The Control of Adenylate Cyclase by Calcium in Turkey Erythrocyte Ghosts

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SUMMARY

The adenylate cyclase of turkey erythrocytes is inhibited by low concentrations of calcium. Calcium binds to the enzyme system so tightly that the enzyme can compete with ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid (EGTA) for the metal. The calcium binding site is shown to be distinct from the magnesium binding sites required for activity. Thus Ca\(^{2+}\) functions as a negative allosteric effector.

Calcium decreases dramatically the \(V_{max}\) of the catecholamine-stimulated activity without affecting the affinity for the hormone or for the substrate ATP. The cooperativity in the response toward Mg\(^{2+}\) dependence (Hill coefficient, \(n_H = 3\)) is also unaffected by Ca\(^{2+}\) whereas the \(S_0.5\) (concentration yielding one-half \(V_{max}\)) for Mg\(^{2+}\) is affected only slightly. The Ca\(^{2+}\) effect is cooperative (\(n_H = 2\)) and therefore brought about by a cluster of Ca\(^{2+}\) binding sites. Mn\(^{2+}\) can substitute for Mg\(^{2+}\) as the enzyme activator but the Mn\(^{2+}\)-activated enzyme is no longer inhibited by Ca\(^{2+}\). The possible physiological significance of the Ca\(^{2+}\) effect is discussed.

Cyclic adenosine 3':5'-monophosphate\(^1\) is now recognized as the "second" or intracellular messenger for many hormones. These hormones interact with specific receptors on the cell membrane thereby activating adenylate cyclase. In addition to these hormones there are other substances which may regulate the activity of adenylate cyclase by interacting with specific regulatory sites. Recent reports (1, 2) suggest that, in the liver cell and turkey erythrocyte, GTP acts as a positive regulatory ligand.

Calcium, at low concentrations, inhibits most adenylate cyclases which have been studied (3). The mechanism by which this inhibition occurs is not understood. Because most tissues contain calcium at concentrations sufficiently high to inhibit adenylate cyclase, it has been suggested that calcium inhibition may be a physiologically significant phenomenon (4). This suggestion is further supported by reports that calcium activates phosphodiesterase (5), an enzyme which degrades cAMP\(^2\) into 5'-AMP. Thus, elevated calcium levels would decrease the production and increase the destruction of cAMP. The present report deals with the kinetic mechanism by which calcium inhibits the epinephrine-stimulated activity of turkey erythrocyte ghost adenylate cyclase.

MATERIALS AND METHODS

\[^{32P}\]ATP and \[^{3H}\]cAMP were purchased from the Radiochemical Centre, Amersham, England. Creatine phosphokinase, creatine phosphate, theophylline, 1-epinephrine, and EGTA were purchased from Sigma. Alumina (neutral) was a product of M. Woelm, Eschwege, West Germany. Bovine serum albumin was purchased from Calbiochem.

Adenylate Cyclase Assay—The activity of adenylate cyclase was assayed according to the method of White and Zinser (6). The assay mixture (0.15 ml) contained 50 to 100 μg of protein, 0.4 mg per ml of theophylline, 1.0 mg per ml of bovine serum albumin, 1.6 mg per ml of creatine phosphokinase, 20 mM creatine phosphate, and 2.3 mM \[^{32P}\]ATP (2 to 3 cpm per pmol) in 40 mM Tris-HCl buffer, pH 7.4. EGTA was also present in the assay mixture, at a concentration of 0.27 mM. The reaction mixture was incubated for 20 min at 37° in a shaking water bath. It was stopped by boiling for 3 min after addition of \[^{3H}\]cAMP (approximately 1000 cpm) as an internal marker. \[^{32P}\]cAMP was isolated by passage through an alumina column and recovery (50 to 70%) corrected according to the recovery of \[^{3H}\]cAMP. Samples were counted in 15 ml of Bray’s solution (7) in a Packard Tri-Carb scintillation counter. We have found that the rate of cAMP production under these conditions is constant for 30 min in the presence of either fluoride or catecholamines. Linearity with time and protein concentration is maintained under all conditions reported in this communication. All activities are reported as picomoles of cAMP produced per min per mg of protein. All experiments were performed in triplicate and the variation was never higher than 5% between samples. Protein concentrations were determined by the method of Lowry et al. (8) using chromatographically pure bovine serum albumin as a standard.

Preparation of Erythrocyte Ghosts—Fresh heparinized whole turkey blood was obtained on the day of the experiment. It was centrifuged at 4° and the serum and Buffy coat (leukocyte layer) removed. The cells were then washed three times with 0.14 M NaCl, each washing consisting of suspension in 5 to 10 volumes of saline (0.9% NaCl solution) followed by 15 min of centrifugation in an International centrifuge at 2200 rpm and 4°. The washed, packed erythrocytes were found to be free of other types of blood cells when examined by phase-contrast microscopy. They were then suspended in 2 volumes of chilled 0.03 M sodium phosphate.

\[^1\]Cyclic adenosine 3':5'-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid

\[^2\]The abbreviations used are, cAMP, cyclic adenosine 3':5'-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid.
pH 7.4, with 1 mM EDTA and 2 mM MgCl₂. The cells were broken in a large Dounce homogenizer (35 strokes) in ice. The cell membranes were separated from nuclei and hemoglobin in discontinuous sucrose gradients (25 and 35%, w/w) where the membranes formed a white band between the 25 and 35% layers. The erythrocyte ghosts were collected and washed twice in 0.14 M NaCl with 1 mM EGTA (pH 7.4). They were suspended in the same solution and assayed immediately. Membranes were stored in 20% in liquid nitrogen in glass containers. This procedure proves to preserve their full activity for at least a month.

**RESULTS**

**Calcium Inhibition of Activity**—As shown in Table I, the epinephrine-stimulated activity, and to a lesser extent fluoride-stimulated activity, was enhanced by the inclusion of EGTA in the reaction mixture. This result immediately suggested that even the trace concentrations of metal ions in distilled water and in the chemical reagents were sufficiently high to inhibit the activity of adenylate cyclase. The specific activity of epinephrine-stimulated adenylate cyclase as a function of increasing calcium concentrations in the assay mixture is shown in Fig. 1. In these assays, done in the presence of 0.27 mM EGTA and 6 mM MgCl₂, 75% inhibition of activity occurred at calcium concentrations below that of the EGTA present. This finding suggests that the affinity of adenylate cyclase for calcium exceeds that of EGTA for calcium. At 1 mM calcium, no epinephrine stimulation occurred with 0.1 mM epinephrine. Half-maximal inhibition occurred at 0.22 mM added calcium. The Hill plot for calcium inhibition is shown in Fig. 2. A Hill coefficient (9) of 2.1 was obtained.

Calcium inhibition of activation by epinephrine is summarized in Figs. 3 and 4, and Table II. Hormone-stimulated V_max was decreased dramatically, whereas the effect of Ca²⁺ on S_0.5 for epinephrine was small and probably insignificant. Also, the Hill coefficient for epinephrine stimulation was affected only slightly (Fig. 4) by the addition of calcium. The effect of Ca²⁺ was fully reversible. Namely, the addition of EGTA to the system reversed Ca²⁺ inhibition.

The possibility that the effects of Ca²⁺ are attributable to an increase in the phosphodiesterase activity is excluded by the observation that CAMP production is linear for 30 min.

**Effect of Calcium on Magnesium Requirement and Epinephrine Activation**—Calcium manganese and magnesium chloride solutions were prepared in double distilled water and their concentrations determined by titration with EDTA using Eriochromschwartz T as the end point indicator.

**Table I**

<table>
<thead>
<tr>
<th>EGTA (mM)</th>
<th>Specific activity</th>
<th>+ Epinephrine</th>
<th>+ NaF</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8 ± 1</td>
<td>161 ± 5</td>
<td></td>
</tr>
<tr>
<td>0.027</td>
<td>32 ± 1</td>
<td>122 ± 35</td>
<td></td>
</tr>
<tr>
<td>0.27</td>
<td>150 ± 5</td>
<td>194 ± 5</td>
<td></td>
</tr>
<tr>
<td>0.53</td>
<td>154 ± 5</td>
<td>190 ± 5</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.** Inhibition of epinephrine-stimulated adenylate cyclase activity by calcium. All assays contained 0.1 mM epinephrine, 0.27 mM EGTA, 2.3 mM ATP, and 6 mM magnesium. In the absence of added calcium, epinephrine-stimulated activity was 138 ± 4 (mean ± S.D.), while in the absence of epinephrine (basal activity) was 2 ± 2 pmol of cAMP per min per mg of protein (mean ± S.D.). Results represent the mean of triplicate determinations.

**Fig. 2.** Inhibition of epinephrine-stimulated adenylate cyclase activity by calcium. Hill plot. Data from Fig. 1 was plotted using a value of 148 pmol of cAMP per min per mg of protein as maximal activity. Y represents the fraction of maximal activity observed.
FIG. 3. Epinephrine activation of adenylate cyclase as a function of calcium concentration. All assays contained 0.27 mM EGTA, 6 mM magnesium, and 2.3 mM ATP. Results represent the mean of triplicate determinations.

FIG. 4. Epinephrine activation of adenylate cyclase as a function of calcium concentration. Hill plot. A double reciprocal plot of the data in Fig. 3 was used to obtain the value of specific activity at saturation with epinephrine for each calcium concentration used. P represents the fraction of maximal activity obtained at each Ca²⁺ concentration.

Table II

Effect of calcium on epinephrine activation

<table>
<thead>
<tr>
<th>Calcium concentration (mM)</th>
<th>Vmax (pmol cAMP/min/mg protein)</th>
<th>Epinephrine concentration, yielding half-maximal activity (M)</th>
<th>Hill coefficient (nH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>150 ± 5</td>
<td>(1 ± 0.2) × 10⁻⁸</td>
<td>1.0</td>
</tr>
<tr>
<td>2 × 10⁻⁴</td>
<td>100 ± 3</td>
<td>(3 ± 0.6) × 10⁻⁷</td>
<td>0.7</td>
</tr>
<tr>
<td>4 × 10⁻⁴</td>
<td>30 ± 1</td>
<td>(2 ± 0.5) × 10⁻⁷</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Fig. 5. The effect of calcium on the Mg²⁺ concentration dependence. All assays contained 0.27 mM EGTA, 2.3 mM ATP, and 0.1 mM epinephrine. Results represent the mean of triplicate determinations.

Fig. 6. Double reciprocal plots for the Ca²⁺ effect on the magnesium concentration dependence.

Discussion

Effect of Ca²⁺ on Epinephrine Stimulation—Our findings indicate that calcium is a powerful inhibitor of adenylate cyclase in the absence of added calcium was 0.26 mM. Addition of calcium, as previously noted, progressively lowered the maximal activity observed. There was no change, however, in the S₀.₅ for ATP when calcium was added.

Prevention of Calcium Inhibition by Manganese—The ability of Mn²⁺ to substitute for Mg²⁺ is shown in Table V. Maximal activity with Mn²⁺ in the absence of either Mg²⁺ or Ca²⁺ was found at a Mn²⁺ concentration of 10 mM. This activity was only 30% of the activity obtained when 6 mM Mg²⁺ was used. When Mn²⁺ was substituted for Mg²⁺ as the metal activator, it was found that Ca²⁺ was no longer capable of inhibiting adenylate cyclase.

Basal Activity—The effects of Ca²⁺, Mg²⁺, Mn²⁺, and ATP on basal activity were tested. It was found that basal adenylate cyclase activity remained 0 ± 2 pmol of cAMP per min per mg (mean ± S.D.) over wide concentration ranges of these agents. Basal activity in our system was never more than 1.5% of the hormone-stimulated activity.
concentration (V_max) was obtained by extrapolating the curves in Fig. 6. This value was used to obtain the Hill coefficient (n_H). Maximal activity at infinitely high magnesium concentration (P = 1) was obtained by extrapolation and magnesium concentration at half-maximal activity. Results represent the mean ± S.D.

Effect of calcium on magnesium dependence

All assays contained 0.1 mM epinephrine, 0.27 mM EGTA, and 2.3 mM ATP. Maximal activity at infinitely high magnesium concentration (V_max) was obtained by extrapolating the curves in Fig. 6. This value was used to obtain the Hill coefficient (n_H) and magnesium concentration at half-maximal activity. Results represent the mean ± S.D.

<table>
<thead>
<tr>
<th>Calcium concentration</th>
<th>V_max (μmol AMP/min/mg protein)</th>
<th>Mg²⁺ concentration at half maximal activity (S_0.5)</th>
<th>Hill coefficient (n_H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>146 ± 4</td>
<td>(1.8 ± 0.3) X 10^-8</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>2 x 10^-4</td>
<td>87 ± 2</td>
<td>(5.5 ± 0.5) X 10^-9</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>3 x 10^-4</td>
<td>53 ± 2</td>
<td>(4.0 ± 0.5) X 10^-9</td>
<td>2.9 ± 0.2</td>
</tr>
<tr>
<td>4 x 10^-4</td>
<td>43 ± 1</td>
<td>(4.5 ± 0.5) X 10^-9</td>
<td>3.1 ± 0.2</td>
</tr>
</tbody>
</table>

Effect of calcium on ATP requirement

ATP concentrations were determined spectrophotometrically using ε₂₆₀ = 15,300. All assays contained 6 mM Mg²⁺, 0.27 mM EGTA, and 0.1 mM epinephrine. Results represent the mean ± S.D.

<table>
<thead>
<tr>
<th>Calcium concentration</th>
<th>ATP concentration at half-maximal activity (S_0.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>(2.6 ± 0.3) X 10^-4</td>
</tr>
<tr>
<td>2.0 x 10^-4</td>
<td>(3.0 ± 0.4) X 10^-4</td>
</tr>
<tr>
<td>3.0 x 10^-4</td>
<td>(2.2 ± 0.3) X 10^-4</td>
</tr>
<tr>
<td>4.0 x 10^-4</td>
<td>(2.0 ± 0.4) X 10^-4</td>
</tr>
</tbody>
</table>

One other possibility should be considered, that calcium inhibits the enzyme by competing with magnesium for ATP and that the Ca²⁺-ATP complex is a powerful inhibitor of the enzyme, as was suggested earlier (3). This possibility seems untenable since, at pH 7.4, the Ca²⁺-ATP dissociation constant is 0.69 mM and Mg²⁺-ATP dissociation constant is 0.38 mM (11). Clearly, when the magnesium concentration is 6 mM and the calcium concentration is 0.3 mM, all of the ATP is in the magnesium form. Therefore, the effect of calcium on the adenylate cyclase itself must be the direct effect of Ca²⁺ and not of the complex CaATP.

Effect of Ca²⁺ on ATP Requirement—The S_0.5 for ATP in the absence of added Ca²⁺ was 0.26 mM. This is similar to the value found in other systems (11). Addition of Ca²⁺ does not alter the S_0.5 for ATP (Table IV). This observation is consistent with the conclusion that Ca²⁺ inhibition of adenylate cyclase is not the result of Ca²⁺ competition with Mg²⁺ for ATP.

Prevention of Ca²⁺ inhibition by Mn²⁺—The expression of adenylate cyclase activity requires the presence of a divalent cation which is usually Mg²⁺. Mn²⁺ can substitute for Mg²⁺...
probably by binding to the Mg\(^{2+}\) sites on the enzyme as well as forming the MnATP complex. The activity observed when Mn\(^{2+}\) is used is only 30% of the activity found in the presence of Mg\(^{2+}\) (Table V). The ability of Mn\(^{2+}\) to substitute for Mg\(^{2+}\) and the resultant lower activity has also been noted in other systems (10). It is interesting however, that once the enzyme is saturated with Mn\(^{2+}\), Ca\(^{2+}\) is no longer capable of inhibiting the enzyme. This suggests that the Ca\(^{2+}\) sites are also occupied by Mn\(^{2+}\) which is not surprising as Mn\(^{2+}\) binding to Caz+ sites has been noted with other enzymes such as trypsin (12) and \(\alpha\)-amylase (13). It would appear that Mn\(^{2+}\) binding to the Ca\(^{2+}\) sites does not, by itself, inhibit activity but does prevent the binding of, and inhibition by, Ca\(^{2+}\).

These data do not, however, eliminate the possibility that (a) Mn\(^{2+}\) binds to a site distinct from the Mg\(^{2+}\) and Ca\(^{2+}\) sites or (b) Mn\(^{2+}\) binds only to the Mg\(^{2+}\) site and indirectly prevents Ca\(^{2+}\) inhibition.

**Structure and Control of Adenylate Cyclase**—We would like to suggest that the turkey erythrocyte-\(\beta\)-receptor-adenylate cyclase complex has specific binding sites for the following ligands: (a) hormone (catecholamines), (b) substrate (Mg\(^{2+}\)ATP), (c) positive allosteric effector (Mg\(^{2+}\)), and (d) negative allosteric effector (Ca\(^{2+}\)). Our findings lead us to conclude that binding of calcium does not affect hormone, MgATP, and Mg\(^{2+}\) binding sites, but inhibits the enzyme by decreasing the catalytic efficiency (\(k_{cat}\)). This allosteric inhibition by Ca\(^{2+}\) is brought about by the interaction of a cooperative Ca\(^{2+}\) cluster with the catalytic site of adenylylate cyclase.

In addition, our findings suggest that the affinity of these allosteric calcium sites for calcium is very high, since they can compete effectively with EGTA for calcium (Fig. 1).

Maximal activity is inhibited to the extent of 75% by 0.27 mM CaEGTA. Clearly, a intracellular (bound + free) calcium concentrations which are 1 mM, this calcium site could be indeed saturated and the enzyme would be in the inhibited state. However, the concentration of unbound calcium available to the adenylate cyclase calcium site is probably much lower than 1 mM (14). The physiological regulation of adenylate cyclase by calcium may, therefore, result from a redistribution of available intracellular calcium. Almost every adenylate cyclase studied to date is inhibited by low concentrations of calcium (3). This suggests that other adenylate cyclases probably contain specific calcium binding sites and that calcium regulation of adenylate cyclase activity may be a general physiological phenomenon.

Little is currently known about intracellular calcium pools and Ca\(^{2+}\) transport in isolated cells (14). This problem is currently under investigation in our laboratory.

**Note Added in Proof**—Recent experiments (15) reveal that external Ca\(^{2+}\) inhibits adenylate cyclase in the intact cell once the calcium ionophor A23187 is incorporated onto the erythrocyte membrane. Furthermore, \(\beta\)-catecholamines stimulate Ca\(^{2+}\) efflux (15) in the intact cell, thus probably activating the enzyme by the removal of Ca\(^{2+}\) from their inhibitory sites.

**REFERENCES**

The control of adenylate cyclase by calcium in turkey erythrocyte ghosts.
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