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 Ca2+ 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 Mg2+ dependence (Hill coefficient, $n_H = 3$) is also unaffected by Ca2+ whereas the $S_{0.5}$ (concentration yielding one-half $V_{max}$) for Mg2+ is affected only slightly. The Ca2+ effect is cooperative ($n_H = 2$) and therefore brought about by a cluster of Ca2+ binding sites. Mn2+ can substitute for Mg2+ as the enzyme activator but the Mn2+-activated enzyme is no longer inhibited by Ca2+. The possible physiological significance of the Ca2+ effect is discussed.

Cyclic adenosine 3′:5′-monophosphate 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 in turkey erythrocyte, GTP acts as a positive regulatory ligand. 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

[...]

1 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% liquid nitrogen in glass containers. This procedure proves to preserve their full activity for at least a month.

**Assays for 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 Eriochromeskraft T as the end point indicator.

**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 (n) of 2.1 was obtained.

Calcium inhibition of activation by epinephrine is summarized in Figs. 3 and 4, and Table II. Hormone-stimulated V₅₀ was decreased dramatically, whereas the effect of Ca²⁺ on S₀.₅ 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 Activation**—The effect of calcium on the magnesium dependence of epinephrine-stimulated activity is shown in Figs. 5, 6, and 7, and summarized in Table III. In addition to decreasing maximal activity, increasing calcium concentrations increased somewhat the S₀.₅ for magnesium. This probably reflects the influence of V₅₀ itself on the S₀.₅, since S₀.₅ is also a function of I',₀₅. Thus, the small change observed in the S₀.₅ for Mg²⁺ may be due to the large change in V₅₀. There was no change in the cooperativity toward Mg²⁺ upon Ca²⁺ addition (Fig. 7 and Table III).

**Effect of Calcium on ATP Requirement**—As shown in Table IV, the ATP concentration required for half-maximal activity (S₀.₅)
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 Ca$^{2+}$ concentration.

TABLE II

<table>
<thead>
<tr>
<th>Calcium concentration</th>
<th>$V_{\text{max}}$</th>
<th>Epinephrine concentration, yielding half-maximal activity ($S_{0.5}$)</th>
<th>Hill coefficient ($n_H$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mm</td>
<td>pmol cAMP/min/mg protein</td>
<td>$[\text{Epinephrine}]_{M}$</td>
<td>$\mu$</td>
</tr>
<tr>
<td>None</td>
<td>150 ± 5</td>
<td>(1 ± 0.2) x 10^{-4}</td>
<td>1.0</td>
</tr>
<tr>
<td>2 x 10^{-4}</td>
<td>100 ± 3</td>
<td>(3 ± 0.6) x 10^{-5}</td>
<td>0.7</td>
</tr>
<tr>
<td>4 x 10^{-4}</td>
<td>30 ± 1</td>
<td>(2 ± 0.5) x 10^{-3}</td>
<td>0.8</td>
</tr>
</tbody>
</table>

FIG. 5. The effect of calcium on the Mg$^{2+}$ 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 Ca$^{2+}$ effect on the magnesium dependence.

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 $S_{0.5}$ for ATP when calcium was added.

Prevention of Calcium Inhibition by Manganese—The ability of Mn$^{2+}$ to substitute for Mg$^{2+}$ is shown in Table V. Maximal activity with Mn$^{2+}$ in the absence of either Mg$^{2+}$ or Ca$^{2+}$ was found at a Mn$^{2+}$ concentration of 10 mM. This activity was only 30% of the activity obtained when 6 mM Mg$^{2+}$ was used. When Mn$^{2+}$ was substituted for Mg$^{2+}$ as the metal activator, it was found that Ca$^{2+}$ was no longer capable of inhibiting adenylate cyclase.

Basal Activity—The effects of Ca$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, 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.

DISCUSSION

Effect of Ca$^{2+}$ on Epinephrine Stimulation—Our findings indicate that calcium is a powerful inhibitor of adenylate cyclase in
concentration (V_\text{max}) was obtained by extrapolating the curves in Fig. 6. This value was used to obtain the Hill coefficient (n_H) representing the mean ± S.D.

Maximal activity at infinitely high magnesium concentration was 2.3 mM ATP. Maximal activity at infinitely high magnesium concentration (P = 1) was obtained by extrapolation of the data in Fig. 5 as plotted in Fig. 6. A, no added calcium; B, 0.2 mM added calcium; C, 0.3 mM added calcium. The concentration of ATP was 2.3 mM and epinephrine was 0.1 mM. Y represents the fraction of maximal activity observed.

**TABLE III**

**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_\text{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_\text{max} (mol cAMP/min/mg protein)</th>
<th>Mg^2+ concentration at half maximal activity (S_0.5)</th>
<th>Hill coefficient (n_H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>=</td>
<td></td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>None</td>
<td>146 ± 4</td>
<td>(1.8 ± 0.3) × 10^{-5}</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>2 × 10^{-4}</td>
<td>87 ± 2</td>
<td>(3.5 ± 0.5) × 10^{-3}</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>3 × 10^{-4}</td>
<td>53 ± 2</td>
<td>(4.0 ± 0.5) × 10^{-3}</td>
<td>2.9 ± 0.2</td>
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<tr>
<td>4 × 10^{-4}</td>
<td>43 ± 1</td>
<td>(4.5 ± 0.5) × 10^{-3}</td>
<td>3.1 ± 0.2</td>
</tr>
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</table>

**TABLE IV**

**Effect of calcium on ATP requirement**

ATP concentrations were determined spectrophotometrically using ε_{260} = 15,300. All assays contained 6 mM Mg^2+, 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) × 10^{-4}</td>
</tr>
<tr>
<td>2.0 × 10^{-4}</td>
<td>(3.0 ± 0.4) × 10^{-4}</td>
</tr>
<tr>
<td>3.0 × 10^{-4}</td>
<td>(2.2 ± 0.3) × 10^{-4}</td>
</tr>
<tr>
<td>4.0 × 10^{-4}</td>
<td>(2.0 ± 0.4) × 10^{-4}</td>
</tr>
</tbody>
</table>

turkey erythrocyte ghosts. The Hill coefficient of 2 for the inhibitory effect of calcium suggests that a cooperative cluster of Ca^2+ sites functions as the "switch off" mechanism. The affinity of this cluster toward Ca^2+ is extremely high and the ghosts are inactive in the absence of excess EGTA. It seems, therefore, that the affinity of these regulatory sites toward Ca^2+ is higher than that of EGTA. The epinephrine concentration (S_0.5) necessary for half-maximal activity is slightly increased and the Hill coefficient for epinephrine activation is slightly decreased by calcium binding (Figs. 3 and 4, and Table I). This effect is small and probably insignificant.

**Effect of Ca^2+ on Mg^2+ Binding**—Magnesium is required for the expression of epinephrine-stimulated activity. The magnesium concentration dependence is cooperative with a Hill coefficient of 3 (Fig. 7 and Table III). A Hill coefficient of 2 for Mg^2+ has been found in other systems (10). At pH 7.4 and at the ATP concentration used, it can be calculated that all of the ATP is present as Mg^2+ATP using the known MgATP stability constants (11).

From the Mg^2+ concentration dependence (Figs. 5, 6, and 7) of hormone-stimulated adenylate cyclase, it appears that the enzyme probably requires at least 2 free Mg^2+ ions and the substrate MgATP. Magnesium therefore has a dual effect: as substrate in the MgATP form, and as a positive allosteric effector in the form of free Mg^2+. The cooperative response toward Mg^2+ is unaltered at increasingly high Ca^2+ concentrations (Figs. 5 and 7, Table III) and S_0.5 for Mg^2+ increases only slightly. One can therefore conclude that Ca^2+ does not compete with Mg^2+ for the same binding site but rather binds to a specific site distinct from the Mg^2+ site.

One other possibility should be considered, that calcium inhibits the enzyme by competing with magnesium for ATP and that the Ca^2+ATP complex is a powerful inhibitor of the enzyme, as was suggested earlier (3). This possibility seems untenable since, at pH 7.4, the Ca^2+ATP dissociation constant is 0.69 mM and Mg^2+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 Ca^2+ and not of the complex CaATP.

**Effect of Ca^2+ on ATP Requirement**—The S_0.5 for ATP in the absence of added Ca^2+ was 0.26 mM. This is similar to the value found in other systems (11). Addition of Ca^2+ does not alter the S_0.5 for ATP (Table IV). This observation is consistent with the conclusion that Ca^2+ inhibition of adenylate cyclase is not the result of Ca^2+ competition with Mg^2+ for ATP.

**Prevention of Ca^2+ Inhibition by Mn^2+**—The expression of adenylate cyclase activity requires the presence of a divalent cation which is usually Mg^2+. Mn^2+ can substitute for Mg^2+
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 Ca$^{2+}$ sites has been noted with other enzymes such as trypsin (12) and α-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-β-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 adenylate 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, l-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.
M L Steer and A Levitzki