Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) Identify Distinct Regulatory Sites on Adenylyl Cyclase (AC) Types VI and VIII and Consolidate the Apposition of Capacitative Cation Entry Channels and Ca\(^{2+}\)-sensitive ACs*

(Received for publication, September 17, 1999, and in revised form, October 10, 1999)

Chen Gu‡ and Dermot M. F. Cooper‡§¶§

From the ‡Neuroscience Program and §Department of Pharmacology, University of Colorado Health Sciences Center, Denver, Colorado 80262

Ca\(^{2+}\)-sensitive adenylyl cyclases may act as early integrators of the two major second messenger-signaling pathways mediated by Ca\(^{2+}\) and cAMP. Ca\(^{2+}\) stimulation of adenylyl cyclase type I (ACI) and adenylyl cyclase type VIII (ACVIII) is mediated by calmodulin and the site on these adenylyl cyclases that interacts with calmodulin has been defined. By contrast, the mechanism whereby Ca\(^{2+}\) inhibits adenylyl cyclase type V (ACV) and adenylyl cyclase type VI (ACVI) is unknown. In this study, Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) were compared to probe the involvement of E-F hand-like domains in both Ca\(^{2+}\)-stimulated and inhibition of ACVIII and ACVI, respectively. HEK 293 cells transfected with ACVIII cDNA and C6–2B glioma cells (where the endogenous adenylyl cyclases is predominantly ACVI) were used to compare the effects of these three cations in in vitro and in vivo measurements. The in vitro data identified two Ca\(^{2+}\)-regulated sites for both ACVIII and ACVI. Strikingly different potency series for these cations at mediating high affinity stimulation and inhibition of ACVIII and ACVI, respectively, effectively rule out the possibility that calmodulin or proteins utilizing similar Ca\(^{2+}\)-binding motifs mediate inhibition of ACVI. On the other hand, the low affinity inhibition that is common to both ACVIII and ACVI showed virtually identical potency profiles for the IIa cation series, indicating a common site of action. Remarkably, whereas Sr\(^{2+}\) was rather ineffective at regulating these cyclases (particularly ACVIII) in vitro, adequate concentrations accumulated in the vicinity of these enzymes as a consequence of capacitative cation entry to partially regulate both of these activities in vivo. This latter finding consolidates earlier observations that Ca\(^{2+}\)-sensitive adenylyl cyclases detect and respond to capacitative cation entry rather than global cytosolic cation concentrations.

Nine distinct isoforms of mammalian adenylyl cyclases have been cloned and characterized (1, 2). More than half of these adenylyl cyclases can be regulated by Ca\(^{2+}\), resulting in either inhibition or stimulation of activity. ACI\(^{1}\) and ACVIII are stimulated by Ca\(^{2+}\), acting through loosely bound calmodulin via identified domains on the enzymes (3–7). By contrast, the molecular mechanism of Ca\(^{2+}\) inhibition of ACV and ACVI is unknown (2). Although Ca\(^{2+}\) inhibits ACV and ACVI over the same (submicromolar) concentration range as it stimulates ACI and ACVIII, it is not known whether the inhibition is mediated directly on the enzymes or via calmodulin or another Ca\(^{2+}\)-binding protein. Examples are available in the literature: for instance phosphorylase kinase, where non-dissociable calmodulin mediates the effect of Ca\(^{2+}\) on enzyme activity (8). In addition, an array of Ca\(^{2+}\)-binding proteins have been described that could potentially play roles in the inhibitory regulation of adenylyl cyclase (2). Of course, it is also possible that Ca\(^{2+}\) binds directly to adenylyl cyclase to regulate its activity, as it does to synaptotagmins and phospholipase A\(^{2}\) (9, 10). In this regard, it had been proposed that the inhibition of ACV activity by Ca\(^{2+}\) is through direct Ca\(^{2+}\)-binding to the C1b region of ACV (11), although recent studies have questioned this conclusion (12). The sensitivity of Ca\(^{2+}\)-stimulable and -inhibitable adenylyl cyclases to the IIa series of divalent cations, Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\), could provide a first step in determining whether they are regulated by similar or different mechanisms, since, for instance, E-F hand Ca\(^{2+}\)-binding motifs display characteristic preferences for these cations (13).

Physiologically, the ability of adenylyl cyclases to be regulated by Ca\(^{2+}\) transitions provides a key point for the integration of Ca\(^{2+}\) and cAMP signaling (14). In intact cells, Ca\(^{2+}\)-sensitive adenylyl cyclases, either endogenously or heterologously expressed, require capacitative Ca\(^{2+}\) entry for their regulation, rather than Ca\(^{2+}\) release from intracellular stores or nonspecific, ionophore-mediated Ca\(^{2+}\) entry through the plasma membrane (15–17). Consequently, the regulation of adenylyl cyclases by divalent cation entry in the intact cell may also provide some insight into selective features displayed by particular adenylyl cyclases in their in vivo setting.

The present studies compared the effects of the IIa series of divalent cations, Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\), in in vitro and in vivo assays on the activities of Ca\(^{2+}\)-sensitive adenylyl cyclases (type VI and VIII). In in vitro assays, quite different potency series were observed by low concentrations of calcium and related cations for the stimulation and inhibition, respectively, of ACVIII and ACVI, which strongly suggested that the inhibition of ACVI is not mediated by calmodulin. By contrast, the inhibition of ACVIII and ACVI that is elicited by high concentrations of Ca\(^{2+}\) and related cations displayed similar potency series, which intimated that it is mediated at a conserved site in all adenylyl cyclases. The in vivo experiments provided a different perspective on the cellular aspects of the regulation of these adenylyl cyclases. Whereas Sr\(^{2+}\) had been relatively weak in in vitro assays, in the intact cell, adequate concentrations of Sr\(^{2+}\) could accumulate via capacitative entry mecha-

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* This work was supported by National Institutes of Health Grant GM 32483 (to D. M. F. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Pharmacology, Box C-236, University of Colorado Health Sciences Center, 4200 E. Ninth Ave., Denver, CO 80262. Tel: 303-315-8964; Fax: 303-315-7097; E-mail: dermot.cooper@UCHC.edu.

¶ The abbreviations used are: ACI, adenylyl cyclase, type I; ACVIII, adenylyl cyclase, type VIII; ACVI, adenylyl cyclase, type VI; ACV, adenylyl cyclase, type V.
nisms to permit regulation of high affinity sites. This latter observation reinforces earlier observations that adenyl cyclase isoforms are far more sensitive monitors of capacitative cation entry than are cytosolic indicators (15, 17).}

**EXPERIMENTAL PROCEDURES**

**Materials**—Thapsigargin, forskolin, and Ro 20–1724 were from Calbiochem (San Diego, CA). [2-3H]Adenine, [3H]cAMP, and [α-32P]ATP were obtained from Amersham Pharmacia Biotech. Fura-2/AM and Fluronic F-127 were obtained from Molecular Probes, Inc. (Eugene, OR). Other reagents were from Sigma (St. Louis, MO).

Cell Culture—Transformation of HEK 293 Cells—C6–2B rat glioma cells were maintained in F-10 medium (Life Technologies, Inc.) with 10% (v/v) bovine calf serum (Gibco) in 75-cm² flasks at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Cells were plated at approximately 70% confluence in 24-well plates for cAMP accumulation experiments. HEK 293 cells were maintained in minimal essential medium with 10% (v/v) fetal bovine serum, penicillin (50 μg/ml), streptomycin (50 μg/ml), and neomycin (100 μg/ml) in 75-cm² flasks at 37 °C in a humidified atmosphere of 95% air and 5% CO₂. Wild type ACVIII and an N-terminal deletion form of ACVIII, termed N₁₀₆glyco, were expressed in HEK 293 cells. Transfections were performed on HEK 293 cells at around 50% confluence, using the calcium phosphate method described by Chen and Okayama (18). Seventeen hours after transfection, the cells from 75-cm² flasks were harvested using phosphate-buffered saline containing 0.06% EDTA, plated onto 24-well culture plates, and incubated for 2 days before CAMP measurements were made.

**Cell Fractionation—**C6–2B rat glioma cells and HEK 293 cells transiently transfected with ACVIII cDNA plasmids, were lysed using a method described previously (19). Crude membranes were prepared following mechanical shearing of the cells by passage through a 22-gauge needle, 10 times, in homogenization buffer containing 2 mM MgCl₂, 1 mM EDTA, 50 mM Tris buffer, pH 7.4, 1 mM MgCl₂, 0.1 mM EGTA, and 0.1 mM protease inhibitors as described (20) with the addition of 52.3 μM/ml phenylmethylsulfonyl fluoride, 52.4 μg/ml benzamidine, and 2 μg/ml pepstatin A. The supernatant from a low speed centrifugation (1,000 rpm, 1 min, SS-34 Sorvall) was pelleted (12,000 rpm, 10 min, SS-34 Sorvall, resuspended in assay buffer (40 mM Tris buffer, pH 7.4, 800 μM EGTA and 0.25% bovine serum albumin), and used immediately. A plasma membrane preparation from N₁₀₆glyco-transfected HEK 293 cells was made using sucrose gradients (7).

**Adenylyl Cyclase Activity Measurements—**The adenylyl cyclase activity in C6–2B rat glioma cell membranes and transfected cell membranes was measured in the presence of the following reaction components: 12 mM phosphocreatine, 2.5 units of creatine phosphokinase, 0.1 mM MgCl₂, 0.1 mM ATP, 70 mM Tris buffer, pH 7.4, 0.04 mM GTP, 1 μCi of [α-32P]ATP, 3-isobutyl-1-methylxanthine (500 μM), and 20 μM forskolin. The supernatant from a low speed centrifugation (1,000 rpm, 1 min, SS-34 Sorvall) was pelleted (12,000 rpm, 10 min, SS-34 Sorvall, resuspended in assay buffer (40 mM Tris buffer, pH 7.4, 800 μM EGTA and 0.25% bovine serum albumin), and used immediately. A plasma membrane preparation from N₁₀₆glyco-transfected HEK 293 cells was made using sucrose gradients (7).

**CAMP Accumulation Measurements—**In intact cells, CAMP accumulation was measured by the method described by Evans et al. (23) as described previously (15) with some modifications. The cells on 24-well plates were incubated (60 min at 37 °C) with [2-3H]adenine (1.5 μCi/well) to label the ATP pool. The cells were then washed once and incubated with a nominally Ca²⁺-free Krebs buffer (900 μM/well) containing 120 mM NaCl, 4.75 mM KCl, 1.44 mM MgCl₂, 11 mM glucose, 25 mM HEPES, and 0.1% bovine serum albumin (fraction V) adjusted to pH 7.4 with 2 M Tris base. The use of Ca²⁺-free Krebs buffer in experiments denotes the addition of the 0.1 mM EGTA to the nominally Ca²⁺-free Krebs buffer. All experiments were carried out at 30 °C in the presence of 100 μM theophylline and 100 μM theophylline, the free cation (1 μM) and Ro 20–1724 (100 μM), which were preincubated with the cells for 10 min prior to a 1-min assay. Cells were preincubated for 4 min with the Ca²⁺-ATPase inhibitor, thapsigargin, at a final concentration of 100 nM. This has the effect of passively emptying intracellular Ca²⁺ stores, establishing a low basal [Ca²⁺], and priming the cells for capacitative Ca²⁺ entry (24). Assays were terminated by addition of triethylamine and the percentage of conversion of [3H]ATP to [3H]cAMP was measured as described previously (22). Means ± S.D. of triplicate determination are indicated. The data were fitted to four-parameter sigmoid curves with the program SigmaPlot 4.0, as with the in vitro adenylyl cyclase curves described above.

**RESULTS**

**Distinct and Common Ca²⁺ Regulatory Sites of ACVIII and ACVII—**The Ca²⁺ stimulation of ACVIII is mediated through loosely bound calmodulin. Therefore, the effects of Ca²⁺, Sr²⁺, and Ba²⁺ at stimulating the activity of ACVIII in vitro would be expected to mimic the binding of these cations by calmodulin. The effects of these cations at stimulating adenylyl cyclase activity in plasma membranes from HEK 293 cells transiently transfected with ACVIII cDNA is examined in the experiment shown in Fig. 1A. The activity of ACVIII was progressively stimulated by increasing (submicromolar) concentrations of free Ca²⁺ (Fig. 1A). Stimulation reached a plateau at 1 μM free Ca²⁺ (Fig. 1A). Sr²⁺ also stimulated ACVIII activity to a similar extent as Ca²⁺, although a free Sr²⁺ concentration of 30 μM was required (Fig. 1A). Stimulation was also elicited by Ba²⁺, although the effects were not evident until the free cation concentration exceeded 100 μM (Fig. 1A). The concentration response curves for all three cations were similar in shape, but were separated by about 40-fold concentration differences (Fig. 1A). The selectivity of ACVIII for these cations (Ca²⁺ > Sr²⁺ > Ba²⁺) agrees with the binding affinities of these cations to calmodulin and some other (E-F hand-containing) Ca²⁺-binding proteins (Table I).

The stimulation of ACVIII activity was followed by an inhibitory phase upon further increasing the free cation concentration (Fig. 1A). The EC₅₀ values for Ca²⁺ and Sr²⁺ are around 100 μM and 1.8 mM, respectively. Ba²⁺ is ineffective. This inhibitory phase is referred to as low affinity inhibition, which is a feature of all of the mammalian adenylyl cyclases, whether they be Ca²⁺-stimulable, Ca²⁺-inhibitable, or Ca²⁺-insensitive in vitro (2).

In order to circumvent the possibility that the high affinity stimulation by Ca²⁺, Sr²⁺, and Ba²⁺ was obscuring or distorting the values for these cations at mediating low affinity inhibition of ACVIII, we exploited a mutation in ACVIII (N₁₀₆glyco) that we described earlier (7). Even in the absence of added calmodulin, ACVIII binds calmodulin tightly via its N terminus, with the result that upon the addition of Ca²⁺, a stimulation of activity is observed. Deletion of amino acids 1–106
ACVIII are the results of fitting the data, using a two-site competition model. Values shown are from an experiment that was repeated at least three times with similar results.

However, it is not known whether the Ca2+ effect evoked by Sr2+—the effects of Ca2+, Sr2+, and Ba2+ on ACVIII and ACVI activities by capacitative entry were compared (Fig. 3). HEK 293 cells transfected with ACVIII cDNA were pretreated with 100 nM thapsigargin to deplete the intracellular Ca2+ stores and prime the cells for capacitative Ca2+ entry, which selectively regulates the activities of Ca2+-sensitive adenylyl cyclases (16, 17). Following thapsigargin pretreatment, adenylyl cyclase activity was measured in response to the inclusion of increasing concentrations of CaCl2, SrCl2, or BaCl2 as indicated. The activity of ACVIII was stimulated by submillimolar external Ca2+ and full stimulation (more than 3-fold) was achieved by 4 mM CaCl2 (Fig. 3). Sr2+ also stimulated HEK 293 cells expressing ACVIII, like Ca2+, except that the plateau for Sr2+ was only approximately one-half of that achieved by Ca2+ (Fig. 3). Ba2+ exerted a modest effect on ACVIII activity with 8 mM external Ba2+ stimulating adenylyl cyclase activity by about 20% (Fig. 3). The inability to exceed the plateau in activity in response to increasing extracellular SrCl2 suggests a ceiling on entry, since the in vitro data indicate that both Ca2+ and Sr2+ can elicit the same maximal stimulation of ACVIII activity.

The effects of Ca2+, Sr2+, and Ba2+ on ACVIII activity through capacitative entry were next compared in C6–2B glioma cells. The experiments were performed on intact C6–2B glioma cells under conditions comparable to those used for HEK 293 cells transfected with ACVIII. Significant inhibition (approximately 20%) of ACVI activity was observed in the presence of 0.5 mM thapsigargin (Fig. 4). The maximal inhibition of ACVI activity by Ca2+ was approximately 45% when the Ca2+ concentration reached 4 mM (Fig. 4). Submillimolar Sr2+ also inhibited ACVI activity (approximately 10% inhibition; Fig. 4). The maximal effect elicited by Sr2+ was approximately 25% inhibition, which was achieved in a similar external concentration range as that of Ca2+ (Fig. 4). Again the fact that Sr2+ could not elicit the same degree of inhibition of ACVI as did Ca2+, suggests a ceiling on Sr2+ entry. No significant effect of Ba2+ on ACVI activity was detected (Fig. 4).

Given that the potency of Sr2+ to regulate ACVIII and ACVI is much less than that of Ca2+ (cf. Fig. 1 and 2), it was quite
To explore the issue of the relative entry of Sr\(^{2+}\) C6–2B glioma cells as in the adenylyl cyclase assays described above. In the absence of extracellular Ca\(^{2+}\), 100 mM thapsigargin induced a small peak (approximately 60 nm), which reflects Ca\(^{2+}\) release from internal Ca\(^{2+}\) stores and extrusion (Fig. 5, A and B). Four minutes later (at 300 s) 2 mM of Ca\(^{2+}\), Sr\(^{2+}\), or Ba\(^{2+}\) were added externally. In HEK 293 cells, capacitative entry of Ca\(^{2+}\) and Sr\(^{2+}\) peaked at near 0.2 and 0.6 mM, respectively, within 60 s and continued to increase with time (Fig. 5A). However, the intracellular Ba\(^{2+}\) concentration reached 0.5 mM within 60 s and continued to increase with time (Fig. 5A). A similar situation arose in C6–2B glioma cells (Fig. 5B), although capacitative entry of Ca\(^{2+}\) and Sr\(^{2+}\) (0.4 and 2 mM, respectively) was more substantial than in HEK 293 cells (Fig. 5B). Indeed, when a detailed comparison is made of Ca\(^{2+}\) and Sr\(^{2+}\) entry as a function of external cation concentration between HEK 293 cells and C6–2B glioma cells, it is clear that much higher levels of Sr\(^{2+}\) are achieved in C6–2B cells (Fig. 5C). These data go some way to accounting for the ability of Sr\(^{2+}\) to regulate cAMP accumu-

TABLE I
Comparison of cation binding affinities of adenylyl cyclase types VI and VIII, and other Ca\(^{2+}\)-binding proteins

<table>
<thead>
<tr>
<th>Cation</th>
<th>Ca(^{2+})</th>
<th>Sr(^{2+})</th>
<th>Ba(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effective ionic radius (Å)</td>
<td>1.06</td>
<td>1.21</td>
<td>1.38</td>
</tr>
<tr>
<td>Adenylyl cyclase type VI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High affinity</td>
<td>0.232 ± 0.015</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Low affinity</td>
<td>221.2 ± 27.9</td>
<td>1070 ± 56</td>
<td>20,000 ± 882</td>
</tr>
<tr>
<td>Adenylyl cyclase type VIII</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High affinity</td>
<td>0.380 ± 0.044</td>
<td>11.96 ± 1.45</td>
<td>478.9 ± 34.2</td>
</tr>
<tr>
<td>Low affinity</td>
<td>132 ± 10</td>
<td>1573 ± 127</td>
<td>18,000 ± 1700</td>
</tr>
<tr>
<td>N(_{21-106})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low affinity</td>
<td>131 ± 20</td>
<td>2380 ± 440</td>
<td>N/A</td>
</tr>
<tr>
<td>EF hands</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphodiesterase via calmodulin</td>
<td>~2.5</td>
<td>~25</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>PL scramblase</td>
<td>28.7 ± 4.1</td>
<td>308 ± 28.6</td>
<td>2783 ± 439</td>
</tr>
<tr>
<td>E. coli galactose-binding protein</td>
<td>1.4 ± 0.1</td>
<td>190 ± 10</td>
<td>12,000 ± 4000</td>
</tr>
<tr>
<td>C2 domain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Synaptotagmin I</td>
<td>5.4</td>
<td>177</td>
<td>254</td>
</tr>
<tr>
<td>Phospholipase A(_{2})</td>
<td>6</td>
<td>100</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

Fig. 2. Inhibitory effects of Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) on the endogenous adenylyl cyclase of C6–2B glioma cells in vitro. Assays were performed on plasma membranes extracted from C6–2B glioma cells stimulated with 20 μM forskolin, in the presence of the indicated free concentrations of Ca\(^{2+}\) (■), Sr\(^{2+}\) (○), and Ba\(^{2+}\) (▲). Relative adenylyl cyclase activities were obtained by normalizing the basal activities with those in the absence of the cations. The curves were the results of fitting the data, using a two-site competition model (for Ca\(^{2+}\)) or single-site competition model (for Sr\(^{2+}\) and Ba\(^{2+}\)). Values shown are from an experiment that was repeated at least three times with similar results.

Fig. 3. Stimulation by Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) of cAMP accumulation of intact HEK 293 cells transfected with ACVIII. Assays (1 min) were performed on intact HEK 293 cells transfected with ACVIII cDNA. The cells were pretreated with 0.5 mM isobutylmethylxanthine, 0.1 mM Ro, 0.1 mM EGTA and 100 nM thapsigargin, as described under “Experimental Procedures.” The ACVIII activities were determined in the presence of 20 μM forskolin and different external concentrations of Ca\(^{2+}\) (■), Sr\(^{2+}\) (○), and Ba\(^{2+}\) (▲). The relative activities were obtained by normalizing the ATP to cAMP conversions with those in the absence of the cations. The curves were obtained by fitting the data points with four-parameter sigmoid model. Data are representative of four experiments with similar results.

unexpected to observe effects of Sr\(^{2+}\) on the adenylyl cyclase activities of intact cells, especially for the C6–2B glioma cells. Extrapolating from the in vitro data, capacitative Ca\(^{2+}\) entry would need to generate a free Ca\(^{2+}\) concentration near the plasma membrane of HEK 293 cells of ~1 μM to maximally stimulate ACVIII activity (Fig. 1). In contrast, to obtain half- and full maximal stimulation by Sr\(^{2+}\), 10 and 50 μM free Sr\(^{2+}\), respectively, would be required (Fig. 1). Therefore, the in vitro data (Fig. 3) suggests that if 1 μM Ca\(^{2+}\) enters the cells during capacitative entry, the entry for Sr\(^{2+}\) would be around 10 μM. Following a similar analysis for the inhibition of ACVIII activity in C6–2B glioma cells, the entry of at least 100 μM Sr\(^{2+}\) would be required to reconcile the in vitro (Fig. 2) and in vivo data (Fig. 4). To explore the issue of the relative entry of Sr\(^{2+}\) versus Ca\(^{2+}\), intracellular cation measurements were made using fura-2.

Measurements of [Ca\(^{2+}\)]\(_{i}\), [Sr\(^{2+}\)]\(_{i}\), and [Ba\(^{2+}\)]\(_{i}\). Using fura-2—Capacitative cation entry was triggered in HEK 293 cells and C6–2B glioma cells as in the adenylyl cyclase assays described
Discussion

The ability of low concentrations of Ca\(^{2+}\) to induce conformational changes in target proteins in the face of great excess concentrations of Mg\(^{2+}\) is a hallmark of Ca\(^{2+}\)-regulated proteins. So-called E-F hand-containing proteins are the most common mechanism for imparting this specificity to protein domains. This quality reflects the ability of Ca\(^{2+}\) to coordinate 7 oxygen atoms and rapidly substitute inner sphere water molecules, compared with the smaller Mg\(^{2+}\) (0.86 versus 1.2 Å for Ca\(^{2+}\)), which coordinates only 6 nitrogen or oxygen atoms and substitutes water more slowly (13). The IIa cations, Sr\(^{2+}\) and Ba\(^{2+}\), possess the high coordination number of Ca\(^{2+}\), and its rapid water substitution, although their larger crystal ionic radii (1.4 and 1.56 Å, respectively) makes them less effective at substituting for Ca\(^{2+}\) in regulating target proteins. This relative affinity of the IIa cations for E-F hands makes a comparison of the potency of Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) a useful device for probing the involvement of this motif in regulating Ca\(^{2+}\)-dependent processes. In the present case, a comparison of the potency of these cations was first made in probing the Ca\(^{2+}\)-mediated stimulation of ACVIII (known to be mediated by calmodulin and, therefore, E-F hands) with the Ca\(^{2+}\)-mediated inhibition of ACVI, in which the nature of the Ca\(^{2+}\)-binding site is unknown.

Increasing concentrations of Ca\(^{2+}\) elicit a biphasic response from ACVIII and ACVI in vitro. The high affinity effect of Ca\(^{2+}\) at regulating ACVIII and ACVI differs in sign, but not in the effective concentrations. The relative selectivity of ACVIII for the IIa series of cations (Ca\(^{2+}\) > Sr\(^{2+}\) > Ba\(^{2+}\)) agrees with the binding affinities of these cations to calmodulin and some other (E-F hand-containing) Ca\(^{2+}\)-binding proteins. Their relative potency (Ca\(^{2+}\), Sr\(^{2+}\), and Ba\(^{2+}\) affinities of 0.3, 12, and 500 μM, respectively) differs somewhat from the series for these cations at activating brain phosphodiesterase via calmodulin (2.5, 25, and >1000 μM, respectively; Ref. 30). The series also differs slightly from the ability of the binding of these cations to alter calmodulin tyrrosine fluorescence, which displayed \(K_a\) values of approximately 2, 8, and ≥50 μM (30). These discrepancies are...
in keeping with the induction of slightly different conformational states of calmodulin by target proteins (31, 32).

The effects of the IIa series cations on ACVIII contrast strongly with their effects on ACVIII. Strikingly, unlike Ca$^{2+}$, Sr$^{2+}$ and Ba$^{2+}$ evoke only a monophasic, low affinity inhibition. Even though a 5% high affinity inhibition by Sr$^{2+}$ could be masked in the apparent monophasic effect, the difference in efficacy between Ca$^{2+}$ and Sr$^{2+}$ suggests a fundamentally different mechanism for inhibition than for stimulation. In the case of effects mediated by E-F hands, Ca$^{2+}$ and Sr$^{2+}$ are usually equally efficacious, although with different potencies (e.g. as with ACVIII stimulation). As alluded to earlier, based on their similar coordination number and rapid water substitution rates, it is counterintuitive for Sr$^{2+}$ to be unable to compete for a Ca$^{2+}$-binding site. This paradox may be reconciled if we consider that Sr$^{2+}$ is competing for a Mg$^{2+}$- rather than a Ca$^{2+}$-binding site. The crystal ionic radii for Mg$^{2+}$, Ca$^{2+}$, Sr$^{2+}$, and Ba$^{2+}$ are 0.89, 1.2, 1.4, and 1.56 Å, respectively (13). Consequently, Sr$^{2+}$ may not always be able to compete for a limited Mg$^{2+}$-binding pocket. Indeed, examples can be found in the literature among other enzymatic processes, where (i) Ca$^{2+}$ and Mg$^{2+}$ are equivalent and Sr$^{2+}$ is impotent, (ii) Ca$^{2+}$ is required and Sr$^{2+}$ cannot substitute, or (iii) Sr$^{2+}$ inhibits and Ca$^{2+}$ is ineffective (33–36). Based on recent kinetic experiments, high affinity inhibition of adenyl cyclase by Ca$^{2+}$ can be at least partly described as a competitive inhibition of activation by Mg$^{2+}$ (12).

Although kinetic experiments cannot define molecular mechanism, in this case, based on the ineffectiveness of Sr$^{2+}$ at mimicking Ca$^{2+}$, the kinetic inference may be correct and the high affinity effects of Ca$^{2+}$ may be exerted via a Mg$^{2+}$- rather than a Ca$^{2+}$-binding site. C2 domains, which are direct Ca$^{2+}$-binding domains on proteins, such as synaptotagmin, phospholipase A$_2$, and protein kinase C, display similar preferences as E-F hands for the IIa series cations (Table I; Ref. 13). It seems fair to conclude that high affinity inhibition of ACVIII is not only not mediated by calmodulin, but also is not mediated by any E-F hand-utilizing protein, such as hippocalcin, neurolacin, etc., or any C2 domain.

Low affinity inhibition by Ca$^{2+}$ is a common feature of mammalian adenyl cyclases (2). Examination of the relative potencies of the IIa series cations indicates no differences between ACVIII and ACVII (Table I), which suggests a common type of mechanisms in both a Ca$^{2+}$-stimulable and Ca$^{2+}$-inhibitable adenyl cyclase. Finally, further evidence is provided that Ca$^{2+}$-sensitive adenyl cyclases detect higher concentrations of cations entering the cell in response to store depletion than do cytosolic monitors of capacitative Ca$^{2+}$ entry.

Acknowledgments—We are grateful to Drs. Kent Fagan and Eric Nelson for help with devising and carrying out some of the experiments and to Dr. Adam Zweifach for useful comments.

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Effects of Ca$^{2+}$, Sr$^{2+}$, and Ba$^{2+}$ on ACVIII and ACVII
Effects of $Ca^{2+}$, $Sr^{2+}$, and $Ba^{2+}$ on ACVIII and ACVI

Ca\textsuperscript{2+}, Sr\textsuperscript{2+}, and Ba\textsuperscript{2+} Identify Distinct Regulatory Sites on Adenylyl Cyclase (AC) Types VI and VIII and Consolidate the Apposition of Capacitative Cation Entry Channels and Ca\textsuperscript{2+}-sensitive ACs
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doi: 10.1074/jbc.275.10.6980

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