Residence of Adenylyl Cyclase Type 8 in Caveolae Is Necessary but Not Sufficient for Regulation by Capacitative Ca$^{2+}$ Entry*  

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Ca$^{2+}$-sensitive adenylyl cyclases (ACs) depend on capacitative Ca$^{2+}$ entry (CCE) for their regulation. Residence of the endogenous Ca$^{2+}$-inhibitable adenylyl cyclase of C6-2B glioma cells in cholesterol-enriched caveolae is essential for its regulation by CCE (Fagan, K. A., Smith, K. E., and Cooper, D. M. F. (2000) J. Biol. Chem. 275, 26530–26537). In the present study, we established that deletion of cellular cholesterol ablated the regulation by CCE of a Ca$^{2+}$-stimulable adenylyl cyclase, AC8, heterologously expressed in HEK293 cells. We considered the possibility that a calmodulin-binding domain in the N terminus of AC8, which is not required for in vitro regulation by Ca$^{2+}$, might play a targeting role. Deletion and mutation of the N terminus did attenuate the enzyme’s sensitivity to CCE without altering its in vitro responsiveness to Ca$^{2+}$/calmodulin. Both N terminus-deleted AC8 and wild type AC8 were expressed at the plasma membrane, as shown by imaging analysis of green fluorescence protein-tagged constructs. However, not only wild type AC8 but also the CCE-insensitive mutants occurred in caveolar fractions of the plasma membranes, even though a Ca$^{2+}$-insensitive adenylyl cyclase, AC7, was excluded from caveolae. Finally, the AC8 mutants were no more responsive to nonphysiological elevation of Ca$^{2+}$ than the wild type. We conclude that (i) not all adenylyl cyclases reside in caveolae, (ii) the calmodulin-binding domain in the N terminus of AC8 does not play a role in caveolar targeting, (iii) the N terminus does play a role in associating AC8 with factors that confer sensitivity to CCE, and (iv) residence of Ca$^{2+}$-sensitive adenylyl cyclases in caveolae is essential but not sufficient for regulation by CCE.

The two major second messenger signaling systems, cyclic AMP and Ca$^{2+}$, are tightly integrated at a number of levels. The earliest opportunity at which Ca$^{2+}$ can influence the cAMP pathway is of course at the level of synthesis, via direct effects on adenylyl cyclases (ACs). Of the currently known mammalian adenylyl cyclases, four are either stimulated or inhibited by physiological increases in [Ca$^{2+}$], (1). In nonexcitable cells, this regulation by [Ca$^{2+}$], is orchestrated by a strict dependence on the entry of Ca$^{2+}$ that is triggered by the emptying of internal Ca$^{2+}$ stores, the so-called capacitative Ca$^{2+}$ entry (CCE) (2). Other means of elevating [Ca$^{2+}$], such as release from stores or ionophore-mediated entry, are ineffective (3–5). The dependence of Ca$^{2+}$-sensitive adenylyl cyclases on regulation by CCE led to the suggestion that adenylyl cyclases and Ca$^{2+}$ entry channels were functionally colocalized (5). Caveolae are plausible platforms for such “functional domains.” These cholesterol- and glycosphingolipid-rich regions of the plasma membrane are marked by the protein caveolin. Their unique lipid composition renders them insoluble in nonionic detergents at low temperature (6), a property that has been exploited in their isolation. These and other cholesterol-rich, detergent-insoluble regions of the plasma membrane, collectively referred to as lipid rafts, have been postulated to be a coordination point for a variety of signaling molecules (6, 7).

We recently demonstrated that the endogenously expressed Ca$^{2+}$-inhibitable adenylyl cyclase, AC6, occurs in caveolae in C6-2B cells (8). This localization is required to ensure the regulation of AC6 by CCE because disruption of the caveolae by cholesterol depletion ablates the Ca$^{2+}$ inhibition of the adenylyl cyclase activity; restoration of the cell cholesterol restores the regulation by CCE (8). Adenylyl cyclases from other sources, including AC6, when heterologously expressed in cardiac myocytes, also occur in caveolae (9–12). Other components of the cAMP signal transduction cascade, including receptors and GTP-regulatory proteins, are also found in caveolae (13–16).

In the present study, we wished to determine whether the Ca$^{2+}$-stimulated adenylyl cyclase, AC8, which we had previously shown to be dependent on CCE for its regulation by Ca$^{2+}$ (4), would also be targeted to caveolae when heterologously expressed in HEK293 cells. To assess whether there was an obligate association between residence in caveolae and susceptibility to regulation by CCE, we evaluated a number of mutations of AC8 with decreased sensitivity to CCE and asked whether the mutant forms were also targeted to caveolae. A calmodulin-binding domain plays a role in targeting nitric oxide synthase to caveolae (17). We had previously identified two calmodulin-binding sites in AC8, one at the C terminus that was critical in mediating the in vitro responsiveness to Ca$^{2+}$ and one at the N terminus that appeared to be of no obvious regulatory significance (18). Consequently, we evaluated the possibility that the noncatalytically significant calmodulin-binding domain in the N terminus of AC8 might play a targeting role. We explored two mutations in the N terminus domain of AC8 that displayed refractory responses to CCE, although in in vitro assays, the mutants displayed equivalent stimulation by Ca$^{2+}$ as wild type AC8. Imaging revealed that a GFP-tagged, N terminus-deleted AC8 was expressed at the plasma membrane, similar to wild type AC8. However, to our surprise,  

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The abbreviations used are: AC, adenylyl cyclase; CCE, capacitative Ca$^{2+}$ entry; TG, thapsigargin; MJ3CD, methyl-j-cyclodextrin; GFP, green fluorescence protein; 2-APB, 2-aminoethoxydiphenyl borate; MBSC, 25 mM 4-morpholinethanesulfonic acid, 150 mM NaCl, 250 mM Na$_2$CO$_3$, and 2 mM EDTA, pH 8; TTBS, Tris-buffered saline plus 0.2% Tween 20.
fractionation of the plasma membrane into caveolar and non-caveolar regions revealed that not only the wild type AC8 but also CCE-insensitive AC8 mutants were located in caveola. A heterologously expressed, Ca\(^{2+}\)-insensitive adenylyl cyclase, AC7, was not targeted to caveola. Furthermore, mutant forms of AC8 were no more susceptible to high, nonspecific elevations in [Ca\(^{2+}\)], than wild type AC8. These results show that (i) not all adenylyl cyclases are targeted to caveola, (ii) the targeting mechanism for recruiting AC8 to caveolar domains does not involve the calmodulin-binding domain in the N terminus, and (iii) most importantly, localization in caveola is not sufficient for the regulation of Ca\(^{2+}\)-sensitive adenylyl cyclases by CCE, although it is necessary.

**EXPERIMENTAL PROCEDURES**

**Materials**—Thapsigargin, forskolin, ionomycin, and SKF96365 were from Calbiochem. Fura-2/AM and pluronic F-127 were from Molecular Probes, Inc. (Eugene, OR). [2-\(^3\)H]Adenine, [\(\alpha\)-32P]ATP, and [\(^3\)H]cAMP were purchased from Amersham Biosciences, Inc. All other reagents were from Sigma.

**Cell Culture**—HEK293 cells were maintained in 13 ml of minimum essential medium (Invitrogen) with 10\% (v/v) fetal bovine serum (Gemini), 1% (v/v) penicillin (100 IU/ml), 1% (v/v) streptomycin (100 \(\mu\)g/ml), 5\% CO\(_2\). Cells were plated on 100-mm dishes at 70\% confluence 1 day prior to transfection. After confluence, the cells were washed with phosphate-buffered saline followed by the addition of thapsigargin (TG; 100 \(\mu\)M) or 2-aminoethoxydiphenyl borate (2-APR; 100 \(\mu\)M; Ref. 20), were also added as indicated. The 340/380 nm light emission ratios were converted to [Ca\(^{2+}\)]\(_i\) using the standard formula (21).

**Mutagenesis**—The deletion mutant SM1, which results in the deletion of the first 106 amino acids of AC8, was constructed by cutting pcDNA3/AC8 plasmid with BspI, blunting the end with T4 DNA polymerase, and ligating it again with NotI, and subcloning the fragment into the EcoRI (blunted with T4 DNA polymerase) and NotI sites of pcDNA3/Hisb (Invitrogen) in frame. 8M34 was constructed using QuikChange mutagenesis, in which WQT (amino acids 38–40) and RFI (amino acids 49–51) were switched to AAA. Both WQT (amino acids 38–40) and RFI (amino acids 49–51) are located in the N-terminal putative calmodulin-binding domain. Therefore, calmodulin would not be expected to bind to the N terminus of SM34. The mutated cDNA constructs were confirmed by sequencing.

**Measurement of cAMP Accumulation in Response to CCE and Depletion of Cellular Cholesterol**—cAMP accumulation was measured in intact cells expressing different AC8 constructs as described previously (18). Unless stated otherwise, cells were preincubated for 10 min with the Ca\(^{2+}\)\(-\)ATPase inhibitor thapsigargin and a final concentration of 8 M. cAMP accumulation was measured over a 1-min period beginning with the addition of forskolin along with other reagents as indicated. Assays were terminated by the addition of 5\% (w/v) final concentration) trichloroacetic acid. Markers were added to monitor recovery of cAMP and ATP. The [\(^3\)H]ATP and [\(^3\)H]cAMP content of the supernatant was quantified as described above (22). Accumulation of cAMP is expressed as the conversion of [\(^3\)H]ATP into [\(^3\)H]cAMP. Results are presented as the means \(\pm\) S.D. of triplicate determinations. Cellular cholesterol was depleted as described previously (8). Briefly, attached HEK293 cells were washed with phosphate-buffered saline followed by incubation in serum-free F-10 media plus 20 \(\mu\)M HEPES, pH 7.4, containing methyl-\(\beta\)-cyclodextrin (M\(\beta\)CD; 15 \(\mu\)M) for 1 h at 37 °C. After cholesterol depletion, cells were washed, and either cAMP accumulation or [Ca\(^{2+}\)]\(_i\) measurements were carried out, as described above.

**Adenylyl Cyclase Activity Measurements**—Determination of adenylyl cyclase activity in vitro was performed as described previously (23) on isolated transfected HEK293 cell membranes (24). The adenylyl cyclase activity of the HEK293 cell membranes was measured in the presence of the following components: 12 \(\mu\)M phosphocreatine, 2.5 units of creatine phosphokinase, 0.1 mM cAMP, 1 mM MgCl\(_2\), 0.1 mM ATP, 70 mM Tris buffer, pH 7.4, 0.04 mM GTP, 1 \(\mu\)Ci of [\(\alpha\)-32P]ATP, 1 \(\mu\)M calmodulin, and 20 \(\mu\)M forskolin, as indicated. Free Ca\(^{2+}\) concentrations were established from a series of CaCl\(_2\) solutions buffered with 200 \(\mu\)M EGTA in the assay (23) using the computer program BAD4 (25). The reaction mixture (final volume, 100 \(\mu\)l) was incubated at 30 °C for 20 min, and the reaction was terminated by the addition of Tris buffer, pH 11, and sonicated with a single 3-s burst on a Fisher model 60 sonicator at 50\% power. The suspension was transferred to a tight-fitting Dounce homogenizer and homogenized with 12 strokes. The sucrose concentration was adjusted to 40\% by the addition of 2 ml of 60\% sucrose in 4-morpholinethanesulfonic acid-buffered saline containing sodium carbonate (MBSC). The extract was placed below a 5–30\% discontinuous sucrose gradient prepared in cold MBSC and centrifuged in a Beckman SW40 rotor at 39,000 rpm for 18 h at 4 °C. After centrifugation, two distinct light scattering bands were visible at \(\sim\)20\% sucrose and 35–40\% sucrose. Each of these bands was removed, distributed over several washes in TTBS, and centrifuged for 6 h at 4 °C to pellet the membranes. The pellets were resuspended in 150 \(\mu\)l of 1\% SDS, sonicated for 3 s at 50\% power, and frozen until use. The protein concentration of each pellet was determined by the BCA assay kit (Pierce).

**Western Blotting**—AC8-specific antibody was kindly provided by Drs. J. J. Cali and J. Krupinski. The polyclonal caveolin antibody was obtained from Transduction Laboratories, and the T7 tag monoclonal antibody was from Novagen. Proteins were resolved with SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane. Adenylyl cyclase immunoreactivity was determined after blocking the membranes in TTBS plus 5\% milk for 1 h, followed by incubation for 2 h in primary antibody diluted 1:15,000 in TTBS plus 1\% milk and several washes in TTBS. Signal detection was achieved using horseradish peroxidase-coupled secondary antibody (Bio-Rad) followed by enhanced chemiluminescence (PerkinElmer Life Sciences) and film detection. Caveolin immunoreactivity was determined after the membranes were blocked in TTBS plus 3\% milk overnight at 4 °C followed by incubation with primary antibody diluted 1:2500 in 1\% milk for 3 h. Signal detection was achieved as described above. The T7-tagged AC7 immunoreactivity was determined after a 30-min block in 3\% bovine serum albumin followed by a 1-h incubation in primary antibody diluted 1:10,000 in 1\% bovine serum albumin. After several washes in TTBS, signal detection was achieved using a horseradish peroxidase-coupled goat anti-mouse antibody from Promega with enhanced chemiluminescence and film detection.

**RESULTS**

**Susceptibility of the Regulation of AC8 by CCE to Cholesterol Depletion**—The inhibition of the endogenously expressed AC6 in C6-2B cells by CCE is dependent on the residence of AC6 in cholesterol-rich, caveolin-containing domains of the plasma membrane (8). In this study, as a prelude to probing the molecular determinants for residence in caveolae and susceptibility to regulation by CCE, we wished to determine whether the heterologously expressed, Ca\(^{2+}\)-stimulable AC8 displayed a similar requirement of caveolar residence for Ca\(^{2+}\) regulation by CCE. As a first step, we asked whether disruption of caveola by depleting cellular cholesterol attenuated the ability of CCE to stimulate AC8. Initially, it was important to determine the effects, if any, of cholesterol depletion on CCE in HEK293 cells. Cellular cholesterol was depleted as described above with M\(\beta\)CD as previously applied to C6-2B cells (8), and CCE was examined (Fig. 1A). These cells were pretreated with the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor TG to passively deplete the internal Ca\(^{2+}\) stores. This prosimizes the cells for CCE (27). Both the untreated and M\(\beta\)CD-treated cells yielded very similar [Ca\(^{2+}\)]\(_i\) rises (\(-\)580 nM at 360 s; subsequent cAMP accumulation assays are measured over a 1-min period corresponding to the 300–360-s interval). There-
alone (pcDNA3) was 0.27 stimulations for control cells transfected with vector was 1.42 stimulations of either untreated or MCD-treated, fura-2-loaded HEK293 cells appear to be affected by cholesterol depletion, as we had previously demonstrated that whereas the two mutants retained some ability to stimulate AC8 in untreated cells transiently transfected with AC8 that had been either untreated (□) or treated with MβCD (□) as described under "Experimental Procedures." Cells were pretreated with 3-isobutyl-1-methylxanthine (100 μM) and compared with wild type AC8 in terms of their sensitivity to Ca2+-sensitive adenylyl cyclase, AC8, contains a calmodulin-binding domain in the N terminus of the protein, which is not required for the regulation of the enzyme by Ca2+ in vitro (18). Because a calmodulin-binding domain appeared to play a role in targeting nitric oxide synthase to caveolae (17), we evaluated the possibility that the equivalent domain in the N terminus of AC8 might play a similar role. Constructs were generated containing point mutations in the AC8 N terminus calmodulin-binding domain (8M34) or a deletion of the first 106 amino acids of the AC8 N terminus (8M1), which includes the helical calmodulin-binding domain between amino acids 34 and 51 (Fig. 2; Ref. 18). These mutants were heterologously expressed in HEK293 cells and compared with wild type AC8 in terms of their sensitivity to Ca2+ in vitro (Fig. 3A). This assay illustrated that both mutants could be stimulated in the same concentration range as the wild type enzyme, with very similar overall activity. The sensitivity of these AC8 constructs to regulation by CCE was next compared in intact HEK293 cells. The results demonstrated that whereas the two mutants retained some ability to be stimulated by CCE, this stimulation was greatly reduced compared with that seen with the wild type AC8 (Fig. 3B).

Expression and Plasmalemmal Targeting of AC8 Mutants—To address the possibility that altered expression might account for the reduction in the mutants' sensitivity to CCE, Western blotting was performed on membranes prepared from the same batches of transfected cells used in the in vitro and in vivo assays. Both the 8M1 and 8M34 mutants and the wild type are expressed in equivalent amounts (Fig. 3C).

Another possible explanation for the decrease in sensitivity of the mutants to stimulation by CCE is that a significant portion of the mutant proteins is retained in the endoplasmic reticulum due to deletion of the N terminus. To evaluate this possibility, imaging was performed to compare GFP-tagged wild type and the N terminus-deleted 8M1 construct. Both constructs were expressed equivalently at the plasma membrane (Fig. 4).

Expression in Caveolar Membranes—Previous work had shown that the Ca2+-inhibitable cyclase, AC6, must be local-
cholesterol depletion (see Fig. 1), we wished to determine whether it was also targeted to caveolae. Upon separating plasma membranes into caveolar and noncaveolar fractions, we found that the vast majority of the AC8 immunoreactivity was localized to caveolar membranes (Fig. 5A, top panel), as indicated by comigration with caveolin immunoreactivity (Fig. 5A, bottom panel). We next examined the localization of the two mutants within the plasma membrane. Quite unexpectedly, the immunoreactivity of both 8M1 and 8M34 was also largely confined to caveolar membranes (Fig. 5A). Because these adenylyl cyclase constructs were so poorly regulated by CCE, we wondered whether all adenylyl cyclase species were targeted to caveolar regions of the plasma membrane, regardless of their susceptibility to CCE. We examined the membrane localization of AC7, an adenylyl cyclase that is not regulated by changes in Ca\(^{2+}\); HEK293 cells were transfected with AC7 cDNA, and membranes were fractionated as described above. AC7 immunoreactivity was clearly excluded from caveolae (Fig. 5B). Thus, it is not a general property of adenylyl cyclases to occur in caveolae. This result also implies that the targeting mechanism of Ca\(^{2+}\)-sensitive adenylyl cyclases to caveolae must lie in a region other than the first 106 amino acids of the N terminus.

**Regulation of AC8 Mutants by Other Forms of Ca\(^{2+}\) Entry**—Wild type AC8 is highly dependent on CCE rather than release from internal stores or ionophore-mediated Ca\(^{2+}\) entry (4). We wondered whether the mutations that rendered AC8 unresponsive to CCE might have made the enzyme responsive to more substantial, albeit non-CCE-mediated, rises in [Ca\(^{2+}\)]. The Ca\(^{2+}\) entry through CCE channels in HEK293 cells is modest, as demonstrated previously. This entry is blocked by treatment with the CCE inhibitors 2-APB (20) and SKF96365 (Fig. 6A). These two compounds had little effect on the much larger Ca\(^{2+}\) entry mediated by the Ca\(^{2+}\)-ionophore ionomycin, indicating...
only minor contributions from CCE (Fig. 6B). The ability of wild type AC8 and the mutants to be stimulated by these various forms of [Ca^{2+}], rise was compared (Fig. 7). Only the wild type AC8 is robustly stimulated by CCE, and this stimulation is reversed with either 2-APB or SKF96365 (Fig. 7A). Ionomycin elicits a modest stimulation of wild type AC8, an effect that is blocked by both 2-APB and SKF96365, which reflects the CCE component accompanying the ionophore-mediated entry (Fig. 7B). This CCE component is triggered by insertion of the ionophore into endoplasmic reticulum membranes (as well as the plasma membranes); the ionophore, like thapsigargin, depletes stores in the absence of extracellular Ca^{2+} (28). The substantial [Ca^{2+}], rise resulting from release from internal stores in response to ionomycin does not stimulate either the wild type or mutant AC8 constructs (Fig. 7C). Together, these results indicate that substantial [Ca^{2+}], rises generated by means other than CCE, which do not stimulate wild type AC8, are also unable to regulate the mutant adenyl cyclases, which suggests that residence of adenyl cyclases in caveolae limits the access of regulatory concentrations of Ca^{2+} arising from sources other than CCE.

**DISCUSSION**

Ca^{2+}-sensitive adenyl cyclases, whether endogenously or exogenously expressed, are exclusively regulated by CCE in intact cells (3–5, 29, 30). This, along with other evidence (25), has led to the hypothesis that Ca^{2+}-sensitive adenyl cyclases and CCE channels are in close proximity. The basis for this colocalization is not fully understood, although residence of adenyl cyclase in caveolae is required in the case of the Ca^{2+}-inhibitable AC6 endogenously expressed in C6-2B cells. Thus, depletion of cellular cholesterol eliminates the regulation by CCE of AC6. Restoration of cellular cholesterol restores the susceptibility to CCE (8). Therefore, it appears that the sequence of AC6 includes the information required for targeting to caveolae, either directly or indirectly via another protein, to result in functional colocalization with CCE sites. The present study evaluated the presence, significance, and molecular determinants of a heterologously expressed, Ca^{2+}-stimulable adenyl cyclase, AC8, in caveolae.

AC8 was chosen, first to determine whether a heterologously expressed, Ca^{2+}-stimulable adenyl cyclase would be found in caveolae, as we had found with endogenously expressed AC6. AC8 was also particularly interesting because it contained two calmodulin-binding sites, one at the C terminus that was critical in mediating the in vitro responsiveness to Ca^{2+} and one at the N terminus that appeared to be of no obvious regulatory significance. This latter site piqued our interest because there was literature in the nitric oxide synthase field suggesting a role for a calmodulin-binding site in the targeting of nitric oxide synthase to caveolae (17). Furthermore, it has been shown that endothelial nitric oxide synthase is dependent on CCE for its Ca^{2+}-dependent activity (31, 32). We first established that, as with AC6, upon depletion of cellular cholesterol, the response of AC8 to CCE was much reduced. We therefore chose to explore two mutants in the N terminus of AC8, one that was a deletion of part of the N terminus, including the calmodulin-binding domain, and another that converted six critical amino acids of the calmodulin-binding motif so that the molecule would be unlikely to bind calmodulin. Both of these mutants appeared potentially insightful in that they were both refractory to CCE, although they were fully responsive to Ca^{2+}/calmodulin in in vitro assays. With these findings, we approached the subcellular localization of AC8 and the mutants. In keeping with our earlier premise on the localization of AC6 to caveolae as an essential attribute to the regulation of AC6 by CCE, we were encouraged to find that AC8 also occurred in caveolae.
ever, we were somewhat taken aback to find that both of the unresponsive mutants were also found in caveolae. Does this mean that localization in caveolae is irrelevant to the regulation of Ca\textsuperscript{2+}-sensitive adenylyl cyclases? Apparently not. Clearly, in the case of AC6 in C6-B2 glioma cells, the enzyme could be regulated by CCE when it occurred in caveolae and was unresponsive when caveolae were disrupted (8). Furthermore, a Ca\textsuperscript{2+}-insensitive adenylyl cyclase, AC7, was excluded from caveolae (present report; Fig. 5). It is also interesting to note that Trp1, a candidate CCE participant (33), is also present in caveolae (34). Therefore, it seems reasonable to propose that localization in caveolae is a necessary feature of the regulation of Ca\textsuperscript{2+}-sensitive adenylyl cyclases by CCE; however, such localization is clearly not sufficient, based on the present results. Additional factors, including protein-protein interactions, which may be mediated by the N terminus, seem to be required to ensure the regulation. It should also be acknowledged that we do not understand why mutating the calmodulin-binding domain in the N terminus perturbs the regulation of AC8 by CCE. This may be directly related to the binding of calmodulin or it may be a secondary effect due to alteration in the overall configuration of the N terminus resulting in inefficient interactions with another protein.

Another intriguing aspect of the regulation and organization of Ca\textsuperscript{2+}-sensitive adenylyl cyclases is their insensitivity to either Ca\textsuperscript{2+} released from stores or nonspecific entry of Ca\textsuperscript{2+} into cells mediated by ionophore, even when these latter [Ca\textsuperscript{2+}], rises are more substantial (as measured by cytosolically distributed fura-2) than CCE. We did wonder whether the unresponsiveness of the AC8 mutants to CCE might be associated with a loss of selectivity for the [Ca\textsuperscript{2+}], rise to which they would respond, given that in \textit{in vitro} measurements there was no difference in response between wild type AC8 and the two mutants. However, no such “nonselective” regulation was revealed. These data then underline two complementary aspects to the regulation of adenylyl cyclases by CCE: first, an intimate association of components that could be mediated by various degrees of adjacency, including direct or indirect protein-protein interactions, along with lipid solubility-enforced colocalization, and second, a compartmentalization that renders the adenylyl cyclase inaccessible to certain elevations in [Ca\textsuperscript{2+}]. Microdomains for both cAMP and Ca\textsuperscript{2+} have been proposed for almost 20 years. Buxton and Brunton (35) showed that global cAMP measurements did not predict the differing efficacies with which different hormones could activate CAMP-dependent protein kinase in cardiac myocytes. Again, in cardiac myocytes, Fischmeister and colleagues (36, 37) demonstrated compartmentalization of CAMP in the activation of voltage-gated Ca\textsuperscript{2+} channels. Rich et al. (38) presented data that were interpreted to suggest that there was a barrier to the diffusion of cAMP from its site of synthesis. Clearly, in the case of Ca\textsuperscript{2+}, diffusion is tightly controlled. Compelling evidence exists for both “nanodomains” of extremely high [Ca\textsuperscript{2+}], ensured by the high concentrations achieved around the mouths of Ca\textsuperscript{2+} channels and “microdomains” of moderately elevated [Ca\textsuperscript{2+}], enforced by mobile and immobile buffers (39). Possibly, the environment surrounding the cytoplasmic facet of caveolae is “shielded” from [Ca\textsuperscript{2+}], rises generated elsewhere by both mobile and immobile Ca\textsuperscript{2+}-binding proteins (40). In this regard, it is also worth noting that ionomycin-mediated transmembrane Ca\textsuperscript{2+} movement in liposomes is inhibited by incorporation of cholesterol into the lipid bilayer (41). Thus, in intact cells, ionomycin-mediated Ca\textsuperscript{2+} entry may be occurring primarily in non-cholesterol-rich regions of the plasma membrane. Alternatively, Mahmoud and Fewtrell (42) point out that ionophores are of far lower Ca\textsuperscript{2+} influx rates than CCE channels. Consequently, many more ionophore molecules are required to achieve the same rate of Ca\textsuperscript{2+} influx. Based on \textit{in vitro} studies, adenylyl cyclases should respond to 1 or 2 \mu M [Ca\textsuperscript{2+}], which is a concentration that can be achieved globally by either mechanism. However, adenylyl cyclases in the intact cell may respond not to the steady-state levels that are achieved in the cytosol but to the transiently elevated [Ca\textsuperscript{2+}], levels achieved by CCE channels. In this regard, it is interesting that adenylyl cyclase regulation by CCE is ablated only by the fast Ca\textsuperscript{2+} chelator, 1,2-Bis(2-aminophenoxy)ethane-N\textsubscript{2}N\textsubscript{2}N\textsuperscript{2}-tetracetate acid, and not by EGTA at the same concentration (5). Furthermore, adenylyl cyclases tagged with aequorin to report the [Ca\textsuperscript{2+}], in their environment report very high concentrations of [Ca\textsuperscript{2+}], in response to CCE (>10 \mu M) versus modest concentra-
sions in response to ionophore. This issue of adenylyl cyclases’ insensitivity to apparently equivalent [Ca\textsuperscript{2+}]_, rises may be most revealing about how these enzymes are organized in the cell.

In summary, the present studies refine the questions we ask in understanding how adenylyl cyclases are regulated by CCE. Clearly, the N terminus does not play an obligatory role in targeting to caveolae; however, it does play an essential role in ensuring the regulation of adenylyl cyclase by CCE channels once the enzyme is in caveolae. Three issues appear particularly pressing: (i) how are adenylyl cyclases targeted to caveolae, (ii) what is the mechanism that ensures regulation of adenylyl cyclases by CCE channels once they are in caveolae, and (iii) how can the inaccessibility of adenylyl cyclases to global [Ca\textsuperscript{2+}] be explained?

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