected. Data not shown indicated that the K_m for cGMP of the enzyme in the ammonium sulfate precipitate was not altered by the addition of EGTA (0.25 mM). Linear $1/v$ *vs.* $1/[S]$ plots were observed both with and without EGTA. In order to further characterize this activation, the enzyme

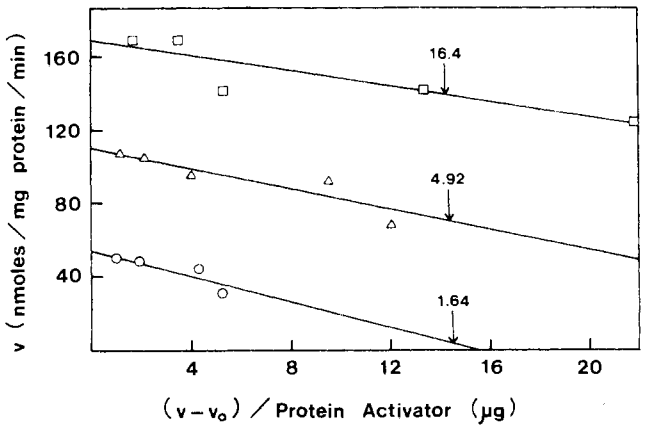

FIGURE 5: Effect of cGMP on affinity of phosphodiesterase for protein activator. Phosphodiesterase (3.8 μg of protein) was assayed in the presence of 1.13 μg of protein activator and 5 mM $CaCl_2$. cGMP concentrations (in μM) are indicated on each curve.

was assayed at a fixed substrate concentration while the concentration of Ca^{2+} and activator protein was varied. In Figure 4A the activation produced by varying the amount of protein activator in the presence of several fixed concentrations of $CaCl_2$ is presented. Ca^{2+} can clearly be seen to be essential for increasing the reaction velocity by the activating factor. In Figure 4B, the activation produced by varying the concentration of $CaCl_2$ in the presence of several fixed concentrations of protein activator is presented. Once again, an absolute requirement for both factors was observed, Ca^{2+} having no effect in the absence of the protein activating factor. The data in Figure 4A indicated that increasing Ca^{2+} concentration tended to decrease the activator constant for the protein activating factor while increasing the reaction velocity. Figure 4B indicated a reciprocal lowering of the activator constant for Ca^{2+} with increasing concentrations of protein activator. In Figure 4, only a fraction of the total data is presented to avoid clutter especially at the lower concentrations of activator and Ca^{2+}. Actually 100 determinations were made and the experiment represents one of duplicate experiments. In order to further quantify these effects, a graphical analysis of the kinetics of dual activation of an enzyme with basal activity was developed. This analysis, which is outlined in Methods, involves the plotting of v *vs.* $(v_{activated} - v_{basal})/[activator]$, *i.e.*, $(v$ *vs.* $(v - v_0)/[A])$. The slopes of such plots will be proportional to the negative value of the activator dissociation constant. This analysis was applied to determine the effect of varying the cGMP concentration on the affinity of the phosphodiesterase for protein activator. $CaCl_2$ (5 mM) was present in all determinations. Figure 5 indicates that the affinity of phosphodiesterase for the protein factor (the negative value of the slope) was constant at different substrate levels. This finding simplified the derivation of rate equations for the kinetic models. Data such as that in Figure 4A and B can now be subjected to a more rigorous kinetic analysis using the procedure outlined in Methods. Figure 6A is a replot of data from an experiment analogous to that in Figure 4A using the v *vs.* $(v - v_0)/[A]$ linearization. This figure indicates that the apparent dissociation constant for the protein activator decreased in the presence of increasing concentrations of Ca^{2+}. A similar treatment of the data from an experiment analogous to that in Figure 4B was applied and the results are shown in Figure 6B. As previously suggested, the apparent dissociation constant for Ca^{2+} was seen to decrease as the amount of protein activator was increased. Both figures were found to have variable intercepts as well as variable slopes. This effect on the intercepts would indicate that the maximal velocity also increases as the concentration of Ca^{2+} and protein activator increase.

As mentioned under Methods, rate equations were also derived for two additional models differing from the one illustrated in Figure 1. The method of Cha (1968) was also used in these derivations. These models represented different routes of forming EApCa from E and, thus, have different numbers of possible enzyme species: (1) E + ApCa $\rightleftharpoons$ EApCa; (2) E + Ap $\rightleftharpoons$ EAp, EAp + Ca $\rightleftharpoons$ EApCa; (3) E + Ca $\rightleftharpoons$ ECa; ECa + Ap $\rightleftharpoons$ ECaAp. When the rate equations for these models are derived, none of them predict the variable intercepts and slopes observed in Figure 6A and B.

Using the rate equation derived for the model given in Figure 1, we can obtain estimates of the individual dissociation constants of the two activators. When the intercepts of the lines in Figure 6A and 6B were plotted as outlined

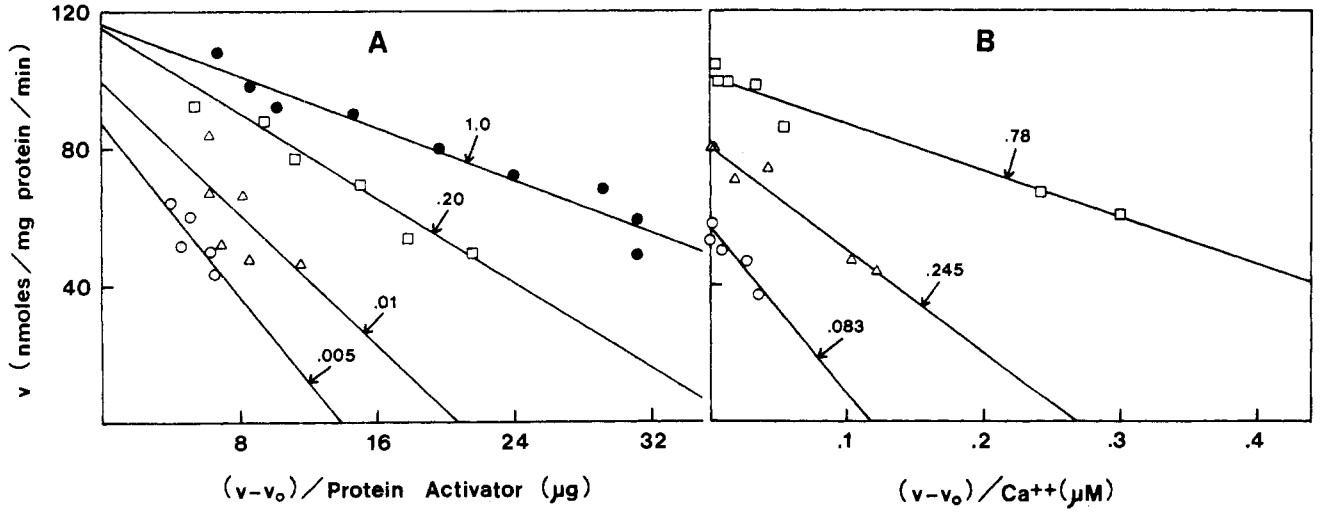

FIGURE 6: (A) The effect of Ca^{2+} on the apparent K_a for protein activator. Phosphodiesterase (3.8 μg of protein) was assayed in the presence of fixed concentrations (in mM) of $CaCl_2$, which are indicated on each curve. Protein activator was varied. (B) The effect of protein activator on the apparent K_a for Ca^{2+}. Phosphodiesterase (3.8 μg) was assayed in the presence of fixed amounts (in μg) of protein activator, which are indicated on each curve. $CaCl_2$ was varied. Substrate was 4.9 μM cGMP.

Table II: Kinetic Parameters Derived from Slope and Intercept Replots of Figure 6.[a]

Parameter	Value	Parameter	Value
K_{Ap}	4 μg	K_{Ca}	80.0 μM
K'_{Ap}	0.2 μg	K'_{Ca}	3.0 μM

[a] All slope and intercept replots were straight lines. Additional data than that presented in Figure 6 were used in the calculations.

under Methods (eq 7 and 8) straight lines were obtained. The negative value of the slope of the replot of the intercepts from Figure 6A represents K_{Ca}'. This dissociation constant for Ca^{2+} was found to be 3.0 μM. When the intercepts of the lines in Figure 6B were similarly plotted, an estimate of 0.2 μg for the values of K_{Ap}' was obtained.

Using the value of K_{Ca}' obtained above, the slopes of the lines in Figure 6A and B can be analyzed using eq 9 and 10 (see Methods). The slope replots of Figure 6A or B produced similar results when subjected to their corresponding analyses. The average value for K_{Ap} determined from the two linear slope replots was 4 μg. The average value of K_{Ca} was estimated to be 80 μM. All of the dissociation constants determined depended upon the purity of the protein activator and enzyme. Extraneous Ca^{2+} binding to the phosphodiesterase or the presence of other proteins in the protein activator preparations would alter the absolute values of the parameters. The values of the constants obtained are presented in Table II. An important relationship can be seen in the values of the constants, namely, $K_{Ap} > K_{Ap}'$ and $K_{Ca} > K_{Ca}'$. This relationship may be indicative of a heterotropic cooperativity between protein activator and Ca^{2+} for their binding to the enzyme.

Discussion

These studies provide further evidence that the stimulation of the cAMP-cGMP phosphodiesterase by protein activating factor is completely dependent on low concentrations of Ca^{2+}. Phosphodiesterase present in ammonium sulfate fractions of bovine cerebral cortex was inhibited 50% by EGTA and strongly stimulated by Ca^{2+}. When such extracts were chromatographed on DEAE-cellulose, the major peak of phosphodiesterase was no longer stimulated by Ca^{2+} nor was it inhibited by EGTA. A second peak of Ca^{2+} sensitive phosphodiesterase was found to represent a mixture of heat-stable protein activator and tailing phosphodiesterase activity from the major peak. Comparatively large amounts of protein activator stimulated phosphodiesterase in the absence of added Ca^{2+}. This stimulation was no longer observed if EGTA (250 μM) was included in the tubes. The activation observed in the absence of added Ca^{2+} was traced to an assay mixture contamination of 5 μM Ca^{2+}. Since protein activator decreased the apparent K_a for Ca^{2+}, the addition of large amounts could cause stimulation of phosphodiesterase at very low Ca^{2+} concentrations. These facts could account for the apparent lack of a Ca^{2+} requirement for the activator in other studies (Cheung 1970, 1971).

A kinetic model for the activation of phosphodiesterase was developed and it is possible that other models may exist which fit the observed data equally well. However, using the data obtained certain models can be ruled out. One such model is the simple binding of Ca^{2+} to the activator protein to form a complex which then binds to the enzyme (Teshimia and Kakiushi, 1974). Such a model would predict that the lines in both Figure 6A and B would have a common intercept. Using the model which fits the observed data (Figure 1) various kinetic constants were determined. The relationship between the constants, $K_{Ap} > K_{Ap}'$ and $K_{Ca}' > K_{Ca}$, is indicative of heterotropic cooperativity between Ca^{2+} and protein activator for their binding to the enzyme. The physiological interpretation of such behavior is that the binding of Ca^{2+} is enhanced in the presence of protein activator and the binding of protein activator is enhanced in the presence of Ca^{2+}. A possible limitation in these results could arise if the concentration of the activator protein approached that of the enzyme resulting in activator depletion. We feel that this is unlikely. The activator protein is purified about tenfold greater than the enzyme preparation and the molecular weight of the activator is about one-fifth that of phosphodiesterase (Teshima and Kakiuchi, 1974). The activator is, therefore, at least 50 times more concentrated on a molar basis than the enzyme. Since the preparations used were not pure, the constants determined are approximations, but it is their relative values which lead to the indication of positive heterotropic cooperativity.

The determination of the constants K_{Ap}, K_{Ca}, K_{Ap}', and K_{Ca}' in the context of the model presented in Figure 1 does not ensure that all these binding steps have physiological significance. The constants determined predict only that if these steps do occur, then the dissociation constants will be the ones given. As for most kinetic analysis, the results do not preclude the possibility of more complex mechanisms which may come to light after extensive purification of phosphodiesterase.

Recent developments during the course of this investigation have strengthened the possibility of the model presented. The binding of Ca^{2+} to protein activator has been measured (Teo and Wang, 1973b). Using $^{45}CaCl_2$ and a purified protein activator from bovine heart, the above authors have determined a dissociation constant of 2.9 μM for the binding of Ca^{2+} to the high affinity site of the protein activator. Thus, the binding of Ca^{2+} to the protein activator is known to occur. Teshima and Kakiuchi (1974) have shown that protein activator can bind to the enzyme in the presence of Ca^{2+}. This result was demonstrated using gel exclusion chromatography. The enzyme was shown not to bind significant amounts of protein activator in the presence of EGTA. However, the technique was possibly not sensitive enough to determine if a very small amount of EAp can form in the absence of Ca^{2+}. Such a complex has been shown to have a relatively high dissociation constant in this presentation and would be difficult to detect by the rather insensitive method of the above authors.

Results presented to date are therefore not inconsistent with a model in which E, EAp, ECa, and EApCa are all capable of being formed. The question still remains as to which sequences of combination of E, Ap, and Ca^{2+} to produce activation of phosphodiesterase have physiological significance. The possibility that long term control of cellular homeostasis could be maintained by regulating the level of protein activator and short term control by regulating the intercellular Ca^{2+} concentration definitely exists.

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Proton Magnetic Resonance Studies of Carbonic Anhydrase. I. Identification of Histidine Resonances†

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ABSTRACT: Nuclear magnetic resonance (nmr) spectra of human carbonic anhydrase B recorded in deuterium oxide reveal seven discrete single proton resonances between 7 and 9 ppm downfield from sodium 2,2-dimethyl-2-silapentane-5-sulfonate. Simplification of spectra by use of Fremy's salt, comparison of peak widths at intersections, and evaluation of the results of inhibition and modification experiments permit determination of the pH dependencies of these resonances. Five of these peaks change position with increasing pH; three move upfield by approximately 95 Hz and two move downfield by 10 and 23 Hz. The first three reflect residues with pK values of 7.23, 6.98, and 6 and can be assigned to the C-2 protons of histidines. The two remaining pH dependent resonances reflect groups with pK values of 8.2 and 8.24. Their line widths and T_1 values are comparable to those of the first group, and they also appear to reflect C–H protons of histidines. Despite the structural and functional similarities of the B and C isozymes of human carbonic anhydrase, few of the low field resonances appear to be common to both. Six histidine C-2 protons are observed in the C enzyme and reflect groups with pK values of approximately 7.3, 6.5, 5.7, 6.6, 6.6, and 6.4. A seventh peak contains two protons and moves upfield with increasing pH without titrating. A final resonance to low field moves downfield with increasing pH and reflects a group with a pK between 6 and 7. Its behavior resembles that of peak 1 of the human B enzyme, and it also appears to be a histidine C–H proton. This peak may reflect a conserved residue in the two isozymes that plays an important role in enzymatic function, as discussed in the following paper.

Carbonic anhydrase is a zinc metalloenzyme of approximately 29,000 molecular weight found throughout the plant and animal kingdoms where it catalyzes the reversible hydration of carbon dioxide. The enzyme consists of a single polypeptide chain with one tightly bound Zn(II) (Lindskog and Malmstrom, 1962) that is essential for catalytic activity and binding of anion and sulfonamide inhibitors. Zn(II) can be replaced by a number of divalent transition metal cations without changing the conformation of the enzyme, but only the cobalt derivative is catalytically active (Linkskog and Malmstrom, 1962; Linkskog *et al.*, 1971; Coleman, 1967). Two major forms of carbonic anhydrase in man and other higher organisms (Nyman, 1961; Rickli *et al.*, 1964) have different activities and are designated the B and C isozymes. They are thought to possess similar three-dimensional structures (Lindskog *et al.*, 1971; Andersson *et al.*, 1972; Coleman, 1971) and active sites (Lindskog and Nyman, 1964; Lindskog *et al.*, 1971; Khalifah, 1971). They demonstrate comparable binding strengths for Zn(II) (Lindskog and Nyman, 1964) and for inhibitors (Taylor *et al.*, 1970; Verpoorte *et al.*, 1967), and the general features of the visible absorption spectra of their cobalt derivatives are nearly identical in the free and inhibited states (Lindskog and Nyman, 1964; Taylor *et al.*, 1970). Both enzymes also catalyze hydration of certain aldehydes (Pocker and Meany, 1965, 1967) and hydrolysis of various esters (Verpoorte *et al.*, 1967; Pocker and Stone, 1967), though at much lower rates.

The pH dependencies of the turnover number of carbonic anhydrase and of the visible absorption spectrum of its cobalt derivative, both of which describe sigmoidal curves with points of inflection near neutrality, are thought to reflect ionization of a group on or near the metal ion controlling catalytic activity (Khalifah, 1971; Kernohan, 1964). A 2-Å crystallographic study of the human C enzyme shows

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