Inhibition by Calcium of Mammalian Adenylyl Cyclases*

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Ca$^{2+}$ regulates mammalian adenylyl cyclases in a type-specific manner. Stimulatory regulation is moderately well understood. By contrast, even the concentration range over which Ca$^{2+}$ inhibits adenylyl cyclases AC5 and AC6 is not unambiguously defined; even less so is the mechanism of inhibition. In the present study, we compared the regulation of Ca$^{2+}$-stimulable and Ca$^{2+}$-inhibitable adenylyl cyclases expressed in SF9 cells with tissues that predominantly express these activities in the mouse brain. Soluble forms of AC5 containing either intact or truncated major cytosolic domains were also examined. All adenylyl cyclases, except AC2 and the soluble forms of AC5, displayed biphasic Ca$^{2+}$ responses, suggesting the presence of two Ca$^{2+}$ sites of high (~0.2 μM) and low affinity (~0.1 μM). With a high affinity, Ca$^{2+}$ (i) stimulated AC1 and cerebellar adenylyl cyclases, (ii) inhibited AC6 and striatal adenylyl cyclase, and (iii) was without effect on AC2. With a low affinity, Ca$^{2+}$ inhibited all adenylyl cyclases, including AC1, AC2, AC6, and both soluble forms of AC5. The mechanism of both high and low affinity inhibition was revealed to be competition for a stimulatory Mg$^{2+}$ site(s). A remarkable selectivity for Ca$^{2+}$ was displayed by the high affinity site, with a $K_i$ value of ~0.2 μM, in the face of a 5000-fold excess of Mg$^{2+}$. The present results show that high and low affinity inhibition by Ca$^{2+}$ can be clearly distinguished and that the inhibition occurs type-specifically in discrete adenylyl cyclases. Distinction between these sites is essential, or quite spurious inferences may be drawn on the nature or location of high affinity binding sites in the Ca$^{2+}$-inhibitable adenylyl cyclases.

Profound physiological significance derives from the regulation of adenylyl cyclase by Ca$^{2+}$, which provides a confluence of two major signaling pathways. For instance, Ca$^{2+}$ stimulation of adenylyl cyclase has been implicated in learning-memory functions (1–3). Compelling evidence for this assertion comes from studies with Aplysia (4) and with Drosophila mutants (5) and more recently mice that have had adenylyl cyclase genes deleted (6). On the other hand, Ca$^{2+}$ inhibition of adenylyl cyclase has been proposed to contribute to oscillations and/or pacemaking in cardiac tissue (7) and the maintenance of endothelial cell permeability (8). A central issue in implicating the Ca$^{2+}$ responsiveness of an adenylyl cyclase in a physiological process concerns the correspondence between the concentrations of Ca$^{2+}$ achieved in response to physiological stimuli versus those required to regulate these adenylyl cyclases in vitro.¹ This issue seems moot with Ca$^{2+}$-stimulable adenylyl cyclases, which are stimulated by Ca$^{2+}$ in vitro and are also stimulated in response to various physiological means of elevating Ca$^{2+}$ in vivo (2, 7, 9). Such correlations are mutually supportive. The situation with Ca$^{2+}$-inhibitable adenylyl cyclases is much more complex. The averaged cytosolic concentration of Ca$^{2+}$ achieved in intact cells upon triggering either capacitative- or voltage-gated Ca$^{2+}$ entry (~1 μM) corresponds to the concentrations reported to be effective at inhibiting these adenylyl cyclases in some in vitro studies (10). However, a wide array of in vitro inhibitory sensitivities have been reported from various sources (10–25). For instance, both monophasic (11–13, 21, 23) and biphasic (10, 14–19, 24) inhibition by Ca$^{2+}$ have been reported, spanning sub- to supramicromolar concentration ranges.

Some of the discrepancies may emanate from somewhat uncontrolled assay conditions, e.g. with respect to pH or free EGTA concentrations, which will confound estimates of free Ca$^{2+}$ (26). However, mixed populations of adenylyl cyclases in selected tissues may also confound analysis. The nine isoforms of mammalian adenylyl cyclases that have been cloned to date (7, 27) possess distinct characteristics including their sensitivity to Ca$^{2+}$, which allows them to be classified as (i) Ca$^{2+}$-stimulated (AC1, AC8, and, possibly, AC3), (ii) Ca$^{2+}$-insensitive (AC2, AC4, and AC7), and (iii) Ca$^{2+}$-inhibited (AC5 and AC6; Refs. 7, 28, and 29). Heterogeneous prepaations of such adenylyl cyclases could give rise to a variety of Ca$^{2+}$ concentration responses. There is also the issue of low affinity Ca$^{2+}$ inhibition, which appears to be a property of all adenylyl cyclases. This inhibition is believed to reflect competition by Ca$^{2+}$ for an allosteric regulatory site for Mg$^{2+}$ (16, 17, 20–22), although the precise mechanism is unknown. This low affinity inhibition by Ca$^{2+}$ can be confounded with high affinity inhibition, particularly if Ca$^{2+}$ concentrations are not rigorously established or controlled (26).

Ideally, well controlled, direct comparisons of expressed adenylyl cyclase isoforms should resolve these ambiguities. Indeed, in membranes from transfected HEK 293 cells, AC1 and AC8 were stimulated by submicromolar concentrations of Ca$^{2+}$, while they displayed inhibition in response to higher concentrations, mirroring what happens in most brain tissues (9). However, again, the situation with the Ca$^{2+}$-inhibitable adenylyl cyclases is less clear. A monophasic inhibition by Ca$^{2+}$ spanning sub- to supramicromolar concentrations was associated with the first description of canine AC5 (25). A second report on rabbit AC5 indicated an inhibition by very low submicromolar (suprananomolar) Ca$^{2+}$ concentrations (30). In the first description of AC6, its activity in transfected cell mem-

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¹ Earlier biochemical studies had established that the in vitro effect of Ca$^{2+}$ was direct, readily reversible, and not secondary to phosphorylation or a variety of potentially Ca$^{2+}$-dependent processes (18, 29, 60, 61).
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branes was inhibited by submicromolar concentrations of Ca$^{2+}$ (31), although higher concentrations were not explored. Recently, a chimeric, soluble form of AC5, comprising its fused cytosolic (C1 and C2) domains was reported to display high affinity inhibition by Ca$^{2+}$, which was lost upon truncation of the C1 region (32). Furthermore, AC2, which is classified as a Ca$^{2+}$-insensitive adenylyl cyclase, displays inhibition in response to high concentrations of Ca$^{2+}$. It therefore seems essential to clearly define the effective concentration ranges for Ca$^{2+}$ inhibition of individual adenylyl cyclases.

Consequently, in the present study, we compared the effects of a comprehensive range of Ca$^{2+}$ concentrations on adenylyl cyclase activity from a variety of sources. Adenylyl cyclase activity in membranes of Sf9 cells expressing AC1, AC2, and AC6 was compared with adenylyl cyclase activity of two selected brain tissues that express respectively AC5 (the striatum; Refs. 33 and 34) and AC1 (the cerebellum; Ref. 35). Also, the full-length and truncated forms of the AC5 C1/C2 chimera were assessed. Unequivocal evidence is provided demonstrating that, with a high affinity, Ca$^{2+}$ inhibits AC6 (and striatal AC5), stimulates AC1 (and cerebellar AC1), and does not modulate AC2. In the supramicromolar concentration range, all adenylyl cyclases tested showed the same low affinity inhibition by Ca$^{2+}$. Further analyses reveal that both the selective high affinity inhibition occurring in AC6 (and AC5) and the low affinity inhibition occurring in all adenylyl cyclase isoforms involve competitive mechanisms with Mg$^{2+}$ activation of the enzyme. As for the two soluble forms of AC5, our results show that both chimeras display only low affinity inhibition and that the site of high (and low) affinity inhibition must be sought by other experimental approaches.

**EXPERIMENTAL PROCEDURES**

**Materials**—Forskolin was from Calbiochem. [32P]$\alpha$-P$\gamma$ATP and [3H]$\mu$AMP were from Amersham Pharmacia Biotech, and other reagents were from Sigma.

**Expression of AC1, AC2, and AC6 in Sf9 Cells**—Recombinant baculoviruses encoding AC1 and AC2 were generously provided by Drs. A. G. Gilman (University of Texas Southwestern Medical Center, Dallas) and R. Yphantis (Mt. Sinai Medical School, New York), respectively (36, 37). For construction of recombinant baculovirus encoding AC6, the entire coding region of mouse AC6 was engineered by polymerase chain reaction into the baculovirus vector, pBlueBacHis2, between KpnI and SalI restriction sites. The culture of Sf9 cells and the production, cloning, and amplification of recombinant baculovirus were performed according to the method of Summers and Smith (38). Sf9 cells were usually infected with 1 plaque-forming unit/cell of baculovirus and were harvested 48 h after infection.

**Preparation of Membranes from Sf9 Cells**—Membranes were prepared from Sf9 cells expressing individual isoforms of adenylyl cyclase, as described previously (39). The cell suspensions were centrifuged, washed with Phillips’s buffer containing protease inhibitors (20 $\mu$g/ml soybean trypsin inhibitor, 4 $\mu$g/ml leupeptin, 12 units/ml kallikrein inactivator, 4 $\mu$g/ml antipain, 52.4 $\mu$g/ml benzamidine, 52.3 $\mu$g/ml phenylmethylsulfonyl fluoride, and 2 $\mu$g/ml pepstatin A). Following centrifugation and subsequent lysis of cells in hypo-osmotic buffer containing protease inhibitors, samples were sheared mechanically by homogenization with a Wheaton Dounce homogenizer with 25 strokes and passage through a 22-gauge needle 10 times. The lysates were centrifuged at 270 $\times$ g for 10 min. The crude membranes were prepared by pelleting this supernatant at 20,000 $\times$ g for 30 min and were washed with 0.3 M of CaCl$_2$ (as determined by the method of Lowry et al. (40) using bovine serum albumin). The preparation was stored in liquid nitrogen.

**Preparation of Soluble Forms of AC5**—The plasmids encoding the soluble forms of AC5 comprising either the C1 or C1a domain linked to the C2 domain (VC1aC2 and VC1aC2, respectively) were kindly provided by Dr. T. B. Patel (University of Kentucky, Lexington; Ref. 32). The proteins were expressed in the Escherichia coli strain TP2000, which is incapable of producing CAMP (42). The expression of VC1aC2 and VC1aC2, cell lysis and assays were performed as described previously (32).

**Preparation of Mouse Cerebellum and Striatal Membranes**—Male mice of the C57Bl/6 strain were decapitated. The brain was removed and placed on dry ice. The cerebellum was detached and homogenized immediately. A transverse section of the forebrain was made at the level of the optic chiasma, and the dorsal striatum was dissected based on the method described by Glowinski and Iversen (43). The tissues were homogenized and centrifuged at 1000 $\times$ g for 10 min in 20 volumes of a cold Tris-sucrose (50 mm, 10%) buffer, pH 7.4, containing 0.8 mm EGTA and a mixture of protease inhibitors as described above. The supernatant was centrifuged at 15,000 $\times$ g for 10 min, and the resulting pellet was washed and centrifuged three times before final suspension in a volume of Tris buffer (50 mm) sufficient to give a concentration of proteins in the range of 1 mg/ml as determined by the method of Lowry (44). Aliquots were immediately frozen in liquid nitrogen until use. Before use for the adenylyl cyclase assay, the samples were further washed and centrifuged two times in 2 ml of a Heps buffer (50 mm), pH 7.4, containing 240 mm EGTA and 0.25% bovine serum albumin.

**Adenylyl Cyclase Activity Measurements**—The adenylyl cyclase activity of infected Sf9 cells or mouse brain tissues (cerebellum and striatum) was measured in the presence of the following components: 12 mmophosphate, 2.5 units of creatine phosphokinase, 0.1 mm cAMP, 1 mm MgCl$_2$, 0.1 mm ATP, 0.04 mm GTP, 0.5 mm isobutylmethylxanthine, 70 mm HEPES buffer, pH 7.4, and 1 $\mu$Ci of [32P]$\alpha$-P$\gamma$ATP. (MgCl$_2$ and ATP concentrations were varied in some experiments, as indicated). Because we were inhibiting adenylyl cyclase activity to low levels (at high [Ca$^{2+}$], particularly at low [Mg$^{2+}$]), maximizing these values was desirable to generate robust kinetic parameters; consequently, basal activity was enhanced with forskolin for all sources except cerebellum and striatum, which displayed elevated basal activity. Forskolin stimulation was employed in some experiments from these latter sources and yielded unchanged parameters (not shown). Calmodulin (1 $\mu$g) was included for the assay of cells expressing AC1 and cerebellar membranes. Free Ca$^{2+}$ concentrations were established from a series of CaCl$_2$ solutions buffered with 60 mm EGTA in the assay and were calculated as described previously in detail (44). The reaction mixture (final volume, 100 $\mu$l) was incubated for 5 min at room temperature. Reactions were terminated with sodium lauryl sulfate (0.5%) containing 1.5 mm cAMP and 22 mm ATP, [3H]$\mu$AMP (~10,000 cpm) was added as a recovery marker, and the [32P]cAMP formed was quantified as described previously (45). Data points are presented as mean activities of triplicate determinations (error bars are indicated when they are larger than the data point symbols in Figs. 1–5). For each assay, at least two adenyl cyclase activities were compared, and a cerebellum Ca$^{2+}$ dose-response curve was determined as an internal control to monitor the accuracy of the Ca$^{2+}$ buffering system.

**Assays for Effect of Ca$^{2+}$ on Mg$^{2+}$ or MgATP$^2$ Requirement**—These assays were conducted as described above except that concentrations of Mg$^{2+}$ or ATP varied. Free Mg$^{2+}$ concentrations were established from a series of MgCl$_2$ solutions buffered with 60 mm EGTA and were calculated as described previously (44). In order to investigate the respective effects of occupancy of high and low affinity regulatory sites for Ca$^{2+}$, two selected concentrations of Ca$^{2+}$ (2.75 and 86 mm) were included in these assays. After calculation, the concentrations of free Ca$^{2+}$ actually ranged from 2.59 to 3.43 mm and from 81.6 to 90.1 mm, respectively, in the presence of the lowest and the highest concentration of Mg$^{2+}$ or ATP.

**Statistics**—Nonlinear regression was used to fit a competition curve with either one or two components to the data (Graphpad, Inplot4). Goodness of fit was quantified by the least-squares method (F-test) and was deemed better suited to one model when $p < 0.05$.

Pairwise Student’s $t$ tests (two-tailed) were performed to compare the means of $K_a$ or $K_i$ values determined with different concentrations of Ca$^{2+}$.

**RESULTS**

**Species-Specific Effects of Ca$^{2+}$ on Adenylyl Cyclase Activity**—The effects of a broad range of Ca$^{2+}$ concentrations on adenylyl cyclase activity were compared in membranes from Sf9 cells expressing AC6 and striatum (which predominantly expresses AC6; Ref. 33). Clearly, Ca$^{2+}$ elicits a biphasic decline in activity with increasing concentration (Fig. 1). In both cases, curve-fitting analyses revealed that the inhibition curves always fitted significantly better to a two-component model ($p < 0.05$,
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Fig. 1. Effects of Ca\textsuperscript{2+} on adenylyl cyclase activity in membranes of cells expressing AC6, AC2, AC1, or soluble forms of AC5 and striatal or cerebellum membranes. Membranes expressing various adenylyl cyclases were assayed in the presence of the indicated free Ca\textsuperscript{2+} concentrations, as described under "Experimental Procedures." Forskolin was included in the assay as follows: 100 \mu m for AC6 and soluble forms of AC5 and 10 \mu m for AC2 and AC1. No forskolin was included in assays of either striatal or cerebellar membranes. Each panel shows a representative experiment that was repeated at least three times with similar results. Data are mean adenylyl cyclase activity (\pm S.E.) of triplicate determinations.

Effects of Ca\textsuperscript{2+} on Mg\textsuperscript{2+} Activation of Adenylyl Cyclase—For a considerable period, it has been believed that low affinity inhibition of adenylyl cyclase activity by Ca\textsuperscript{2+} reflected competition for a Mg\textsuperscript{2+} activation site (11, 16–18, 20–22). However, this issue has not been addressed in the light of current information on adenylyl cyclase diversity. Consequently, the effects of occupancy of high affinity and low affinity Ca\textsuperscript{2+} inhibition sites on the activation by Mg\textsuperscript{2+} of various adenylyl cyclases were investigated. The effects of two concentrations of free Ca\textsuperscript{2+}, corresponding to the plateau region of the first inhibitory phase (2.75 \mu m; cf. Fig. 1) and within the second phase (86 \mu m; cf. Fig. 1) of the Ca\textsuperscript{2+} inhibition curves, on the activation by Mg\textsuperscript{2+} of AC1, AC2, AC6, and striatal adenylyl cyclase were determined. Concentration-activity curves were generated using varying concentrations of Mg\textsuperscript{2+} (Fig. 2). The results showed that Mg\textsuperscript{2+} increased adenylyl cyclase activity concentration-dependently, as expected. The two selected concentrations of Ca\textsuperscript{2+} inhibited the Mg\textsuperscript{2+} stimulation in membranes expressing AC6. Similar effects were observed in striatal membranes. In contrast, the stimulation by Mg\textsuperscript{2+} was inhibited only by the high Ca\textsuperscript{2+} concentration (86 \mu m) in membranes expressing AC2. Similar assays were also performed with AC1 membranes. Only the effect of the high concentration of Ca\textsuperscript{2+} was investigated (in the absence of calmodulin), since at low concentrations AC1 would be stimulated by Ca\textsuperscript{2+}. Indeed, in this condition, Ca\textsuperscript{2+} also reduced the activation produced by Mg\textsuperscript{2+} in AC1. Reciprocal plots of velocity versus Mg\textsuperscript{2+} concentration are shown in Fig. 3. The linear regression analyses performed on these data indicate that, in AC6 and striatal membranes, both 2.75 and 86 \mu m Ca\textsuperscript{2+} inhibited Mg\textsuperscript{2+} activation competitively; i.e. the intercept (1/V\textsubscript{max}) was unchanged by Ca\textsuperscript{2+}, whereas the slope (K\textsubscript{c}/V\textsubscript{max}) increased (Fig. 3, Table II). The inhibition detected in AC2 was also competitive at the high concentration of Ca\textsuperscript{2+} (Fig. 3, Table II). Finally, Ca\textsuperscript{2+} also demonstrated competitive inhibition at 86 \mu m Ca\textsuperscript{2+} in AC1 membranes. The mean K\textsubscript{c} for Mg\textsuperscript{2+} was about 1 \mu m in all of the adenylyl cyclases examined in the absence of Ca\textsuperscript{2+} (Table II). However, in the presence of 2.75 \mu m Ca\textsuperscript{2+}, this value was significantly increased (by about 2-fold), with Ca\textsuperscript{2+}-inhibitable adenylyl cyclases. This increase reached 6–8-fold and occurred in all adenylyl cyclase isofoms when 86 \mu m Ca\textsuperscript{2+} was included in the assays (Table II).

Effects of Ca\textsuperscript{2+} on Mg\textsuperscript{2+} Requirement in AC6, AC2, AC1, or Striatum—Since Mg\textsuperscript{2+} not only activates adenylyl cyclase, but also participates in the formation of the cyclase substrate, MgATP\textsuperscript{2-}, we investigated the effects of inhibition by Ca\textsuperscript{2+} (3.41 and 89 \mu m) on the relationship between activity and substrate (MgATP\textsuperscript{2-}) concentration. In these experiments, the concentration of free Mg\textsuperscript{2+} was held constant (10 \mu m) so that it could be determined whether the antagonistic effect of Ca\textsuperscript{2+} on Mg\textsuperscript{2+} activation reported in Fig. 2 (and Table I) might reflect some effect on the participation of Mg\textsuperscript{2+} in the substrate

| TABLE I | High and low affinity effects of calcium |
|---------|---------------------------------------|
|          | High affinity | Low affinity |
| AC6 in SF9 cells (n = 6) | 0.148 ± 0.039 | 0.060 ± 0.005 |
| AC2 in SF9 cells (n = 9)  | 0.066 ± 0.013 | 0.032 ± 0.004 |
| AC1 in SF9 cells (n = 7)  | 0.122 ± 0.021 | 0.058 ± 0.002 |
| Striatum (n = 3)           | 0.079 ± 0.030 | 0.054 ± 0.010 |
| Cerebellum (n = 20)        | 0.094 ± 0.012 | 0.055 ± 0.010 |
| VC1C2 (n = 7)              | None           | 0.081 ± 0.017 |
| VC1aC2 (n = 6)             | None           | 0.097 ± 0.017 |

Values are mean ± S.E. of effective concentrations of calcium giving 50% of the response for the high affinity and the low affinity sites, respectively, as determined from curve-fitting analyses of experiments analogous to those depicted in Fig. 1. The number of determinations performed on each adenylyl cyclase is indicated (n).
formation. The concentration-response curves for MgATP stimulation of adenyl cyclase activity were determined in membranes expressing AC6, AC2, and AC1, respectively, and in striatum membranes in the absence of Ca$^{2+}$ (●) and in the presence of 2.75 μM Ca$^{2+}$ (□) or 86 μM Ca$^{2+}$ (△). Forskolin was included in the assay as follows: 25 μM for AC6 and 10 μM for AC2 and AC1. No forskolin was added in striatal assays. Data are means ± S.E. of triplicate determinations from an experiment that was repeated twice (striatum) or three times (AC6, AC2, and AC1) with similar results.

**Fig. 2.** Effects of Ca$^{2+}$ on the stimulation of Mg$^{2+}$ of adenyl cyclase activity. Adenyl cyclase activity was determined in membranes expressing AC6, AC2, and AC1, respectively, and in striatum membranes in the absence of Ca$^{2+}$ (●) and in the presence of 2.75 μM Ca$^{2+}$ (□) or 86 μM Ca$^{2+}$ (△). Forskolin was included in the assay as follows: 25 μM for AC6 and 10 μM for AC2 and AC1. No forskolin was added in striatal assays. Data are means ± S.E. of triplicate determinations from an experiment that was repeated twice (striatum) or three times (AC6, AC2, and AC1) with similar results.

**Fig. 3.** Double reciprocal plots of effects of Ca$^{2+}$ on Mg$^{2+}$ stimulation of adenyl cyclase activity. Plots were made from the data presented in Fig. 2 and analyzed by linear regression analysis. Free Ca$^{2+}$ concentrations in the assay were as follows: 0 (●), 2.75 μM (□), and 86 μM (△).

**TABLE II**

Kinetic parameters for Mg$^{2+}$ and MgATP as a function of Ca$^{2+}$ concentrations

Values are means ± S.E. of $K_a$ and $K_m$ values extracted from linear regression analyses performed on the double reciprocal plots of Mg$^{2+}$ and MgATP$^{2-}$ stimulation of adenyl cyclase activity. The number of assays performed for each adenyl cyclase is indicated (n).

|            | Mg$^{2+}$ ($K_a$) | MgATP ($K_m$) |
|------------|-------------------|---------------|
|            | 0 μM Ca$^{2+}$    | 2.75 μM Ca$^{2+}$ | 86 μM Ca$^{2+}$ | 0 μM Ca$^{2+}$ | 3.41 μM Ca$^{2+}$ | 89 μM Ca$^{2+}$ |
| AC6 (n = 3) | 1.02 ± 0.12       | 1.77 ± 0.36   | 8.68 ± 1.23   | 68.30 ± 3.35   | 69.69 ± 4.59     | 46.27 ± 10.34    |
| Striatum (n = 2) | 0.91 ± 0.21       | 2.09 ± 0.61   | 8.11 ± 4.70   | 46.16 ± 2.72   | 43.92 ± 1.07     | 34.73 ± 0.25     |
| AC2 (n = 3)  | 1.02 ± 0.25       | 1.07 ± 0.28   | 6.92 ± 1.52   | 77.26 ± 2.83   | 76.37 ± 1.02     | 45.11 ± 6.45     |
| AC1 (n = 3)  | 1.32 ± 0.06       | ND            | 6.10 ± 0.76   | 383.7 ± 26.3   | ND                | 241.0 ± 20.1     |

* A significant difference ($p < 0.05$) with respect to control (no added Ca$^{2+}$) as determined by a paired Student's t test (two tails).
89 μM Ca²⁺, although the effect on \( V_{\text{max}} \) was most prominent.

Overall, these analyses are consistent with the predominant effect of Ca²⁺ being to antagonize stimulation by Mg²⁺, along with a minor noncompetitive effect on substrate utilization.

**DISCUSSION**

The stimulation of adenylyl cyclases AC1 and AC8 by Ca²⁺ is understood to occur in the submicromolar concentration range and to be mediated by calmodulin (9, 46–48). In addition, the calmodulin binding sites are known for both AC1 and AC8 (49, 50). By contrast, even the concentration ranges over which Ca²⁺ inhibits adenylyl cyclases are not unambiguously understood; even less so is the mechanism of inhibition. There have been a variety of studies reporting monophasic inhibition (11–13, 21, 23) as well as some showing evidence for two distinct binding sites of low and high affinity for Ca²⁺, giving rise to biphasic inhibition (14–19). When AC5 and AC6 were identified, they were characterized as being Ca²⁺-inhibited adenylyl cyclase isoforms (25, 31). When expressed in intact cells, these cyclases are inhibited by capacitative Ca²⁺ entry (51). The mRNAs of these species are also expressed in tissues, such as the heart, in which the adenylyl cyclase activity is inhibited by Ca²⁺ (52, 53). To resolve the confusion that attends Ca²⁺ inhibition of adenylyl cyclase, examination of material expressing a single isoform would seem to be a rational procedure to understand the Ca²⁺ regulation of adenylyl cyclase. Consequently, in the present study we first compared the regulation of a presumably Ca²⁺-stimulable and a Ca²⁺-inhibitable adenylyl cyclase expressed in SF9 cells with tissues that predominantly express these activities in the mouse brain. It was gratifying to note that the response to Ca²⁺ of SF9 cell membranes expressing AC1 and AC6 was virtually identical to the response of cerebellar and striatal membranes, respectively, which are a major source of the analogous adenylyl cyclase mRNAs. Such findings indicate that no co-factor or post-translational modification is lacking between moths and mice to alter adenylyl cyclase responsiveness. Consequently, it seems reasonable to characterize further the Ca²⁺-regulation in membranes from either source.

The present studies clearly indicate that Ca²⁺ inhibits AC6 (and AC5) over precisely the same concentration range as it stimulates AC1. Specifically, our results demonstrate clearly that, in both adenylyl cyclases, the effects of Ca²⁺ progress in a biphasic manner, strongly suggesting interactions with two distinct regulatory binding sites of low and high affinity. Inter-
action with sites of high affinity for Ca\(^{2+}\) (\(-0.15 \, \mu M\)) confer opposite regulation in AC6 (inhibition) and AC1 (stimulation), whereas interaction with sites of low affinity (\(-0.06 \, \text{mM}\)) inhibits both isoforms. The effects of Ca\(^{2+}\) on striatal adenyl cyclase activity were identical to those detected in AC6. Previous reports had found that the basal adenyl cyclase activity in the striatum was higher but less stimulated by Ca\(^{2+}\), or insensitive to Ca\(^{2+}\), as compared with other brain areas, in the presence of calmodulin (54). More recently, the adenyl cyclase activity of the rat striatum was shown to exhibit a daily oscillation with a peak occurring around 10:00–12:00 a.m., during which Ca\(^{2+}\) inhibition was selectively manifest (55). Accordingly, we dissected striatum during this critical phase, and in agreement with the latter findings, our data showed that Ca\(^{2+}\) potently inhibits the striatal adenyl cyclase activity. Finally, comparisons with AC2 demonstrated that, in sharp contrast, this adenyl cyclase was insensitive to low concentrations of Ca\(^{2+}\). Nevertheless, like the other adenyl cyclases examined here, AC2 was inhibited when Ca\(^{2+}\) reached submillimolar levels. Together, these findings demonstrate that the various isoforms of adenyl cyclases behave differently when Ca\(^{2+}\) interacts at high affinity binding sites, whereas they respond similarly when Ca\(^{2+}\) binds low affinity sites.

Next, we compared the mechanisms that yielded high affinity inhibition in AC6 and striatal adenyl cyclase, compared with the low affinity inhibition that is common to all adenyl cyclases. Previous studies of adenyl cyclase activity in various tissues had proposed that high concentrations of Ca\(^{2+}\) reduced activity depending on the substrate concentration in a noncompetitive manner (11, 12, 16, 17, 21, 56). When competition between Ca\(^{2+}\) and Mg\(^{2+}\) was considered, more contradictions were encountered, even between studies carried out on the same tissue. Specifically, some studies showed that Ca\(^{2+}\) inhibition of adenyl cyclase occurred noncompetitively with Mg\(^{2+}\) (11, 19, 23), while others found that high concentrations of Ca\(^{2+}\) compete with Mg\(^{2+}\), whereas lower concentrations do not (56). In other studies, it was proposed that Ca\(^{2+}\) inhibits adenyl cyclase by competing with Mg\(^{2+}\) and suggested that a common metal site bind both ions (16, 17, 20–22). Here, we show that high affinity inhibition by Ca\(^{2+}\) of either AC6 or striatal adenyl cyclase does not involve competition with the MgATP\(^{2-}\) substrate. Instead, high affinity inhibition reflects competition by low concentrations of Ca\(^{2+}\) for the Mg\(^{2+}\) activation in both AC6 and striatal adenyl cyclase, so that the affinity for Mg\(^{2+}\) is reduced selectively in these isoforms. On the other hand, low affinity inhibition produced by Ca\(^{2+}\) in AC6, AC1, and AC2, as well as in the striatal adenyl cyclase, also stems largely from a competitive action on the Mg\(^{2+}\) activation of the enzyme, with a minor effect on substrate utilization.

Recent deletion analyses have suggested the existence of a Mg\(^{2+}\) binding site essential for catalysis and distinct from the ATP-bound cation (57). Along with insight gained from crystallographic studies (58), the latter findings led these authors to conclude that a conserved aspartate residue (corresponding to Asp-396 in the C1a region of canine AC5) plays a critical role in coordinating catalytic Mg\(^{2+}\) ions. Specifically, this Mg\(^{2+}\) was predicted to facilitate the nucleophilic attack of the 3’-hydroxyl group of ATP on the α-phosphate. It is conceivable that Ca\(^{2+}\) competes with Mg\(^{2+}\) at this site, which is largely conserved among all adenyl cyclases, to yield low affinity inhibition. By contrast, the site of high affinity inhibition by Ca\(^{2+}\) of AC5 and AC6 would be expected to be mediated by a region unique to AC5 and AC6.

In the latter context, Scholich et al. (32) reported recently that Ca\(^{2+}\) inhibited a soluble form of AC5, composed of the C1 and C2 regions of the enzyme, but not a shorter form lacking the unconserved C1b domain. These findings were taken to suggest that the 112-amino acid C1b region in AC5 mediated high affinity Ca\(^{2+}\) inhibition of the enzyme activity. In agreement with this study, we also found that Ca\(^{2+}\) would inhibit the C1C2 protein. However, since we were conducting our experiments in parallel with Ca\(^{2+}\)-stimulated cerebellar adenyl cyclase, as an internal control of estimated Ca\(^{2+}\) concentrations, it was clear that the inhibition occurred with submillimolar concentrations of Ca\(^{2+}\), not with submicromolar concentrations. Furthermore, in our experiments, the truncated form of C1C2, namely the C1aC2 construct, displayed the same low affinity inhibition by Ca\(^{2+}\). Based on these results, it seems unlikely that the Ca\(^{2+}\) site responsible for high affinity inhibition resides within the cytosolic domains of AC5. However, it is possible that the soluble chimera expressed in *Escherichia coli* does not adopt the structural configuration of the native ACV. Alternatively, the absence of other domains might explain the absence of high affinity inhibition in the VC1C2 protein. In any case, it is reasonable to conclude that further studies are needed to clarify the structural features conferring Ca\(^{2+}\) inhibition on adenyl cyclases.

The present study has clearly separated the high and low affinity effects of Ca\(^{2+}\). With a high affinity, Ca\(^{2+}\) stimulates AC1 and cerebellar membrane adenyl cyclase in a manner that requires dissociable calmodulin. Again, with a high affinity, Ca\(^{2+}\) inhibits AC6 expressed in S9 cells and striatal adenyl cyclase independently of added calmodulin. These low concentrations of Ca\(^{2+}\) do not affect AC2 expressed in S9 cells. With a low affinity, Ca\(^{2+}\) inhibits all adenyl cyclases, including AC1, AC2, AC6, striatal AC5, and soluble forms of AC5, intact or with the C1b region deleted. Kinetically, the mechanism of both high and low inhibition by Ca\(^{2+}\) appears similar and appears to involve competitive inhibition for a stimulatory Mg\(^{2+}\) site(s). The inhibition does not involve competitive mechanisms for the substrate MgATP\(^{2-}\). A K\(_i\) value of \(-0.2 \, \mu M\) for the high affinity inhibitory site, in the face of a 5000-fold excess of Mg\(^{2+}\), suggests a remarkable selectivity for Ca\(^{2+}\). This selectivity for Ca\(^{2+}\) over Mg\(^{2+}\) is analogous with the specificity of E-F-hand-containing proteins for Ca\(^{2+}\) (59). However, the nature or location of this site remains elusive. No E-F hand or other obvious Ca\(^{2+}\)-binding motif is present in AC5 or AC6 (31). In addition, earlier biochemical studies appeared to eliminate the participation of calmodulin in the inhibition; for instance, neither calmodulin antagonists nor EGTA washing modulated the effect (60), although this does not exclude the possibility that a tightly bound calmodulin is involved (as with phosphorylase kinase). On the other hand, the low affinity site appears to represent a more conventional competition between Ca\(^{2+}\) and Mg\(^{2+}\), which, as with a number of metabolic enzymes, shows an approximately 10-fold difference in effective concentrations of the two cations (62, 63). Given that averaged, cytosolic concentrations of Ca\(^{2+}\) rarely reach 50 \(\mu M\), except transiently around the mouths of ion channels, it is difficult to imagine that this inhibition is of any physiological utility. On the other hand, it is important to take pains to distinguish this inhibition from high affinity inhibition, or quite spurious inferences may be drawn on the nature or location of high affinity binding sites.

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