A Critical Interplay between Ca\(^{2+}\) Inhibition and Activation by Mg\(^{2+}\) of AC5 Revealed by Mutants and Chimeric Constructs

Biao Hu‡, Hiroko Nakata‡, Chen Gu§, Tonny de Beer, and Dermot M. F. Cooper¶

From the Department of Pharmacology, University of Colorado Health Sciences Center, Denver, Colorado 80262

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Adenyl cyclase type 5 (AC5) is sensitive to both high and low affinity inhibition by Ca\(^{2+}\). This property provides a sensitive feedback mechanism of the Ca\(^{2+}\) entry that is potentiated by cAMP in sources where AC5 is commonly expressed (e.g. myocardium). Remarkably little is known about the molecular mechanism whereby Ca\(^{2+}\) inhibits AC5. Because previous studies had showed that Ca\(^{2+}\) antagonized the activation of adenyl cyclase brought about by Mg\(^{2+}\), we have now evaluated the Mg\(^{2+}\)-binding domain in the catalytic site as the potential site of the interaction, using a number of mutations of AC5 with impaired Mg\(^{2+}\) activation. Mg\(^{2+}\) activation exerted contrasting effects on the high and low affinity Ca\(^{2+}\) inhibition. In both wild type and mutants, activation by Mg\(^{2+}\) decreased the absolute amount of high affinity inhibition without affecting the \(K_i\) value, whereas the \(K_i\) value for low affinity inhibition was decreased. These effects were directly proportional to the sensitivity of the mutants to Mg\(^{2+}\). Parallel changes were noted in the efficacies of Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) in the mutant species, suggesting a simple mutation in a shared domain. Strikingly, forskolin, which activates by a mechanism different from Mg\(^{2+}\), did not modify inhibition by Ca\(^{2+}\). Deletion of the N terminus and the C1b domain of AC5 and a chimera formed with AC2 confirmed that the catalytic domain alone was responsible for high affinity inhibition. We therefore conclude that both low and high affinity inhibition by Ca\(^{2+}\) are exerted on different conformations of the Mg\(^{2+}\)-binding sites in the catalytic domain of AC5.

\(\text{Ca}^{2+}\) in submicromolar concentrations exerts type-specific effects on adenyl cyclases in \textit{in vitro} assays (1, 2). The cation stimulates AC1\(^2\) and AC8 in a calmodulin-dependent manner and inhibits AC5 and AC8 apparently independently of calmodulin (3). These effects are mirrored in intact cells when \([\text{Ca}^{2+}]_i\) is elevated by physiological means. Consequently, this regulation has been proposed to provide a device for harmonizing the activities of cAMP and Ca\(^{2+}\) signaling pathways (1). At submicromolar concentrations, Ca\(^{2+}\) inhibits all adenyl cyclases. The significance of this latter effect is unknown. Early kinetic analyses of uncharacterized adenyl cyclase activity had led to the suggestion that this low affinity inhibition of adenyl cyclases represented competition by Ca\(^{2+}\) for an allosteric Mg\(^{2+}\) regulatory site (4, 5). Low affinity inhibition was recently established to be a general property of representatives of various identified adenyl cyclase (6). Kinetic analyses revealed that for each adenyl cyclase, the low affinity inhibition could be described as a competition between Ca\(^{2+}\) inhibition and Mg\(^{2+}\) activation. Mg\(^{2+}\) has long been known not only to participate in the substrate (MgATP\(^{2-}\)) but also to activate adenyl cyclase at concentrations beyond those required to maximize the concentration of metal-chelated substrate (7).

X-ray crystallographic analysis of an adenyl cyclase catalytic core derived from the AC5 C1a and AC2 C2a\(^2\) domains revealed two metal-binding sites (8). One site (the “A site”) appears to form a tetrahedral coordination sphere, with the \(\alpha\)-phosphate of ATP, two carboxylate oxygens from two aspartate residues, and a water molecule, whereas the other (the “B site”) forms an octahedral coordination sphere (an oxygen from the same aspartates as in the A site, an unesterified oxygen from each of the \(\alpha\), \(\beta\), and \(\gamma\)-phosphates of ATP, and the backbone carbonyl of Ile397). It appears as though the A site enables the catalysis of the attack by the 3′-hydroxyl of ribose on the \(\alpha\)-phosphate. Whether one or the other of these sites corresponds to the allosteric Mg\(^{2+}\) regulatory site suggested by kinetic analyses is not clear. However, this structure furnishes a context in which to ask whether these sites might provide the mechanism whereby Ca\(^{2+}\) exerts some of its effects on adenyl cyclase. Zimmerman \textit{et al.} (9) described a number of mutants of AC5 in the immediate vicinity of the catalytic site in which the regulation by Mg\(^{2+}\) was altered. We have used these mutants to determine whether there is a link between Mg\(^{2+}\) regulation and sensitivity to inhibition by Ca\(^{2+}\). Because AC5 is also susceptible to inhibition by submicromolar concentrations of Ca\(^{2+}\), we were also able to address possible interplay between low and high affinity inhibition by Ca\(^{2+}\) in these mutants and the role, if any, played by Mg\(^{2+}\). Our studies showed contrasting effects of Mg\(^{2+}\) activation on the inhibition that was elicited by high and low concentrations of Ca\(^{2+}\) in each of the mutants; activation by Mg\(^{2+}\) decreased the absolute amount of high affinity inhibition, without affecting the \(K_i\) value, whereas the \(K_i\) value for low affinity inhibition was decreased. In contrast, adenyl cyclases are divided into the following domains: N terminus (NT); first transmembrane cassette (TM1); the first cytosolic domain (C1), which is separated into C1a, a highly conserved catalytic domain, and C1b, which is less conserved and not involved in catalysis; the second transmembrane cassette (TM2); and the second cytosolic domain (C2), which is separated into C2a, a highly conserved catalytic domain, and C2b, which is less conserved. The two catalytic domains can be separately expressed and combined to yield a fully functional catalytic unit (31, 32).
activation by forskolin, which acts at a different site than Mg²⁺, did not modify inhibition by Ca²⁺. Overall, these results suggest considerable overlap between the site of action of Mg²⁺ and Ca²⁺ in the catalytic domain of adenylyl cyclase. To exclude other cytosolic domains of AC5, the N terminus and C1b domain were deleted, and a chimera was made between AC5 and the Ca²⁺-insensitive AC2. We conclude that both low and high affinity inhibition by Ca²⁺ reflects direct competition by Ca²⁺ for the different conformations of the catalytic site of AC5 that are induced by Mg²⁺.

**EXPERIMENTAL PROCEDURES**

**Materials**— Forskolin was from Calbiochem. [³H]cAMP and [α-³²P]ATP were obtained from Amersham Biosciences. Other reagents were from Sigma.

**Cell Culture and Preparation of Cell Membranes**—Recombinant baculoviruses encoding wild type and mutant AC5 were generously provided by Dr. R. Taussig (University of Texas Southwestern Medical Center, Dallas, TX) (9). Baculovirus encoding AC2 was provided by Dr. R. Iyengar (Mt. Sinai University, New York, NY). AC5Nt and the chimeric AC5Hind in baculovirus were prepared as described below. The culture of Sf9 cells and the amplification of recombinant baculovirus were performed according to the method of Summers and Smith (10). Sf9 membranes containing individual adenylyl cyclase isozymes were prepared according to the method of Summers and Smith (11). The culture of Sf9 cells and the amplification of recombinant baculovirus were performed according to the method of Summers and Smith (11).

**Adenylyl Cyclase Activity Measurements**—The adenylyl cyclase activity of purified Sf9 or HEK293 plasma membranes (generally 5–10 µg of protein/assay) was measured in the presence of the following components (final concentrations): 12 mM phosphocreatine, 25 units/ml creatine phosphokinase, 0.1 mM cAMP, 0.1 mM ATP, 0.04 mM GTP, 0.125 mM isobutylmethylxanthine, 1–2 µCi of [α-³²P]ATP, 100 µM forskolin (as indicated), 70 mM HEPES buffer, pH 7.4, and the various concentrations of MgCl₂ indicated. Free Ca²⁺, Mg²⁺, Sr²⁺, and Ba²⁺ concentrations were established from a series of CaCl₂, MgCl₂, SrCl₂, and BaCl₂ solutions buffered with 200 µM EGTA as described (11). The reaction mixture (final volume, 100 µl) was incubated at 30 °C for 20 min. The reactions were terminated with sodium lauryl sulfate (0.5%), and [³H]cAMP was added as a recovery marker. The [³H]cAMP formed was quantified as described previously (11). The data points are presented as the mean activities ± S.D. of triplicate determinations from representative experiments that were performed up to three times on at least two separate membrane preparations. The protein concentrations were determined by the Lowry method (12). Background activities of uninfected Sf9 membranes were around 8–10 pmol/mg/min at 1 mM MgCl₂. The activity was poorly responsive to both Ca²⁺ and Mg²⁺ and was not deducted from the indicated values.

**Data Analysis**— Nonlinear regression was used to fit a competition curve with either one or two components to the data using the GraphPad Inplot4 program. Goodness of fit was quantified by the least squares method (F test) and was deemed better suited to a particular model when p < 0.05.

**Construction of Adenylyl Cyclase Chimeras and Mutants**—To make chimeras, QuickChange mutagenesis (Stratagene) was used to engineer complementary restriction sites in dog AC5 cDNA and rat AC2 cDNA in pBlueBacHis2B (Invitrogen) cloned between the Kpn1-EcoRI and BgII-EcoRI sites, respectively. Primers A (5'-CACCCAGAAGGACGAGGAGTGTTGAAAAGGCAATGATC-3') and B (5'-GATCATGCGCTTTTCAAGTCCGGCTCTCTGTTG-3') were used to generate the AC2Hind model when Nt and the C1b domain were deleted, and a chimera was made between AC5 and the Ca²⁺-insensitive AC2. We conclude that both low and high affinity inhibition by Ca²⁺ reflects direct competition by Ca²⁺ for the different conformations of the catalytic site of AC5 that are induced by Mg²⁺.

**Schematic representation of the chimeras and deletions used in the present study.** The major cytosolic and transmembrane domains of AC5 and AC2 are indicated. The position of the amino acid where restriction sites have been engineered to allow the construction of chimeras and deletions as described under "Experimental Procedures" are also shown.

**Fig. 1. Schematic representation of the chimeras and deletions used in the present study.** The major cytosolic and transmembrane domains of AC5 and AC2 are indicated. The position of the amino acid where restriction sites have been engineered to allow the construction of chimeras and deletions as described under “Experimental Procedures” are also shown.
domain of AC5; Fig. 1) was generated by cutting pBlueBacHis2B-AC5Sal with BamHI and subcloning into pcDNA3.0HisA. AC5NtTm1C1 (containing the N-terminal domain, the first transmembrane domain, the C1a domain, and the C1b domain of AC5; Fig. 1) was generated by cutting pBlueBacHis2B-AC5Sal with BamHI and subcloning into pcDNA3.0HisA in frame between BamHI and XhoI. The half molecule AC5Tm2C2 (containing the second transmembrane domain and the C2 domain of AC5; Fig. 1) was constructed by cutting off the Sal site and active site of adenylyl cyclase. The active site occurs at the interface of the C1a and C2a subunits of adenylyl cyclase, which are represented as orange spheres (8). Forskolin, shown in stick representation and in red, binds in the cleft that contains the active site. The ATP analog (DAD, in yellow) and two Mg²⁺ ions (represented as orange spheres) are bound in the adenylyl cyclase active site in this crystal structure. Right panel, location of the mutated amino acids. The panel shows a ribbon representation of the active site and location of the four active site mutations. The two Mg²⁺ ions are displayed as orange spheres, whereas DAD and the amino acids which are mutated are shown in stick representation. Each residue is colored by atom (carbon, yellow; nitrogen, blue; oxygen, red; sulfur, pink). Each of the four mutations occurs proximate to the Mg²⁺ binding sites. Cys⁴⁴¹ and Tyr⁴⁴² are the two residues just after Asp⁴⁴⁰, which is of prime importance to Mg²⁺ binding and adenylyl cyclase activity. The Arg⁴³⁴ and Phe⁴²³ are located more distally from the active site. Both residues contact Tyr⁴⁴², suggesting that mutations of these amino acids might transmit their effects on Mg²⁺ activation through this residue. Both panels were generated with Visual Molecular Dynamics software (34) and POVray (www.povray.org).

RESULTS

AC5 Mutants—Clearly defined mutants that display a range of Mg²⁺ sensitivities provide a direct means for exploring the relationship between the activation by Mg²⁺ of adenylyl cyclase and its susceptibility to inhibition by Ca²⁺. Crystal structure analysis of the catalytic core derived from the AC5 C1a and C22 C2a (VC1a/IIC2a) complex has revealed the key roles played by Asp⁴⁴⁰ and Asp⁴⁰⁶ in coordinating the Mg²⁺ ions involved in the adenylyl cyclase reaction (8). Not surprisingly, mutation of these amino acids results in an inactive enzyme (9). However, mutation of adjacent amino acids, which impinge on the ability of these aspartate groups to bind Mg²⁺, could be insightful. Taussig and co-workers (9) generated mutations in the catalytic domain of AC5 by random mutagenesis, which showed disparate responses to Mg²⁺. Two of the mutations, C441R and Y442H, are near Asp⁴⁴⁰. In the wild type enzyme, Cys⁴⁴¹ is located on the edge of the active site, next to Asp⁴⁴⁰ and on an opposite strand from Asp⁴⁰⁶ (Fig. 2). Substitution of an Arg at this position would allow its positively charged amino group to be in a position to establish electrostatic interaction with Asp⁴⁰⁶ and Glu⁴¹⁸. Such an interaction, and any conformational changes it would induce, would destabilize or hinder Mg²⁺ binding. Tyr⁴⁴² is close to Asp⁴⁴⁰ and is tightly packed between Arg⁴³⁴ and Phe⁴²³. Any change in backbone conformation of Tyr⁴⁴² could readily propagate to the nearby Asp⁴⁴⁰. The second group of mutations, F423L and R434S, are more distant from the catalytic site (Fig. 2). Disruption of the packing of these residues could be expected to bring about a conformational distortion, which would result in reduced sensitivity to Mg²⁺. These mutants therefore seemed like useful tools to evaluate possible sites of action for Ca²⁺.

Mg²⁺ Sensitivity of AC5 Mutants—To identify the mutants that were most likely to be useful in terms of their regulation by Mg²⁺, detailed dose-response curves of the adenylyl cyclase activities expressed in plasma membranes from appropriate baculovirus-infected Sf9 cells were evaluated. Even a superficial analysis of these data revealed that the response to Mg²⁺ had gone from 0.6 mM in the wild type to ~2 mM in the mutant type F423L and R434S and to 7.5 mM in Y442H and C441R, respectively (Fig. 3).

Ca²⁺ Sensitivity of AC5 Mutants—This range of sensitivities to Mg²⁺ provided us with the opportunity to test whether the alteration in sensitivity to Mg²⁺ was accompanied by altered properties of inhibition by Ca²⁺ (Fig. 4). We first performed
detailed Ca$^{2+}$ dose-response curves on each of these mutants at 1 mM Mg$^{2+}$, which is both our standard assay concentration and also approximates intracellular levels of the cation (14, 15). Strikingly, the responses fell into three groups. Wild type AC5 was inhibited in a clearly biphasic manner by Ca$^{2+}$, with a high affinity $K_i$ value that appeared to be $-0.1 \mu M$ and a low affinity $K_i$ value that appeared to be $-30 \mu M$. The mutations F423L and R434S showed a response in which the high affinity component was reduced in amount relative to the low affinity component, although both high affinity values were similar to wild type. By contrast, Y442H and C441R showed a muted biphasic or only a monophasic low affinity inhibition by Ca$^{2+}$ (Fig. 4).

Ca$^{2+}$ Sensitivity of AC5 Mutants as a Function of Activation by Mg$^{2+}$—Given that the adenyl cyclases tested above had exhibited different sensitivities to Mg$^{2+}$ (cf. Fig. 3), we wished to evaluate the possibility that at Mg$^{2+}$ concentrations that elicited equivalent relative activities the response to Ca$^{2+}$ might be comparable for each species. Thus, we compared the response to Ca$^{2+}$ at a number of Mg$^{2+}$ concentrations. We first examined wild type AC5. A dose-response to Ca$^{2+}$ was performed at 0.3, 0.8, and 3 mM Mg$^{2+}$. These concentrations corresponded to $-25$, 50, and 75% of the maximal stimulation by Mg$^{2+}$. In all cases, a biphasic response to Ca$^{2+}$ was evident, but as [Mg$^{2+}$] increased, the relative amount of high to low affinity components decreased (Fig. 5A), declining from 55 to 26% (Table I). At the same time, the $K_i$ for the low affinity component was increased (from 32 to 163 $\mu M$) as the $K_i$ increased (Table I). The most direct interpretation of these results is that Mg$^{2+}$ not only competitively antagonizes low affinity inhibition by Ca$^{2+}$, but it also noncompetitively antagonizes high affinity inhibition (because the high affinity $K_i$ is unchanged). This conclusion is effectively the complement to our recent finding with both AC6 and striatal adenyl cyclase (the latter assumed to be AC5) that high affinity inhibition by Ca$^{2+}$ competitively antagonizes activation by Mg$^{2+}$, whereas low affinity inhibition by Ca$^{2+}$ is a combination of competitive and noncompetitive antagonism of Mg$^{2+}$ activation (6).

We next examined the Ca$^{2+}$ sensitivity of the F423L mutant (which was intermediate in its sensitivity to Mg$^{2+}$) as a function of [Mg$^{2+}$]. In this case, the concentrations required to elicit $-25$, 50, and 75% maximal stimulation were 0.8, 3, and 6 mM (cf. Fig. 3). As had been seen in the first experiment with this mutant, which used relatively low [Mg$^{2+}$], there was a muted biphasic response to Ca$^{2+}$ at the lowest [Mg$^{2+}$] (13% high affinity versus 87% low affinity; Table I), but at higher [Mg$^{2+}$], high affinity inhibition was eliminated (Fig. 5B). These results are again consistent with a noncompetitive antagonism by Mg$^{2+}$ of high affinity inhibition by Ca$^{2+}$. In the case of low affinity inhibition, as with the wild type AC5, the $K_i$ value was increased (from 130 to 519 $\mu M$) as the [Mg$^{2+}$] increased (Table I). In the case of mutant C441R (the mutant with the lowest sensitivity to Mg$^{2+}$), at low [Mg$^{2+}$] only monophasic, low affinity inhibition by Ca$^{2+}$ was evident. At a range of [Mg$^{2+}$] corresponding to 25, 50, and 75% stimulation (viz. 3, 8, and 16 mM), the response to Ca$^{2+}$ remained monophasic (Fig. 5C), and the $K_i$ value increased from 187 to 1268 $\mu M$ (Table I).

Characterization of the Ca$^{2+}$-binding Sites in Wild Type AC5 and AC5 Mutants Using the Ila Cation Series—The foregoing data are consistent with the existence of two binding sites for Ca$^{2+}$, whose relative abundance and affinity is controlled by Mg$^{2+}$ to different extents. To probe the nature of these two putative Ca$^{2+}$-binding sites and whether their properties were altered by Mg$^{2+}$, we used the divalent cation series Ca$^{2+}$, Sr$^{2+}$, and Ba$^{2+}$. The Ila series of cations, Ca$^{2+}$, Sr$^{2+}$, and Ba$^{2+}$, have progressively increasing atomic radii (1.2, 1.4, and 1.56 Å, respectively), which can be used to characterize a Ca$^{2+}$-binding site. For instance, in the case of the best known biological Ca$^{2+}$-binding site, the so-called E-F hand, these cations are all capable of mimicking the actions of Ca$^{2+}$, although with decreasing affinities (16). On the other hand, in the case of a binding site for Mg$^{2+}$, whose crystal ionic radius is only 0.85 Å and which requires only four ligands for binding, the larger cations would be expected to be ineffective. We recently showed
that the sensitivity of the closely related AC6 to Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\), is a telling indicator of the difference between high and low affinity inhibition by Ca\(^{2+}\). It turned out that Sr\(^{2+}\) elicited little if any high affinity inhibition, whereas both Sr\(^{2+}\) and Ba\(^{2+}\) displayed low affinity inhibition (17). Consequently, we compared the wild type and mutant AC5 species to determine whether the same relative effects of Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) were preserved, as an indication of whether or not the metal-binding sites had been distorted. With the wild type, as expected, Ca\(^{2+}\) yielded a biphasic response, Sr\(^{2+}\) a minor component of high affinity inhibition as well as low affinity inhibition, and Ba\(^{2+}\) just low affinity inhibition (Table II). It is notable that AC5, like AC6, shows little high affinity response to Sr\(^{2+}\); however, it is a significant component. In this case it is probably detectable because the Mg\(^{2+}\) concentration used in the assay is not highly activating. The fact that the high affinity inhibition by Sr\(^{2+}\) is less in magnitude than that of Ca\(^{2+}\) probably reflects the decreased ability of Sr\(^{2+}\) to induce the high affinity inhibited state.

In both the F423L and C441R mutations, in which there had that the sensitivity of the closely related AC6 to Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) is a telling indicator of the difference between high and low affinity inhibition by Ca\(^{2+}\). It turned out that Sr\(^{2+}\) elicited little if any high affinity inhibition, whereas both Sr\(^{2+}\) and Ba\(^{2+}\) displayed low affinity inhibition (17). Consequently, we compared the wild type and mutant AC5 species to determine whether the same relative effects of Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) were preserved, as an indication of whether or not the metal-binding sites had been distorted. With the wild type, as expected, Ca\(^{2+}\) yielded a biphasic response, Sr\(^{2+}\) a minor component of high affinity inhibition as well as low affinity inhibition, and Ba\(^{2+}\) just low affinity inhibition (Table II). It is notable that AC5, like AC6, shows little high affinity response to Sr\(^{2+}\); however, it is a significant component. In this case it is probably detectable because the Mg\(^{2+}\) concentration used in the assay is not highly activating. The fact that the high affinity inhibition by Sr\(^{2+}\) is less in magnitude than that of Ca\(^{2+}\) probably reflects the decreased ability of Sr\(^{2+}\) to induce the high affinity inhibited state.

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### Table I

| Cation | Total Mg\(^{2+}\) | High affinity | Low affinity |
|--------|-----------------|---------------|--------------|
|        | (mM)            | (µM)          | (µM)         |
| AC5    |                 |               |              |
| 0.3    | 0.069 (55%)     | 32 (45%)      |
| 0.8    | 0.058 (38%)     | 43 (62%)      |
| 3.0    | 0.060 (26%)     | 163 (74%)     |
| F423L  |                 |               |              |
| 0.8    | 0.164 (13%)     | 130 (57%)     |
| 3.0    | ND              | 396 (100%)    |
| 6.0    | ND              | 519 (100%)    |
| C441R  |                 |               |              |
| 3.0    | 0.181 (11%)     | 187 (89%)     |
| 8.0    | ND              | 593 (100%)    |
| 16.0   | ND              | 1268 (100%)   |

### Table II

Comparison of the kinetic parameters of the inhibition by Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) of wild type AC5 and two AC5 mutants

Adenylyl cyclase activity was determined in Sf9 membranes containing the wild type AC5 in the presence of 0.6 mM MgCl\(_2\), the F423L mutant in the presence of 2 mM MgCl\(_2\), and the C441R mutant in the presence of 6 mM MgCl\(_2\), in the presence of varying concentration of Ca\(^{2+}\), Sr\(^{2+}\), or Ba\(^{2+}\). 100 µM forskolin and 100 µM ATP were included in the assay. The kinetic parameters were extracted from at least three similar experiments. ND indicates that high affinity inhibition was not detectable.
Interaction between Ca\(^{2+}\) and Mg\(^{2+}\) in Regulating AC5

been little or no high affinity inhibition by Ca\(^{2+}\), there is no indication of high affinity inhibition by any of the other IIa cations (Table II). These results confirm that a reduced ability of submicromolar concentrations of Ca\(^{2+}\) to antagonize activation by Mg\(^{2+}\) is inevitably associated with an inability of the less efficacious IIa cations to antagonize activation by Mg\(^{2+}\). This result implies that the presumed change in the Mg\(^{2+}\)-binding site(s) brought about by the mutagenesis has parallel consequences for the effects of the all IIa cations.

Ca\(^{2+}\) Sensitivity of AC5 as a Function of Activation by Forskolin—In the context of the early contention (see the Introduction) that Mg\(^{2+}\) acted at an allosteric site to activate adenylyl cyclase, the possibility could be entertained that the apparent antagonism between Mg\(^{2+}\) and Ca\(^{2+}\) actually reflects not a direct antagonism between cations for the same (or part of the same) binding site, but an antagonism between an activated state induced by Mg\(^{2+}\) and the inhibited state induced by Ca\(^{2+}\). To evaluate this possibility, rather than progressively activating the enzyme by increasing concentrations of Mg\(^{2+}\), we established an activation curve for forskolin at a fixed Mg\(^{2+}\) concentration (1 mM; Fig. 6A). Based on crystallographic analysis, forskolin binds to a distinct locus from MgATP\(^{2+}\) and activates adenylyl cyclase by quite a different molecular mechanism. The diterpene binds to the catalytic core at the opposite side from the catalytic site, fitting into a hydrophobic pocket and stabilizing the association between the C1a and C2a domains (Fig. 1 and Ref. 8). Analogous to the experiments performed with Mg\(^{2+}\), Ca\(^{2+}\) dose-response experiments were conducted at concentrations of forskolin that yielded progressively increasing activity states of -25, 50, and 75% of the maximum, corresponding to 0.5, 5, and 50 \(\mu\)M, respectively. Quite clearly, the amount and affinities for both low and high affinity inhibition by Ca\(^{2+}\) were virtually unchanged, regardless of the degree of activation elicited by forskolin (Fig. 6B). These data show that Ca\(^{2+}\) does not compete with forskolin binding and, in fact, argue again that the targets for the action of Ca\(^{2+}\) are primarily Mg\(^{2+}\)-binding site(s).

Ca\(^{2+}\) Sensitivity of AC5 and AC2 Constructs—The foregoing data strongly suggest that inhibition by Ca\(^{2+}\) of adenylyl cyclase involves direct competition for Mg\(^{2+}\)-binding sites or some of the residues participating in Mg\(^{2+}\) binding. To address the possibility that regions of AC5 other than the C1a domain were involved, chimeras were constructed between AC5 and AC2 at the C1a-C1b junction, and the N terminus of AC5 was also deleted. When the Ca\(^{2+}\) sensitivity of these constructs propagated in Sf9 cells was compared, it was clear that AC5 and AC5NT yielded virtually identical inhibitory profiles in response to Ca\(^{2+}\): -40–45% high affinity inhibition (Fig. 7 and Table III). By contrast, AC2 displayed only low affinity inhibition. The chimera AC52Hind, whose N-terminal domain, the
first transmembrane domain, and the C1a domain came from AC5, whereas the C1b domain, the second transmembrane domain, and the C-terminal domain come from AC2 (see "Experimental Procedures"; Fig. 1), preserved intermediate levels of high affinity inhibition (Fig. 7 and Table III). These data indicate that no region of AC5, other than the C1a, is required to mediate high inhibition by Ca\(^{2+}\).

**Ca\(^{2+}\)-Sensitivity of Truncated AC5 Molecules**—In the experiments described above with the chimeric AC52Hind, it might be argued that the C1b domain, even of a Ca\(^{2+}\)-insensitive adenylyl cyclase, plays a facilitating role in permitting inhibition to be observed. To address this possibility, we tried to apply to AC5 our finding that adenylyl cyclase AC8 cDNA can be divided into two halves, which when expressed together in HEK293 cells, assemble, traffic appropriately to the plasma membrane, and display full functional activity (18). Consequently, we hoped that half molecules of AC5 would behave similarly. In fact, an earlier study had demonstrated that bisected molecules of AC5, when co-expressed in SF9 cells, yielded robust, forskolin-stimulable adenylyl cyclase activity (19). In the present case, when either of the half molecules AC5NtTm1C1a (containing the N-terminal domain, the first transmembrane domain, and the C1a domain of AC5; Fig. 1) or AC5NtTm1C1 (containing the N-terminal domain, the first transmembrane domain, and the C1a and the C1b domains of AC5; Fig. 1) were expressed along with the half molecule AC5Tm2C2 (containing the second transmembrane domain and the C2 domain of AC5; Fig. 1), characteristic AC5 activity was observed. We carried out Ca\(^{2+}\)-dose-response curves of HEK293 cell plasma membranes transfected with these various combinations of partial adenylyl cyclase cDNAs. Control-transfected HEK293 plasma membranes displayed only low affinity inhibition by Ca\(^{2+}\), whereas cells expressing combined partial adenylyl cyclase cDNAs showed both high (0.37–0.61 \(\mu\)M) and low (130–160 \(\mu\)M) affinity inhibition in approximately equal amounts (31–34% high versus 69–66% low affinity, respectively; Fig. 8). From curve fitting of a number of similar experiments, there was no difference in either the sensitivity or the relative amount of high versus low affinity inhibition by Ca\(^{2+}\) in either combination. These data clearly show that the C1b domain plays no role in permitting high affinity inhibition.

**DISCUSSION**

The Ca\(^{2+}\)-inhibited adenylyl cyclases, AC5 and AC6 are discretely expressed in a number of tissues where their susceptibility to inhibition by physiological rises in Ca\(^{2+}\) appears to provide a device for acutely balancing the activities of the Ca\(^{2+}\) and cAMP signaling systems. For instance, in cardiac tissue, the Ca\(^{2+}\)-inhibitable AC5 and AC6 mediate the actions of the sympathetic nervous system on cardiac contractility (2). The susceptibility to inhibition by Ca\(^{2+}\) of these adenylyl cyclases provides a delicate feedback by voltage-gated Ca\(^{2+}\) channels on the cAMP signal that modulates their activity (20). A Ca\(^{2+}\)-inhibitable adenylyl cyclase also plays a key role in maintaining endothelial cell permeability.\(^3\) A number of other physiological examples have been discussed elsewhere on the potential usefulness of the susceptibility to inhibition by Ca\(^{2+}\) of adenylyl cyclases (21). However, remarkably little is known on the molecular mechanisms whereby inhibition by Ca\(^{2+}\) is mediated.

Guillou et al. (6) showed that both submicromolar and supramicromolar concentrations of Ca\(^{2+}\) antagonized activation of adenylyl cyclase by Mg\(^{2+}\). However, those data could not address the site of action of Ca\(^{2+}\), because, for instance, analogous data are seen with the inhibition of adenylyl cyclase mediated by G\(_\alpha\), which antagonizes the activity state induced by G\(_\beta\) with little direct, physical interaction between the binding sites of the two G proteins (22). Consequently, in the present study we took the approach of looking at conservative amino acid mutations around the catalytic site of AC5, which likely impinged on the ability of key aspartate groups to bind Mg\(^{2+}\), resulting in altered activation by Mg\(^{2+}\). We then used these mutants to determine whether there was an interdependence between Mg\(^{2+}\) activation and inhibition by Ca\(^{2+}\). Indeed, an apparently obligate interdependence was encountered. Low affinity inhibition by Ca\(^{2+}\) was a straightforward direct competitive inhibition between Ca\(^{2+}\) and Mg\(^{2+}\) for activation of adenylyl cyclase; higher [Ca\(^{2+}\)] could always inhibit Mg\(^{2+}\) activation, and higher [Mg\(^{2+}\)] could always overcome Ca\(^{2+}\) inhibition. Thus, this process may involve a direct competition between Ca\(^{2+}\) and Mg\(^{2+}\) for the same site on the cyclase, e.g., the A site described from the crystal structure, which promotes nucleophilic attack by the 3'-OH of the ribose on the α-phosphate (see Introduction; Fig. 2). In the case of high affinity

\[^{3}\]Cioffi, D., Moore, T. M., Schaack, J., Creighton, J. R., Cooper, D. M. P., and Stevens, T. (2002) J. Cell. Biol. 157, 1267–1278.
inhibition by Ca\(^{2+}\), this inhibition is maximal at minimal degrees of Mg\(^{2+}\) activation, i.e. as activation by Mg\(^{2+}\) increases, the amount of high affinity inhibition by Ca\(^{2+}\) is diminished, whereas the affinity is unaltered. This is a hallmark of non-competitive inhibition, indicating an allosteric interaction between Mg\(^{2+}\) and Ca\(^{2+}\). The simplest interpretation of these data is that low [Ca\(^{2+}\)] induces a different inactive enzyme state. Mg\(^{2+}\) noncompetitively antagonizes this effect by stabilizing an active state. It should be acknowledged in passing that kinetic analysis of complete, complex reactions for which detailed atomic awareness of the component reactions is emerging, provides only an intuitive overview of the process. In the present case, a more detailed kinetic analysis of the interaction between Ca\(^{2+}\) and Mg\(^{2+}\) as a function of substrate concentration and an evaluation, for instance, of the potential role of Ca\(^{2+}\) in stabilizing the release of the reaction product, pyrophosphate, could throw further light on the reaction mechanism analogous to the detailed description of P site-mediated inhibition of adenylyl cyclase (23).

The results with the mutated AC5 species establish the intimacy of the interaction between Mg\(^{2+}\) and Ca\(^{2+}\). As stated above, just because Ca\(^{2+}\) antagonizes Mg\(^{2+}\) activation, this does not mean that their site of interaction is shared. However, the conservative mutations that perturb Mg\(^{2+}\) binding strongly support the intimacy of this interaction. Indeed, one might argue that if the Ca\(^{2+}\) site was distant from the Mg\(^{2+}\) site, then mutations that decreased the potency of Mg\(^{2+}\) might actually increase the efficacy of Ca\(^{2+}\). The opposite is true. It appears as though the mutations represent a state that would have been induced by higher concentrations of Mg\(^{2+}\), which results in a decreased residual affinity for Mg\(^{2+}\). As a result of the endogenous higher activity state of the mutants, the amount of high affinity inhibition that Ca\(^{2+}\) can induce is already reduced. However, further activation by Mg\(^{2+}\) can still overcome the high affinity inhibition that is expressed, decreasing the relative amount of high versus low affinity inhibition, as occurs in the wild type.

The results with the IIa series of cations, Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\), show parallel changes in the ability of these cations to induce high and low affinity inhibition as a function of mutagenesis. Thus, in the wild type AC5, which shows prominent high affinity inhibition by Ca\(^{2+}\), inhibition by Sr\(^{2+}\) is clearly discernable. The fact that this latter inhibition is less in magnitude than that of Ca\(^{2+}\) correlates with the lower potency of Sr\(^{2+}\) at Ca\(^{2+}\) sites (16). In the mutants where the overall affinity for Mg\(^{2+}\) is already reduced and, as a result, the amount of high affinity inhibition available to Ca\(^{2+}\) is reduced, unless the Ca\(^{2+}\)-binding site had been grossly distorted, it would be expected that Sr\(^{2+}\) would be quite impotent at inducing high affinity inhibition. The parallel loss in inhibitory efficacy of Sr\(^{2+}\) therefore strongly suggests that the mutations induce a simple change in the Mg\(^{2+}\)-binding sites, which translates into simple shifts in the potency of the IIa cations.

The consequences of the activation induced by forskolin on the inhibitory profile elicited by Ca\(^{2+}\) are enlightening. Unlike with Mg\(^{2+}\), increasing activity by forskolin had absolutely no effect on either the affinity or relative distribution of the inhibitory states induced by Ca\(^{2+}\). This finding clearly demonstrates that it is not the activity states per se that predispose or not toward high affinity inhibition. This result suggests that although equivalent activities can be induced by different effectors (forskolin and Mg\(^{2+}\)), these correspond to nonequivalent conformations. Thus, at a fixed [Mg\(^{2+}\)], with increasing forskolin, although activity increases, the occupancy (or conformation) of the Mg\(^{2+}\) site(s) does not change and consequently, the inhibitory response to Ca\(^{2+}\) does not change. This result strongly suggests direct competition between Mg\(^{2+}\) and Ca\(^{2+}\) for common ligating oxygen atoms.

A recent paper indicating selective inhibitory effects of micromolar [Zn\(^{2+}\)] on AC5 and AC6, but not on AC2 (24), prompts some consideration of whether inhibition by Zn\(^{2+}\) might have something to do with the relative sensitivity of AC5 and AC2 to high affinity inhibition by Ca\(^{2+}\). At first glance the inhibition by the two cations appears to occur by fundamentally different mechanisms, because in contrast with the inhibition by Ca\(^{2+}\) described in the present study, (i) the inhibition by Zn\(^{2+}\) is not affected in the slightest by either Mg\(^{2+}\) or Mn\(^{2+}\) and (ii) pre-activation of AC5 by forskolin, for example, counteracts inhibition by Zn\(^{2+}\), again unlike the situation with Ca\(^{2+}\) and forskolin described presently. However, it is conceivable that Zn\(^{2+}\), albeit with lower affinity, could more persistently occupy the site or part of the site(s) that can be occupied by Ca\(^{2+}\). This issue seems worthy of further exploration, particularly given that one of the Mg\(^{2+}\) sites proposed from the crystal structure was actually occupied by Zn\(^{2+}\) (8).

Even though the present studies clearly indicate an intimate interdependence between Ca\(^{2+}\) inhibition and Mg\(^{2+}\) activation, does this mean that the metal-binding sites identified in the crystal structure are likely site(s) of the inhibitory action of Ca\(^{2+}\)? Binding of Ca\(^{2+}\) in site A, site B, or a combination of these sites could lead to conformational differences that would result in enzymatic inhibition. It is not difficult to imagine that the low affinity Ca\(^{2+}\) site is a direct competition for either the A or the B Mg\(^{2+}\)-binding site. The ability of the IIa cations to elicit low affinity inhibition could suggest that this is really a Mg\(^{2+}\)-binding site involving four ligating atoms, for which the IIa cations compete poorly. On the other hand, although the high affinity site may be a variant of the low affinity site, such that the additional two or more ligating atoms needed to confer high selectivity for Ca\(^{2+}\) are exposed during conformational changes associated with the activation cycle, it seems reasonable to at least consider the possibility that it is located elsewhere. The Clb domain was proposed as the site of high affinity inhibition in a study using truncated forms of the cytosolic domains of AC5 linked by 14 amino acids (25). A later study using the same constructs contradicted those conclusions (6). In the present investigation, we also showed that there was no difference in sensitivity to Ca\(^{2+}\) of co-expressed half molecules of AC5, lacking or not the Clb domain. The present study also showed no loss in Ca\(^{2+}\) sensitivity upon deleting the N terminus from AC5. Finally, the construction of a chimeric construct between AC5 and AC2 at the C1a-C1b boundary retained high affinity Ca\(^{2+}\) inhibition. Therefore, by simple elimination, no cytosolic region of AC5 other than the C1a domain is a viable candidate for mediating high affinity inhibition.4

A number of experimental approaches might be considered in attempting to pinpoint the site of action of Ca\(^{2+}\). Our present and previous data have revealed a tightly knit connection between the Mg\(^{2+}\) and Ca\(^{2+}\) sites in AC5 and AC6. These results identify sites A and B or their immediate vicinity as viable loci for the sites of the inhibitory actions of calcium. Site-directed mutagenesis around the catalytic domain is unlikely to yield further information, because mutations that directly affect Mg\(^{2+}\) binding are, not surprisingly, inactive (9). Nevertheless, conservative mutations that impinge on the amino acids involved in Mg\(^{2+}\) binding (informed by the crystal structures

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4 The formal possibility should not be completely discounted that calmodulin might play a role in mediating these high affinity effects distally from the catalytic site, even though early investigations appeared to exclude a role for calmodulin. For instance, extensive washing of membranes with EGTA and calmodulin antagonists, such as trifluoperazine, failed to diminish the effect (11, 33).
available) might reinforce the present conclusions. The possibility that additional parts of the AC5 sequence might play some role in facilitating Ca$^{2+}$ inhibition could be determined from chimeric constructs between the Ca$^{2+}$-inhibitable AC5 and the Ca$^{2+}$-insensitive AC2 along the lines applied in the present study. However, given that the present studies point so strongly toward the C1a domain, new crystal structure analysis between the C1a and C2a from AC5, rather than the C1a from AC5 coupled with the C2a of AC2, as is currently available, might be the most direct means for resolving this issue.

A physiological implication of the present findings is that ambient [Mg$^{2+}$] will dictate the degree to which inhibition by Ca$^{2+}$ will occur in intact cells. High affinity inhibition by Ca$^{2+}$ seems to be the only physiological option for inhibiting adenylyl cyclase, because elevations in [Ca$^{2+}$], are not considered to exceed 1 μM, and high affinity inhibition is antagonized by high [Mg$^{2+}$] (present studies). The fact that AC5 and AC6 are seen to be inhibited upon the entry of Ca$^{2+}$ accords with the in vitro sensitivity observed herein, because cellular [Mg$^{2+}$] is estimated to be between 0.6 and 1 mM (14, 15). However, cellular mechanisms that modulated cytosolic [Mg$^{2+}$] might exert profound influences on how adenylyl cyclases could be inhibited by elevation in [Ca$^{2+}$].

In closing, we have developed a clear kinetic picture of how both low and high affinity inhibition of adenylyl cyclase by Ca$^{2+}$ are mediated. The molecular mechanism clearly requires two Ca$^{2+}$-binding sites, although their precise locations are not known. It seems reasonable to expect that the low affinity site will overlap with the Mg$^{2+}$-binding sites identified from the crystal structure, whereas high affinity inhibition will be mediated on a distinct conformation, which may overlap with the first site. If it turned out that high affinity inhibition of AC5 directly involved a Mg$^{2+}$-binding site in the catalytic domain, it would seem reasonable to ask why so-called Ca$^{2+}$-insensitive adenylyl cyclases, such as AC2 and AC7, do not display high affinity Ca$^{2+}$ inhibition (see e.g. Ref. 6), given that the catalytic domains of adenylyl cyclases are highly homologous (up to 65% amino acid identity) and are assumed to be similar in tertiary structure. This latter assumption may be a little simplistic, given the rather divergent basal activities of different adenylyl cyclase species (26) and therefore, presumably, the conformational transitions associated with catalytic activity. Indeed, it is not difficult to imagine that the Mg$^{2+}$-binding sites and adjacent amino acids in the catalytic domain of Ca$^{2+}$-insensitive adenylyl cyclases may just never quite adopt the conformation that allows Ca$^{2+}$ to bind with high affinity. Detailed, comparative crystallographic analysis seems to be the only unambiguous means for solving this problem. In intact cells, an additional factor conferring sensitivity to elevations in [Ca$^{2+}$] is that Ca$^{2+}$-sensitive adenylyl cyclases (AC6 and AC8) need to be localized in caveolae to be regulated by capacitative Ca$^{2+}$ entry (27, 28). (Capacitative Ca$^{2+}$ entry is the mode of elevation of [Ca$^{2+}$], that is essential for the regulation of Ca$^{2+}$-sensitive adenylyl cyclases in nonexcitable cells (29, 30).) At least one Ca$^{2+}$-insensitive adenylyl cyclase, AC7, is excluded from caveolae (28). Therefore, subcellular localization, sensitivity to Mg$^{2+}$, and quite subtle structural features of the catalytic domain may all contribute to rendering adenylyl cyclases sensitive to Ca$^{2+}$.

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Although it should be borne in mind that several lines of evidence suggest that Ca$^{2+}$-sensitive adenylyl cyclases are located close to capacitative Ca$^{2+}$ entry channels (29, 30), which might allow [Ca$^{2+}$] to reach even higher concentrations in the immediate domain of adenylyl cyclase, both forms of inhibition are diminished by elevating [Mg$^{2+}$] in vitro.