Regulation of the Ca\textsuperscript{2+}-inhibitable Adenyl Cyclase Type VI by Capacitative Ca\textsuperscript{2+} Entry Requires Localization in Cholesterol-rich Domains

The endogenous Ca\textsuperscript{2+}-inhibitable adenyl cyclase type VI of C6-2B glioma cells is regulated only by capacitative Ca\textsuperscript{2+} entry and not by a substantial elevation of [Ca\textsuperscript{2+}], from either intracellular stores or via ionophore-mediated Ca\textsuperscript{2+} entry (Chiono, M., Mahey, R., Tate, G., and Cooper, D. M. F. (1995) J. Biol. Chem. 270, 1149–1155; Fagan, K. A., Mons, N., and Cooper, D. M. F. (1998) J. Biol. Chem. 273, 9297–9305). The present studies explored the role of cholesterol-rich domains in maintaining this functional association. The cholesterol-binding agent, filipin, profoundly inhibited adenyl cyclase activity. Depletion of plasma membrane cholesterol with methyl-\(\beta\)-cyclodextrin did not affect forskolin-stimulated adenyl cyclase activity and did not affect capacitative Ca\textsuperscript{2+} entry. However, cholesterol depletion completely ablated the regulation of adenyl cyclase by capacitative Ca\textsuperscript{2+} entry. Repletion of cholesterol restored the sensitivity of adenyl cyclase to capacitative Ca\textsuperscript{2+} entry. Adenyl cyclase catalytic activity and immunoreactivity were extracted into buoyant caveolar fractions with Triton X-100. The presence of adenyl cyclase in such structures was eliminated by depletion of plasma membrane cholesterol. Altogether, these data lead us to conclude that adenyl cyclase must occur in cholesterol-rich domains to be susceptible to regulation by capacitative Ca\textsuperscript{2+} entry. These findings are the first indication of regulatory significance for the localization of adenyl cyclase in caveolae.

Ca\textsuperscript{2+}-sensitive adenyl cyclases provide an acute focus for harmonizing the activity of Ca\textsuperscript{2+} and cAMP signaling (1). We have previously shown an absolute requirement for capacitative Ca\textsuperscript{2+} entry (CCE),\textsuperscript{1} rather than Ca\textsuperscript{2+} release from stores or ionophore-mediated Ca\textsuperscript{2+} entry, as the means whereby Ca\textsuperscript{2+}-sensitive adenyl cyclases are regulated by Ca\textsuperscript{2+}. This regulation persists whether the cyclases are endogenously or heterologously expressed in non-excitable cells (2–4). These findings are now being extended in a number of other systems (5–7). These and other data led us to propose that adenyl cyclases and CCE channels are localized in the same functional domain of the plasma membrane (4). The mechanism for this functional colocalization is currently unknown.

A role for cytoskeletal elements in maintaining the association between CCE sites and Ca\textsuperscript{2+}-sensitive adenyl cyclases was ruled out by disruption of either actin or microtubule filaments, without a consequence for the regulation of Ca\textsuperscript{2+}-sensitive adenyl cyclases by CCE (4). An alternative mechanism for this colocalization could be the preferential insertion of these two proteins into cholesterol-rich microdomains within the plasma membrane, such as caveolae. Caveolae, which were first described by Palade and co-workers in the 1950s (8), are flask-shaped invaginations of the plasma membrane that are rich in cholesterol and glycosphingolipids (9). Caveolae and, in broader terms, detergent-insoluble, glycosphingolipid-enriched domains (DIGs),\textsuperscript{2} have been postulated to provide a platform for the concentration of signaling molecules (9, 10). A number of the components of the cAMP-signaling cascade have been localized to caveolae, including several G-protein-coupled receptors as well as heterotrimeric G-proteins (for review see Ref. 11). Adenyl cyclase activity has also been reported in low density membrane fractions, from both 549 lymphoma and fibroblast cells (12). Western blotting revealed that heterologously expressed ACIII, ACIV, and ACV in SF9 cells were also localized to caveolin-enriched membrane fractions (13). In cardiac tissue, ACV was communoprecipitated with caveolin-3 (a resident protein of caveolae) (13). Furthermore, caveolin modulated the activity of several of these components, such as the regulatory subunit of protein kinase A (14) and adenyl cyclase in a type-specific fashion. For instance, caveolin inhibited ACIII and ACV but did not affect ACII (15). The presence of the ganglioside GM1 (the receptor molecule for the entry into cells of cholera toxin, which ADP-ribosylates G-protein subunits) in caveolae is a further indication that cAMP signaling may concentrate in these structures (16).

The identity of the channel(s) responsible for CCE is not known. Consequently, the subcellular localization of CCE is not directly discernible. However, it is notable that several proteins involved in Ca\textsuperscript{2+} homeostasis, such as an InsP\textsubscript{3} receptor-like molecule and Ca\textsuperscript{2+}-ATPase, are found in caveolae (17, 18). In endothelial cells, it has also been shown that agonist-evoked release of intracellular Ca\textsuperscript{2+} originates near caveolin-rich cell edges (19).

In the present study, we have evaluated the role of caveolae

\textsuperscript{1} Several terms have been coined to describe cholesterol-rich domains, which are also rich in glycosphingolipids. These characteristics render them Triton-insoluble at 4 °C and buoyant in sucrose gradients. In this paper, the term cholesterol-rich domain includes "rafts," caveolae, "DIGs" (detergent-insoluble, glycosphingolipid-enriched domain), and "DRMs" (detergent-resistant membranes).

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in maintaining the colocalization of CCE sites and the Ca2+-inhibitable ACVI in C6-2B glioma cells. Initial experiments showed that adenylyl cyclase activity was sensitive to cellular treatment with filipin, a cholesterol-binding agent, which suggested a potential localization in cholesterol-rich membrane domains. Electron microscopic analysis revealed morphologically identifiable caveolae along the plasma membrane. Extraction of the cells with the detergent, Triton X-100, followed by density gradient fractionation revealed that a portion of adenylyl cyclase immunoreactivity comigrated with caveolin immunostaining in buoyant membrane fractions that were consistent with cholesterol-rich membranes. Adenylyl cyclase activity also occurred in these low density fractions. Depletion of membrane cholesterol with methyl-β-cyclodextrin (MβCD) resulted in the retention of both adenylyl cyclase immunoreactivity and enzymatic activity in heavier, Triton-soluble fractions. In whole-cell experiments, disruption of caveolae by cholesterol depletion, using MβCD, eliminated the ability of CCE to regulate adenylyl cyclase, without significantly affecting CCE. The regulation of adenylyl cyclase by CCE could be restored by replenishment of membrane cholesterol. These results indicate that the functional colocalization of CCE channels and adenylyl cyclase activity in C6-2B cells is heavily dependent on the residence of ACVI in caveolae.

**EXPERIMENTAL PROCEDURES**

**Materials—**Thapsigargin and forskolin were from Calbiochem. [2-3H]Adenine, [3H]cAMP, and [3H]-PiATP were obtained from American Pharmacia Biotech. Fura-2/AM and pluronic F-127 were from Molecular Probes, Inc. (Eugene, OR). Permanox plastic dishes and EPON resin for electron microscopy studies were obtained from Electron Microscopy Sciences (FT. Washington, PA). All other reagents were from Sigma.

**Cell Culture—**C6-2B rat glioma cells were maintained in 13 ml of F-10 medium (Life Technologies, Inc.) with 10% (v/v) bovine calf serum (Gemini) 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 100-mm tissue culture dishes 2 days prior to use.

**Cholesterol Depletion, Repletion, and Assay—**Cellular cholesterol was depleted in attached C6-2B cells by washing twice with PBS, followed by incubation in F-10 medium (without glucose, HEPES, pH 7.4) containing 10 mM CaCl2 for 1 h. The cells were then washed twice with PBS and used in either cell fractionation or cAMP accumulation experiments. Cholesterol repletion was performed exactly as described previously (20). Briefly, cholesterol-MβCD complexes were formed by the addition of cholesterol (6 mg dissolved in 20 μl of 2:1 isopropanol:chloroform) to a stirred MβCD solution (200 mg dissolved in 2.2 ml of H2O) maintained at 80 °C, until clear. The complexes were then added to serum-free F-10 media to a final concentration of 0.2 μM for 45 min at 37 °C. Cholesterol was measured by gas chromatography-mass spectrometry with coprostane as an internal standard, essentially as described (21).

**Measurement of cAMP Accumulation—**cAMP accumulation in intact cells was measured as described previously (22) with some modifications. C6-2B cells on 100-mm dishes were incubated in F-10 medium (90 ml at 37 °C) with [2-3H]adenine (20 μCi/dish) to label the ATP pool. The cells were then washed once, detached with phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4, pH 7.4) containing EDTA (0.03%), and resuspended in 1.2 ml of 60% sucrose in TNE. The cell pellet was resuspended in 60 μl of 1% SDS for Western blotting. An additional aliquot of the non-precipitated fraction was used for protein determination using the Pierce BCA assay kit. 10 μl of each precipitated fraction was used for cavelin immunoreactivity determination. Proteins were resolved using SDS-polyacrylamide gel electrophoresis and transferred to polyvinyldene difluoride membrane. The membranes were blocked in 3% milk overnight followed by incubation with cavelin antibody (Transduction Laboratories) at a final dilution of 1:2500 in TTBS (Tris-buffered saline plus 0.1% Tween 20) with 1% milk. Signal detection was achieved using horseradish peroxidase-coupled secondary antibody (Bio-Rad) followed by enhanced chemiluminescence (Enhanced Chemiluminescence, NEN Life Science Products) and film detection. Adenylyl cyclase immunoreactivity in the precipitated fractions was determined using a protocol from Kelly et al. (23) with the following modifications. Dithiothreitol was added to 20 μl of each fraction at 100 μl final concentration. The samples were heated at 80 °C for 20 min and cooled to room temperature, and then 0.1 mM of the suspension was loaded onto 7.5% SDS-GE gels for electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes, washed in TTBS, and dried. The membrane was rewet and blocked in 10% milk for 2 h followed by incubation with adenylyl cyclase antibody (Santa Cruz Biotechnology) at a final dilution of 1:500 in TTBS plus 1% milk. Signal detection was achieved using horseradish peroxidase-coupled secondary antibody followed by en-
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**Fig. 1. Effect of increasing filipin concentrations on adenylyl cyclase activity in intact C6-2B cells.** cAMP accumulation was measured in intact C6-2B cells as described under “Experimental Procedures.” Cells were incubated at 37 °C in Ca\(^{2+}\)-free Krebs buffer for 1 h in the presence of the indicated filipin concentration. Adenylyl cyclase activity was stimulated with forskolin (10 \(\mu M\)) and isoproterenol (10 \(\mu M\)) with cAMP accumulation measured over a 1-min assay. The data shown are representative of three similar experiments.

**Fig. 2. Effect of cholesterol depletion and subsequent repletion on the ability of CCE to inhibit ACVI activity in C6-2B cells.** cAMP accumulation in C6-2B cells was measured following incubation in serum-containing media (open bars, \(n = 3\)) or serum-free media containing either 15 mM MβCD (depletion; hatched bars, \(n = 4\)) or MβCD-cholesterol complexes (following depletion; cross-hatched bars, \(n = 2\)) as described under “Experimental Procedures.” Cells were pre-treated with TG (100 nM) 4 min prior to a 1-min assay that included forskolin (10 \(\mu M\)) and [Ca\(^{2+}\)]\(_o\) (as indicated) with the resultant conversion of [\(\beta\)H]}ATP to [\(\beta\)H]}cAMP depicted as the percent of the [Ca\(^{2+}\)]\(_o\) = 0 condition, with their associated standard errors. (100% values: control, 0.58 ± 0.04; MβCD, 0.59 ± 0.05; MβCD/cholesterol, 0.52 ± 0.04). Asterisks denote significantly different values from the relevant MβCD-treated values, as judged by Student’s test (\(p < 0.05\)).

**RESULTS**

**Effects of Filipin on Adenylyl Cyclase Activity—** Determination of adenylyl cyclase activity in vitro was performed as described previously (27), with modifications. Specifically, aliquots (75 \(\mu l\)) from each gradient fraction were assayed for adenyllyl cyclase activity in the presence of the following components: 12 mM phosphocreatine, 1 unit of creatine kinase, 0.1 mM cAMP, 2 mM MnCl\(_2\), 0.1 mM ATP, 0.5 mM 3-isobutyl-1-methylxanthine, 50 \(\mu M\) forskolin, 25 mM Tris, pH 7.5, and 1 \(\mu l\) of [\(\alpha\]-32P]}ATP. The reaction mixture (final volume, 400 \(\mu l\)) was incubated at 30 °C for 30 min. Reactions were terminated with sodium lauryl sulfate (0.5%); [\(\beta\)H]}cAMP was added as a recovery marker, and the [\(\beta\)H]}cAMP formed was quantitated according to the standard Dowex/alumina methodology (28). Data points are presented as mean activities ± S.D. of duplicate determinations.

**Effect of Cholesterol Depletion Using MβCD on the Ability of CCE to Regulate ACVI—** The potential contribution of cholesterol to the functional colocalization of Ca\(^{2+}\)-sensitive adenylyl cyclases and CCE sites was assessed by cholesterol depletion experiments using MβCD. MβCD is non-invasive and extracts cholesterol via its hydrophobic core (29). C6-2B cells were incubated in media without serum containing varying amounts of MβCD at 37 °C. Following this treatment, the cells were washed, and the ability of CCE to inhibit the endogenous ACVI was assessed (Fig. 2). Intracellular Ca\(^{2+}\) stores were depleted in cells maintained in Ca\(^{2+}\)-free Krebs buffer by treatment with thapsigargin (TG). (TG is a sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)}-ATPase inhibitor that causes the passive release of Ca\(^{2+}\) from the stores by a leak mechanism (30). This depletion of the Ca\(^{2+}\)} stores primes the cell for CCE upon addition of [Ca\(^{2+}\)]\(_o\) Adenylyl cyclase activity was assayed over 1 min,

Forskolin-stimulated activity was chosen since it would be expected that the established presence of hormone receptors and G-proteins in cholesterol-rich domains (see Ref. 11 for review) could result in some attenuation of hormone-stimulated activity overlaid on reversal of CCE-mediated inhibition of adenylyl cyclase. These two effects could obscure the issue of the importance of the lipid milieu to the regulation of adenylyl cyclase by CCE.
promising of CCE. This issue was addressed in experiments with Fura-2-loaded populations of C6-2B cells that had been treated (or not) with MβCD, as in Fig. 2 (see “Experimental Procedures”). CCE was stimulated in cells maintained in Ca²⁺-free Krebs buffer by depletion of intracellular Ca²⁺ stores followed by the addition of [Ca²⁺]₀ (Fig. 4). The depletion of intracellular Ca²⁺ stores by TG (100 nM; 60 s) produced a characteristically modest [Ca²⁺], rise (~60–80 nM) which was slightly smaller and slower in the cholesterol-depleted cells. At 300 s, [Ca²⁺]₀ (4 mM) was added, yielding a robust [Ca²⁺], rise that was similar between control and MβCD-treated cells. The control and MβCD-treated cells reached peak [Ca²⁺], values of ~550 and 500 nM, respectively. (Note, cAMP accumulation was measured over the 1-min period corresponding to 300–360 s in the Ca²⁺ trace, where there is no discernible difference between the two conditions; see Fig. 2.)

Since MβCD had little effect on either CCE or forskolin-stimulated adenylyl cyclase activity, but substantially reduced the ability of CCE to regulate the cyclase, it seemed reasonable to conclude that, for their functional interaction, the CCE channel and the adenylyl cyclase might be localized within a cholesterol-rich domain. Caveolae are rich in cholesterol as well as sphingolipids and could provide an identifiable, morphological domain for the localization of ACV₁ (31). Caveolae are not found in all cell types. Consequently, electron microscopy was used to determine whether C6-2B cells possess morphological structures resembling caveolae. Caveolae could clearly be seen, as indicated by the invaginations of the plasma membrane (not shown). The diameter of these structures was ~50–100 nm, which is consistent with previously reported dimensions of caveolae (32, 33). Additionally, the distribution of invaginated caveola was irregular along the plasma membrane, consistent with the presumed transient nature of these structures (9). Confirmation of caveolar morphology in these cells stimulated our further investigations of the role of caveolae in maintaining the functional association between CCE sites and adenylyl cyclase.

The molecular nature of the CCE channel is not known, and its subcellular localization cannot be determined by immunohistochemistry, for example. However, given that the CCE channel and adenylyl cyclase are functionally colocalized (4) and that this interaction is susceptible to cholesterol disruption, the localization of ACV₁ in cholesterol-rich domains could be pursued using cell fractionation techniques. Caveolae and cholesterol-rich regions in general can be isolated by several methods, including mechanical disruption (sonication) or detergent insolubility. In the present case, the insolubility of caveolae in Triton X-100 at 4 °C, a property due to the high content of cholesterol and sphingolipids present in caveolae (31), was exploited using a protocol similar to that of Lisanti et al. (25). In this procedure, membrane proteins that occur in phospholipid-rich regions are solubilized by the detergent; membrane proteins that are in cholesterol-rich regions are not solubilized but are separated from the solubilized proteins by their greater buoyancy (25) (cell debris and non-solubilized material sediment). C6-2B cells were resuspended in TNE buffer containing protease inhibitors with or without 1% Triton X-100 and kept on ice for 30 min (see “Experimental Procedures”). Following homogenization, the samples were brought to 40% sucrose and placed beneath a 5–30% discontinuous sucrose gradient and centrifuged. The gradients were then fractionated and analyzed. The Western blots for both caveolin

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Fig. 3. Determination of the cholesterol content of C6-2B cells following MβCD treatment. C6-2B cells were treated with increasing MβCD concentrations for 1 h at 37 °C as described under “Experimental Procedures.” Control cells were maintained in serum-containing media prior to cholesterol determinations. Following MβCD incubation, the cells were washed twice with phosphate-buffered saline, detached with phosphate-buffered saline containing EDTA (0.03%), and pelleted. The cells were extracted with methanol, and the cholesterol content was determined using mass spectrometry, as described under “Experimental Procedures.”

Fig. 4. Capacitative Ca²⁺ entry in C6-2B cells following cholesterol depletion with MβCD. Cellular cholesterol was depleted with MβCD (15 mM), and [Ca²⁺]ᵢ measurements were made in populations of C6-2B cells as described under “Experimental Procedures.” After incubation either in the presence or absence of MβCD (as indicated), cells were resuspended in nominally Ca²⁺-free Krebs buffer. Cells were pretreated with EGTA (0.1 mM) followed by the addition of TG (100 mM; 60 s) and [Ca²⁺]₀ (4 mM; 300 s) with the resultant [Ca²⁺], rise shown. Results are representative of two similar experiments.

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4 Isolation of caveolae by sonication also worked well, as judged by Western blotting for caveolin. However, sonication was extremely detrimental to adenylyl cyclase activity, even with very low power bursts (data not shown).
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**Fig. 5. Fractionation of C6-2B cells following Triton X-100 solubilization.** Fractions were isolated as described under “Experimental Procedures.” The left panel illustrates Western blot immunoreactivity for adenylyl cyclase (top) and caveolin (bottom) from fractions generated from cells in the absence of 1% Triton X-100. Note that no immunoreactivity for either protein is seen outside of the pellet fraction (fraction 11). The right panel demonstrates the movement of both caveolin and adenylyl cyclase immunoreactivity into lower sucrose concentration fractions following treatment with 1% Triton X-100. The majority of the immunoreactivity for both proteins is seen in fractions corresponding to a sucrose percentage of 15–20% (fractions 4 and 5). Note that the highest intensity bands for both proteins occur in the same fraction. Results are representative of three experiments with similar results.

and adenylyl cyclase from cells not solubilized with Triton X-100 are shown (Fig. 5). Both caveolin and adenylyl cyclase staining was found only in the cell pellet. Upon Triton X-100 solubilization, the majority of caveolin immunoreactivity occurred at 15–20% sucrose (fractions 4 and 5). Much of the adenylyl cyclase staining also moved to lighter sucrose densities following solubilization, comigrating with caveolin (Fig. 5).

Adenylyl cyclase activity was also measured in the fractions following detergent solubilization as above (Fig. 5). As expected from the immunoblotting data, the non-detergent-treated cells exhibited adenylyl cyclase activity only on the bottom of the gradient, whereas Triton X-100 treatment caused a migration of some adenylyl cyclase activity to lower sucrose densities, coinciding with the caveolin staining (Fig. 6). When the specific activity was examined (Fig. 6, inset), the adenylyl cyclase activity migrating at low sucrose concentrations following detergent treatment was much higher than that seen at higher sucrose concentrations (fraction 5, 130 pmol/mg-min versus fraction 9, 10 pmol/mg-min). Fig. 6B shows the protein profile along with the sucrose concentrations for each of the fractions. Triton extraction caused only a modest redistribution of total protein to the low density fractions. However, a substantial amount of adenylyl cyclase activity was found there. These results indicate that much of the adenylyl cyclase is localized within Triton-insoluble membrane domains.

To determine whether the attenuating effects of MβCD on CCE regulation of ACV1 would be reflected in altered sedimentation of adenylyl cyclase, cells were fractionated following MβCD treatment. Prior to the Triton X-100 treatment of the cell suspension, attached cells were incubated in serum-free media with or without MβCD for 1 h at 37 °C. Following homogenization, the sample was again brought to 40% sucrose, placed at the bottom of a 5–30% discontinuous sucrose gradient, and centrifuged. The gradient was fractionated as above, and aliquots of the individual fractions were subjected to Western blot analysis (Fig. 7). As in Fig. 5, Triton X-100 solubilization resulted in the majority of caveolin staining migrating to lighter fractions (15–20% sucrose) with a small portion remaining in the cell pellet (data not shown). Similarly, much of the adenylyl cyclase staining also moved to lower sucrose densities. When cells were depleted of cholesterol using MβCD, adenylyl cyclase staining was confined to the pellet (Fig. 7).

Therefore, the analogous MβCD treatment that had reduced the ability of CCE to inhibit adenylyl cyclase activity in whole-cell experiments also precluded the adenylyl cyclase staining from moving to lighter sucrose concentrations. Examination of the adenylyl cyclase activity in the same fractions mirrored the immunoblot data\(^5\) (Fig. 8). Triton X-100 treatment released a portion of the cyclase activity to lighter densities, whereas MβCD treatment blocked this effect. Comparing the specific activities (Fig. 8, inset), Triton X-100 treatment alone resulted in substantial activity in buoyant fractions, whereas MβCD treatment prior to Triton X-100 solubilization precluded adenylyl cyclase activity from floating into lighter fractions.\(^6\)

**DISCUSSION**

The present study has explored the role of cholesterol-rich domains in sustaining the functional colocalization between a Ca\(^{2+}\)-sensitive adenylyl cyclase and CCE channels in C6-2B cells. The adenylyl cyclase activity was potently inhibited by filipin, a cholesterol-binding agent. Fractionation following Triton X-100 extraction of the cells revealed a comigration of caveolin and adenylyl cyclase activity and immunoreactivity in buoyant, Triton-insoluble membrane fractions. The occurrence of the adenylyl cyclase (and caveolin) in these buoyant fractions was precluded by depletion of cellular cholesterol using MβCD. In intact cells, cholesterol depletion eliminated the ability of CCE to regulate the cyclase, without affecting Ca\(^{2+}\) entry due to CCE. The effect of CCE on adenylyl cyclase was restored by replenishing cellular cholesterol. Therefore, the presence of a Ca\(^{2+}\)-sensitive adenylyl cyclase in caveolae seems important for its regulation by physiological Ca\(^{2+}\) entry through CCE channels.

These results are analogous to the findings by Pike and Miller (20) in which disruption of cholesterol-rich domains with MβCD in A431 cells led to an inhibition in EGF- and bradykinin-stimulated PtdInsP\(_2\) turnover, accompanied by a loss of EGF receptor, G\(_\alpha\), and PtdInsP\(_2\) from the low density membrane domains. The authors also saw a graded response to cholesterol depletion. Depleting cellular cholesterol by only 20% resulted in an approximate 50% loss in EGF- or bradykinin-stimulated PtdInsP\(_2\) turnover. Furthermore, replacement of cellular cholesterol led to a reestablishment of hormone-stimulated PtdInsP\(_2\) hydrolysis. Similarly, cholesterol deple-

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\(^5\) Adenylyl cyclase activity is apparent in fractions 9 and 10, but adenylyl cyclase immunoreactivity is not apparent in these same fractions. It turns out that the relatively high Triton X-100 concentration used in the membrane solubilization procedure interfered with efficient trichloroacetic acid precipitation of the solubilized protein. When SDS is included during the trichloroacetic acid precipitation, at a ratio of Triton X-100 to SDS of 0.67, as described by Chang (54), adenylyl cyclase immunoreactivity is observed in fractions 9 and 10 (data not shown, n = 3).

\(^6\) Note that the relative amount of adenylyl cyclase activity in the caveolin-enriched fractions following Triton X-100 solubilization is lower when compared with the heavier fractions. This result contrasts with the data presented in Fig. 6 where the amount of cyclase activity was similar in fractions 5 and 9. The difference in these experiments was that in Fig. 8, cells were incubated in serum-free media for 1 h to facilitate the removal of cholesterol by MβCD prior to Triton X-100 solubilization. The apparent redistribution might reflect a decrease in cellular cholesterol due to a lack of low density lipoprotein in serum-free media, which delivers cholesterol to the cell (53).
tion with cyclohextrin resulted in the loss of morphologically recognizable caveolar structures in 3T3-L1 adipocytes, and a concomitant loss in insulin receptor signaling, which could be reversed by exogenous replenishment of cholesterol (34).

Therefore, cholesterol-rich domains may not only concentrate signaling molecules but also underpin functional coupling between them.

By both Western analysis and activity measurements, ACVI occurred in buoyant Triton-insoluble membrane fractions that also displayed caveolin immunostaining. However, only a portion of the cyclase was localized in these domains. Nevertheless, the subpopulation of adenylyl cyclase in the Triton-insoluble membrane fractions seemed responsible for the majority of the Ca\(^{2+}\)-regulated adenylyl cyclase activity in vivo. This conclusion stems from the fact that the same cholesterol-depleting protocol, using M\(_{\text{b}}\)CD, resulted in both an elimination of inhibition by CCE of adenylyl cyclase in whole-cell experiments along with a loss of adenylyl cyclase activity and immunostaining from the Triton-insoluble, low density membrane fractions. This observation raises the possibility that there are two pools of ACVI, one in cholesterol-rich domains that can be regulated by CCE, whereas the other is excluded from cholesterol-rich domains and is not regulated by CCE. Of course, the present findings also suggest that at least some CCE channels are preferentially found in cholesterol-rich domains.

The two pools of adenylyl cyclase may be dynamic and interchangeable with the proper stimulus. In this regard, the B2 bradykinin and the m2 muscarinic receptors localize within caveolae in an agonist-stimulated fashion (35–37). The reverse appears to be the case for the EGF and adenosine A1 receptors, which are rapidly moved out of caveolae upon binding EGF (38) or adenosine A1 receptor agonists (39), respectively. Certain

\(^7\) C6-2B cells predominantly express ACVI; therefore, different AC isoforms are probably not involved (56).
isoforms of protein kinase C are also dynamically localized to caveolae. Protein kinase Ca, unlike protein kinase Ce, associates with caveolae in a Ca\textsuperscript{2+}-dependent manner (40). eNOS provides an elegant example of regulated caveolar localization. When eNOS is present in caveolae, its activity is inhibited by binding caveolin. Ca\textsuperscript{2+} stimulation of eNOS causes the enzyme to translocate from caveolae. As [Ca\textsuperscript{2+}]\textsubscript{i} returns to basal levels, eNOS activity is inhibited as it moves back into caveolae and reassociates with caveolin (41). eNOS is also acylated by the fatty acids myristate and palmitate, which promote its residence in caveolae (42, 43) (as does the acylation of several other molecules, including G-proteins (44)). It would be interesting in future studies to determine whether the presence of adenylyl cyclases on this form of Ca\textsuperscript{2+} rise, it does not explain how cyclases cannot respond to a significant release of Ca\textsuperscript{2+} from intracellular stores (2). Conceivably, an array of mobile and immobile Ca\textsuperscript{2+} buffers (53) could serve both to contain the Ca\textsuperscript{2+} signal emanating from the CCE channel and to exclude Ca\textsuperscript{2+} arising from cytosolic sources. Much remains to be known of the microenvironment surrounding critical signaling molecules.

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