Ca\(^{2+}\) stimulation of adenylyl cyclase type 8 (AC8) is mediated by calmodulin (CaM). An earlier study identified two CaM binding sites in AC8; one that was apparently not essential for AC8 activity, located at the N terminus, and a second site that was critical for Ca\(^{2+}\) stimulation, found at the C terminus (Gu, C., and Cooper, D. M. F. (1999) J. Biol. Chem. 274, 8012–8021). This study explores the role of these two CaM binding domains and their interaction in regulating AC8 activity, employing binding and functional studies with mutant CaM and modified AC8 species. We report that the N-terminal CaM binding domain of AC8 has a role in recruiting CaM and that this recruitment is essential to permit stimulation by Ca\(^{2+}\) in vivo. Using Ca\(^{2+}\)-insensitive mutants of CaM, we found that partially liganded CaM can bind to AC8, but only fully liganded Ca\(^{2+}\)/CaM can stimulate AC8 activity. Moreover, partially liganded CaM inhibited AC8 activity in vitro. The results indicate that CaM pre-associates with the N terminus of AC8, and we suggest that this recruited CaM is used by the C terminus of AC8 to mediate Ca\(^{2+}\) stimulation.

The ubiquitous protein, calmodulin (CaM),\(^2\) mediates many of the wide ranging physiological effects of the second messenger, Ca\(^{2+}\). Although a modest sized protein (16.7 kDa), the bi-lobed structure of CaM provides enough interfaces, and conformational changes in response to the binding of four Ca\(^{2+}\) ions, to permit a range of mechanisms whereby it can modulate cellular activity (1). Each lobe of CaM contains two EF-hand Ca\(^{2+}\) binding motifs that are joined by a flexible linker (2). CaM can associate with target proteins before it has bound Ca\(^{2+}\) (apo-CaM), when it is partially bound to Ca\(^{2+}\) or fully loaded with Ca\(^{2+}\) (3). In addition, the two lobes can act independently and bind to different regions within one or more proteins (4). Two broad CaM binding domain (CaMBD) classifications have arisen. The first is the "IQ motif," with the sequence: (F/L/V/I)Q(R/K)XnXXX(R/K). IQ motifs are commonly, but not always, Ca\(^{2+}\)-independent CaMBDs. The second type of CaMBD usually binds CaM in a Ca\(^{2+}\)-dependent manner and is characterized by amphipathic helices with conserved hydrophobic amino acids. This type of CaMBD carries a net positive charge and consists of aromatic residues at either end and basic residues, particularly arginines, in between (5).

One important physiological role for CaM is its regulation of adenylyl cyclases (ACs), providing a critical link between the Ca\(^{2+}\) - and cAMP-signaling pathways (6). Four of the nine known ACs are Ca\(^{2+}\)-sensitive; in particular, adenylyl cyclase type 8 (AC8) is stimulated by Ca\(^{2+}\)/CaM through a mechanism that is not yet resolved. AC8 has two CaMBDs, an amphipathic helix in the N terminus and an IQ-like motif at the C terminus (7), but their role in Ca\(^{2+}\)/CaM regulation of AC8 is not known. What is clear is that the C-terminal CaMBD is essential for Ca\(^{2+}\) stimulation of AC8. Mutagenesis studies revealed that removal of the C terminus of AC8 produced a Ca\(^{2+}\)-insensitive enzyme, whereas removal of the N terminus of AC8 did not affect Ca\(^{2+}\)/CaM stimulation in vitro (7). This observation raises questions concerning the purpose of the additional CaMBD in the N terminus of AC8.

In the intact cell, Ca\(^{2+}\)-sensitive ACs are regulated by capacitative Ca\(^{2+}\) entry (CCE). CCE is a universally encountered mechanism of Ca\(^{2+}\) entry in non-excitable cells that is triggered by the emptying of IP\(_3\)-sensitive Ca\(^{2+}\) stores (8). Ca\(^{2+}\)-sensitive ACs show a unique dependence on CCE for their regulation by Ca\(^{2+}\) in vivo (6). Part of the dependence of AC8 on CCE relies on its presence in caveolae or lipid rafts (9). Caveolae are small invaginations within the lipid bilayer that are enriched with cholesterol and glycosphingolipids (10). Because Feron and colleagues (11) showed that a CaM-binding protein within nitric-oxide synthase contributed to its targeting to caveolae, the possibility was considered that the N terminus of AC8 might also serve a raft/caveolar-targeting purpose. However, deletion of the N-terminal CaMBD did not affect raft targeting, although the response to CCE was greatly diminished (12). No further attempt to understand the role of the N-terminal CaMBD of AC8 has been made, although the speculation was advanced that the N terminus of AC8 might be a CaM-tethering site (7). CaM certainly can pre-associate with some of its target proteins, prior to a rise in [Ca\(^{2+}\)\(_i\)]. For example, L, P/Q, and R type voltage-gated Ca\(^{2+}\) channels (VGCCs) all pre-associate with CaM in resting cells (13), and L type VGCCs can tether CaM (14).

Thus, in the present study, we explore the role played by the N-terminal CaMBD of AC8 in light of its putative function as a tether for CaM. We examine the significance of such tethering in CaM recruitment and consider how this role might be reconciled with the purpose of the second CaMBD located at the C terminus of AC8. We have used mutants of both AC8 and CaM to specifically target the interaction between the two proteins in functional assays and in binding studies. Our results allow the proposal of a tethering and recruiting role for the N terminus of AC8, which is both elegant and conservative in the use of a limiting amount of a universal mediator within the cell.

**EXPERIMENTAL PROCEDURES**

**Materials**—Thapsigargin and forskolin were from Calbiochem. \(^{[3H]}\)Adenine, \(^{[3H]}\)cAMP, \([\alpha-32P]\)ATP, glutathione-Sepharose 4B, ECL Western blotting analysis system, and horseradish peroxidase-conjugated goat anti-rabbit IgG were purchased from Amersham Biosciences. Horseradish peroxidase-conjugated anti-mouse IgG was from...
Promega (Madison, WI), Fura-2, Fura-FF, and Ca\(^{2+}\) standard solutions were obtained from Molecular Probes (Leiden, Netherlands). AC8 antibody was a gift from Dr. J. J. Cali, as previously described (15). CaM monoclonal antibody was from Upstate. All other reagents were obtained from Sigma (Poole, UK) unless stated otherwise.

**Cell Culture—**HEK293 cells were obtained from European Collection of Cell Cultures and were maintained in minimum essential medium supplemented with 50 \(\mu\)g/ml penicillin, 50 \(\mu\)g/ml streptomycin, and 100 \(\mu\)g/ml neomycin (all from Invitrogen), 10% (v/v) fetal bovine serum, and 2 mM L-glutamine. Cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO\(_2\).

**Transfection of HEK293 Cells—**Cells were grown overnight on 92-mm diameter dishes and transfected the following day at ~50% confluence, according to the calcium phosphate method described previously (16). Transfection reagent contained CaCl\(_2\) (250 mM), HEPES transfection buffer (274 mM NaCl, 10 mM KCl, 1.4 mM Na\(_2\)HPO\(_4\), 15 mM D-glucose, 42 mM HEPES, pH 7.4), and cDNA (2 \(\mu\)g for single transfections, 1.5 \(\mu\)g of AC8/AC8M1 with 2 \(\mu\)g of CaM/pCDNA3.1 for cotransfections). Cells were washed twice with phosphate-buffered saline (PBS, 12.1 mM Na\(_2\)HPO\(_4\), 4 mM KH\(_2\)PO\(_4\), 130 mM NaCl, at pH 7.4) 8–12 h after transfection.

**Preparation of HEK293 Cell Membranes—**Membranes were prepared 48 h after transfection by the following procedure. Cells were lysed in homogenization buffer (2 mM MgCl\(_2\), 50 mM Tris, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 \(\mu\)g of DNase I (from Amersham Biosciences), pH 7.4) by repeatedly passing the cell suspension through a 22-gauge needle. The cell lysate was then centrifuged at 200 × g (5 min, 4 °C). The supernatant was collected, and the process was repeated. The supernatant fractions from each homogenization were pooled and then centrifuged (23,000 × g, 15 min, 4 °C). Where the non-membrane fraction was required for Western blotting analysis, the supernatant was saved and supplemented with SDS (1% w/v). The membrane pellet was resuspended in either 1% SDS for Western blotting analysis or assay buffer (40 mM Tris, 1 mM EGTA, 0.25% (w/v) bovine serum albumin, pH 7.4) for adenyl cyclase activity measurements. Protein content was determined as previously described (17).

**Adenyl Cyclase Activity Measurements—**Adenyl cyclase activity was determined as described previously (18). Briefly, membranes prepared from transfected HEK293 cells were assayed in the presence of the following components: 12 mM phosphocreatine, 1.4 mM MgCl\(_2\), 0.04 mM GTP, 0.1 mM [\(^{32}\)P]ATP, 2.5 units of creatine phosphokinase, 70 mM Tris buffer, pH 7.4, 0.5 mM 3-isobutyl-1-methylxanthine, 250 \(\mu\)M EGTA, 1 \(\mu\)M CaM (as indicated), 0.5 \(\mu\)Ci of [\(^{32}\)P]ATP and Ca\(^{2+}\) (see “Determination of Free Ca\(^{2+}\) Concentrations” below). The reaction mixture (100 \(\mu\)l) was incubated at 30 °C for 20 min and terminated with the addition of 100 \(\mu\)l of stopper solution (0.5% w/v SDS, 22 mM ATP, 1.5 mM cAMP). \(^{32}\)P[cAMP (~3000 cpm)] was added as a recovery marker. The \(^{32}\)P[cAMP] formed was quantified as previously described (19).

**Determination of Free Ca\(^{2+}\) Concentrations—**Free Ca\(^{2+}\) concentrations were initially established from a series of CaCl\(_2\) solutions buffered with 250 \(\mu\)M EGTA in the assay, using the computer program BAD4 (20). Free Ca\(^{2+}\) concentration was then confirmed by spectrofluorometric measurements (PerkinElmer Life Sciences LS 50B spectrofluorometer) at 340 and 380 nm, the excitation wavelengths of the Ca\(^{2+}\)-sensitive dyes Fura-2 or Fura-FF. The Ca\(^{2+}\) concentration of each solution was calculated from the 340/380 nm fluorescence ratio by applying the Grynkiewicz equation (21).

**Measurement of Intracellular cAMP Accumulation—**cAMP accumulation was measured in transiently transfected HEK293 cells, 48 h after transfection. Cells were incubated (2 h, 37 °C) in minimum essential medium containing [\(^{3}H\)]adenine (0.5 \(\mu\)Ci/well) to label the ATP pool. Cells were washed once and incubated in 900 \(\mu\)l of nominally Ca\(^{2+}\)-free Krebs buffer (120 mM NaCl, 4.75 mM KCl, 1.44 mM MgSO\(_4\), 25 mM HEPES, 11 mM d-glucose, pH 7.4), supplemented with bovine serum albumin (1 mg/ml) for 20 min at 30 °C. Cells were preincubated with EGTA (100 \(\mu\)M) and 3-isobutyl-1-methyloxanthine (100 \(\mu\)M) for 10.5 min and thapsigargin (100 nM) for 4 min, to passively deplete endoplasmic reticulum Ca\(^{2+}\) stores. cAMP accumulation was measured over a 1-min period, started by addition of forskolin (10 \(\mu\)M) and various CaCl\(_2\) concentrations. The reaction was terminated by the addition of ice-cold trichloroacetic acid (5%, w/v). Samples were incubated at 4 °C at least 30 min before addition of unlabelled cAMP (1 \(\mu\)M), unlabelled ATP (0.65 \(\mu\)M), and [\(^{32}\)P]ATP (~5000 cpm), used as a recovery marker. Cells were centrifuged (4000 × g, 6 min), and the [\(^{3}H\)]cAMP and [\(^{32}\)P]ATP content of the supernatant was determined as described previously (19).

**Whole Cell [Ca\(^{2+}\)], Measurements—**[Ca\(^{2+}\)], were measured in cell populations using a PerkinElmer Life Sciences LS 50B spectrofluorometer as previously described. Briefly, cells were detached with PBS containing 0.01% EDTA and loaded with 2 \(\mu\)M Fura-2/AM plus 0.02% Pluronic F-127 for 45 min at room temperature. The cells were then washed twice, aliquoted into samples containing ~1 × 10\(^6\) cells, and finally resuspended in 3 ml of nominally Ca\(^{2+}\)-free Krebs buffer and placed in a stirred cuvette just prior to the experiment. For zero-Ca\(^{2+}\) Krebs buffer, 100 \(\mu\)M EGTA was added. Test substances were added from 100-fold concentrated stocks. Fura-2 340/380 nm fluorescence ratios were converted to [Ca\(^{2+}\)], values as described previously (21).

**Western Blotting—**Proteins were resolved using 7.5 and 12% w/v SDS-polyacrylamide gels for AC8 and CaM, respectively. AC8 gels were prepared in the presence of 8 M urea. Proteins were transferred to a polyvinylidene difluoride membrane. The polyvinylidene difluoride membrane was incubated in TBS (20 mM Tris, pH 7.5, 150 mM NaCl) containing 5% w/v skimmed milk powder for 30 min, followed by two 10-min washes in TBS supplemented with 0.05% (v/v) Tween 20 (TTBS). Membranes were incubated overnight at room temperature with anti-AC8 polyclonal antibody (1:5000) or anti-CaM monoclonal antibody (1:5000) in TTBS containing 1% skimmed milk powder (antibody buffer). The membranes were washed (3 × 10 min) in TTBS and then incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5000 dilution of stock) or goat anti-mouse IgG conjugated to horseradish peroxidase (1:3000) in antibody buffer for 1 h. Finally, the membranes were washed in TTBS (2 × 15 min), rinsed in TBS, and visualized with ECL reagent. The light intensity of each band was detected and quantified on the Gene Gnome Western blot imaging system (Syngene Bio Imaging) using Gene Snap software. The AC8 antibody detects both wild-type AC8 and the deletion mutant, AC8M1, and each protein is detected as two bands, representing the glycosylated and non-glycosylated forms of the enzyme. The CaM antibody detects the wild type and all mutant species of CaM.

**Production and Purification of His-tagged CaM—**The CaM mutant constructs were a gift from J. H. Caldwell (University of Colorado Health Sciences Center, Denver, CO). CaM mutants contain aspartate to alanine substitutions in either 1) the first and second EF-hand motifs (CaM\(_{12}\)) rendering the N lobe of CaM insensitive to Ca\(^{2+}\), 2) the third and fourth EF-hand motifs (CaM\(_{34}\)) rendering the C lobe of CaM insensitive to Ca\(^{2+}\), or 3) All four EF-hand motifs (CaM\(_{1234}\)) rendering both lobes of CaM insensitive to Ca\(^{2+}\). To obtain fragments containing either wild-type CaM or the desired CaM mutant, the cDNA was PCR-amplified and then digested using BamHI and Ncol restriction enzymes. The
CaM cDNA fragment was then ligated into the vector pQE30 (Qiagen, UK), which had also been digested with BamHI/NcoI. This added a His tag to the N terminus of each CaM construct. His-tagged CaM was produced in *Escherichia coli* cell cultures, which were induced at *A*$_{600}$ = 0.4 to produce the CaM protein with 2 molar isopropyl β-D-thiogalactopyranoside at 37°C. The *E. coli* cells were harvested by centrifugation (6,000 × *g*, 10 min) and resuspended in wash buffer (20 mM Tris, pH 7.6, 150 mM NaCl, 10 mM imidazole), and supplemented with lysozyme. Samples were gently mixed for 5 min at room temperature before sonication. The bacterial cell lysates were centrifuged (25,000 × *g*, 20 min, 4°C), and the supernatants were applied to a Talon metal affinity resin column (BD Biosciences). After several washes with wash buffer, the His-tagged CaM was eluted with sample buffer (10 mM Tris, 100 mM imidazole, pH 7.6). Protein concentration was determined following the method of Smith and colleagues (17).

**Preparation and Purification of GST and GST Fusion Proteins—**GST fusion proteins of the N terminus (GST-AC8Nt) and C terminus (GST-AC8Ct) of AC8 were generated. The N terminus of AC8 up to amino acid A97 was PCR-amplified from the full-length rat cDNA with primers that introduced an EcoRI site at the 5′-end. The product was digested with EcoRI/Sall and ligated into the pGEX4T-1 vector (Amersham Biosciences), which had also been digested with EcoRI/Sall. The C terminus of AC8, from amino acids Asp1106 to Pro1248 was generated following the same method. The fusion proteins were produced using bacterial cell cultures, which were induced and then harvested as described above (see “Production and Purification of His-tagged CaM”). Cell pellets were resuspended in PBS and supplemented with 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture (20 μM 4-(2-aminophenoxy)benzene sulfonyl fluoride, 10 μM EDTA, 1.3 mM benzamidine, 140 mM EDTA, 10 mM leupeptin, 3 mM aprotinin) and lysozyme. After sonication and addition of Triton X-100 (1% (v/v)), cell lysates were centrifuged (25,000 × *g*, 20 min, 4°C), and the supernatant was loaded onto glutathione-Sepharose beads. The GST-AC8 peptide bound to the beads was washed with PBS to remove contaminants and stored at 4°C until required.

**GST Pulldown Assay—**GST, GST-AC8Nt, or GST-AC8Ct glutathione-Sepharose beads were added to PBS supplemented with 1% (v/v) Triton X-100, 0.5 mM CaM, and either 20 μM CaCl$_2$ or 200 μM EGTA for high and low Ca$^{2+}$ condition, respectively. Samples were rotated for 2 h at 4°C, centrifuged (10,000 × *g*, 5 min, 4°C), and washed three times in PBS. Samples were analyzed by SDS-PAGE and Western blotting, using a CaM antibody, as described above (see “Western Blotting”).

**RESULTS**

The N Term of AC8 Recruits CaM to Mediate Ca$^{2+}$ Stimulation—It has been noted previously that the amphiphilic helix CaMBD within the N terminus of AC8 is not of obvious significance in *in vitro* adenyl cyclase activity assays. Deletion of the first 106 N-terminal amino acids of AC8 yielded an enzyme (AC8M1) that was fully stimulated by Ca$^{2+}$/CaM *in vitro* (7). The present study followed on from this observation by initially comparing the Ca$^{2+}$ stimulation of full-length AC8 and AC8M1 and their dependence on exogenous CaM. To address CaM recruitment by AC8, we evaluated our ability to deplete membrane CaM by our washing procedure, which used 1 mM EGTA. Washing the membranes in EGTA reduced the CaM content of the membrane preparation by ~80%, compared with washing in EGTA-free assay buffer (Fig. 1A). The activity of EGTA-washed membranes prepared from cells transfected with either AC8 or AC8M1 or untransfected cells was then compared *in vitro*. AC activity was stimulated by a range of [Ca$^{2+}$] in the presence and absence of exogenously applied CaM (1 μM). AC activity is a measure of cAMP converted from ATP. The maximum activity value produced by Ca$^{2+}$ stimulation depends on transfection efficiency. The greater the expression level of AC8, the higher the maximum production of cAMP that can be attained. Untransfected HEK 293 cells have a low basal activity, which is slightly inhibited by Ca$^{2+}$ in a CaM-independent manner, due to the presence of endogenous Ca$^{2+}$-inhibitable CaB in HEK293 cells (Fig. 1). Efficient transfection of AC8 is detected by the robust -fold stimulation of basal activity in response to Ca$^{2+}$/CaM, not the maximum activity value. The -fold stimulation ranged from 5- to 11-fold (mean = 7.9 ± 2; *n* = 7) for AC8 and 5- to 13-fold (mean = 8.0 ± 2.5; *n* = 7) for AC8M1. Fig. 1 shows a representative experiment of Ca$^{2+}$ stimulation of AC8 (Fig. 1B) and AC8M1 (Fig. 1C). The -fold stimulation in this example was 11-fold and 13-fold, respectively, and is in good agreement with earlier work (7). A Western blot confirms similar expression levels for each construct (Fig. 1E). The pattern of Ca$^{2+}$ stimulation was very similar for both constructs, with mean half-activation (*K*$_{d}$ value) occurring at 0.8 μM ± 0.2 and 1.2 μM ± 0.2 Ca$^{2+}$ (*n* = 3) for AC8 and AC8M1, respectively. Without exogenous CaM, Ca$^{2+}$ stimulation of AC8 was retained compared with untransfected cells but was reduced to only a 4-fold increase over basal activity (Fig. 1B). Typical Ca$^{2+}$ stimulation of AC8 in the absence of CaM ranged from 2.3- to 4-fold over basal (mean = 3.1 ± 0.7; *n* = 7). The mean *K*$_{d}$ value was 1.7 μM ± 0.2 (n = 3). The persistence of Ca$^{2+}$ stimulation of AC8 suggests that residual CaM had been retained by AC8, despite the extensive washing with EGTA. In contrast, AC8M1 activity was not enhanced above basal levels in the absence of added CaM (Fig. 1C, open circles), implying that any associated CaM had been removed. This finding suggests that, (i) the N terminus of AC8 associates with endogenous CaM and (ii) the CaM retained by the N terminus of AC8 can be utilized by the C terminus to mediate Ca$^{2+}$ stimulation *in vitro*, in the absence of added CaM. These data imply that the N terminus could be an excellent device for recruiting CaM.

If indeed the N terminus serves to recruit CaM to AC8, the CaMBD within the N terminus is the most likely site. However non-contiguous sequences surrounding a CaMBD can be involved in CaM interaction (22), so we considered the possibility that other amino acids of the N terminus could also participate. To delimit the CaM recruitment site specifically to the obvious CaMBD, a second AC8 mutant (AC8M34) was explored. The helical CaMBD of AC8 is located between residues 34 and 51. In AC8M34, 6 of these amino acids, specifically tryptophan, glutamine, threonine, arginine, phenylalanine, and isoleucine, have all been substituted to alanine, rendering the CaMBD very unlikely to bind CaM (12). The AC8M34 mutant was expressed in HEK293 cells, and activity was measured in EGTA-washed membrane preparations. AC8M34 was stimulated by CaCl$_2$ in the presence and absence of CaM. The Ca$^{2+}$-concentration effect profile of AC8M34 was the same as AC8M1, with 7-fold Ca$^{2+}$ stimulation in the presence of exogenous CaM (Fig. 1D). The *K*$_{d}$ value was higher (1.9 μM) than the mean *K*$_{d}$ for AC8M1 (1.2 μM) but not significantly so. AC8M34 activity was not stimulated above basal levels in the absence of CaM. The similar dependence of AC8M1 and AC8M34 on CaM for Ca$^{2+}$ stimulation establishes that the N-terminal CaMBD is the principal CaM binding site within the N terminus of AC8.

The N Term of AC8 Is Essential for Ca$^{2+}$ Stimulation *in Vivo*—The N terminus of AC8 evidently binds to CaM, which can then be used to activate AC8. However, this recruitment is redundant *in vitro* when CaM is provided and is in excess. *In vitro* measurements do not reflect the environment that AC8 experiences within the intact cell, where free CaM levels are regulated and limited (23). Our initial results prompted the question of whether AC8M1 would be active in the intact cell, where
the free CaM concentration is restricted. To resolve this issue, whole cell cAMP accumulation was measured in response to CCE, using HEK293 cells that had been transiently transfected with either AC8 or AC8M1 and compared with untransfected cells. EGTA and 3-isobutyl-1-methylxanthine were added to chelate extracellular Ca\(^{2+}\) and inhibit phosphodiesterase activity, respectively. The sarcoplasmic reticulum Ca\(^{2+}\)-ATPase was inhibited with thapsigargin to passively deplete intracellular Ca\(^{2+}\) stores, priming the cells for CCE upon addition of Ca\(^{2+}\) to the extracellular medium (see “Experimental Procedures”). Fig. 2A (panel (i)) shows the increase in [Ca\(^{2+}\)]\(_i\) following activation of CCE. AC8 was stimulated by Ca\(^{2+}\) in a concentration-dependent manner compared with untransfected cells, which did not respond to CCE. AC8M1, however, had lower basal activity and was not significantly stimulated by the addition of up to 4 mM Ca\(^{2+}\) (Fig. 2A, panel (ii)). These data reveal that the N terminus of AC8 is essential for Ca\(^{2+}\) stimulation and support the proposal that the N terminus is needed to recruit CaM for AC8 activation.

We next addressed the relative importance of CaM recruitment by the N terminus. Because application of exogenous CaM permits full activity of AC8M1 in vitro, we wondered whether overexpression of CaM in vivo would rescue AC8M1 activity, thereby overcoming the absence of CaM recruited by the N terminus. AC8 and AC8M1 were co-transfected with cDNA-encoding CaM. Western blotting analysis assessed the relative expression levels of AC8, AC8M1, and CaM. When CaM was transfected, global CaM expression increased by 50–100%, compared with untransfected cells (Fig. 2B). The expression of AC8 or AC8M1 was not affected when co-expressed with CaM (Fig. 2C). Under these conditions, CCE produced an equivalent dose-dependent increase.
Calmodulin Regulation of AC8

Partial Liganded Ca2+/CaM Can Bind to AC8—The C-terminal CaM-binding domain (CaMBD) of AC8 is essential for Ca2+ sensitivity, but it is presently unknown how these two CaMBDs co-operate to mediate Ca2+ stimulation of AC8. One possibility is that a single CaM molecule is recruited by the N terminus of AC8 and then passed over to the C terminus. However, before we can speculate further, a greater understanding of how CaM interacts with each individual CaMBD might illuminate the overall mechanism of Ca2+/CaM activation of AC8. CaM is a dynamic molecule, with two lobes that can act independently and bind to different sequences within a target protein. The presence or absence of Ca2+, at least partially, contributes to how and where CaM binds (4). Each lobe contains two EF-hand Ca2+ binding motifs, specifically residues 20–31 and 56–67 in the N lobe, and residues 93–104 and 129–140 in the C lobe (24). To discover how CaM associates with the two CaMBDs of AC8, we prepared three recombinant CaM mutants from E. coli to determine whether CaM with Ca2+ bound to a single lobe or neither lobe could bind to either CaMBD of AC8. CaM1234 contains an aspartate to alanine substitution mutation in the first position of each EF-hand motif i.e. at positions 20, 56, 93, and 129, so that each of the four binding motifs are altered, rendering them at least 100-fold less sensitive to Ca2+ (25, 26). The N lobe mutant of CaM contains the substitution mutation at amino acid 20 and 56 only (CaM12), whereas the C lobe mutant contains a substitution at amino acid 93 and 129 only (CaM134) (27, 28).

We performed pulldown binding studies with the three CaM mutants and the two CaMBDs of AC8 by generating a GST fusion protein of either the N terminus (GST-AC8Nt) or C terminus (GST-AC8Ct) of AC8. Each fusion protein was incubated with wild-type CaM (CaMwt), CaM12, CaM134, or CaM1234 in the presence of high or low Ca2+ (see “Experimental Procedures”). CaMwt bound to the N terminus of AC8 in both high and low Ca2+ conditions, but binding appears to be greater at the higher Ca2+ condition (Fig. 3A). This Ca2+-dependent binding is not unexpected considering that the CaMBD within the N terminus of AC8 is an amphipathic helix, a motif that predominantly exhibits Ca2+-dependent binding (5). Interestingly, CaM12, which can only bind Ca2+ at the C lobe, also bound to the N terminus of AC8 in a Ca2+-enhanced manner. Under all conditions, binding of CaMwt and CaM134 to the N terminus was significant compared with binding to the GST alone control (see Fig. 3A). Therefore, CaM only requires Ca2+ at its C lobe to bind to the N terminus of AC8. CaM134 and CaM1234 did not bind to the N terminus over a 1-min assay, using HEK293 cells transiently transfected with AC8 and pcDNA3.1 vector, AC8M1, and pcDNA3.1 or untransfected cells. Data are plotted as mean ± S.D. and are representative of five separate experiments, with similar results. B, cytosolic fractions from cells assayed for cAMP accumulation were loaded on SDS-PAGE (25 µg/lane) and probed using a CaM antibody. Bands were quantified by measuring the relative light intensity of each signal (as described under “Experimental Procedures”). C, in A, panel (ii), except cells expressing AC8 and pcDNA3.1 (black bars); AC8 and CaM (gray bars); AC8M1 and pcDNA3.1 (white bars); or AC8M1 and CaM (hatched bars).
Calmodulin Regulation of AC8

**A**

| CaMwt | CaM12 | CaM14 | CaM34 |
|-------|-------|-------|-------|
| GST-AC8Nt | GST-AC8Ct | GST-AC8Nt | GST-AC8Ct |
| High Ca²⁺ | High Ca²⁺ | Low Ca²⁺ | Low Ca²⁺ |

**B**

| CaM12 | CaM14 | CaM1234 |
|-------|-------|---------|
| GST-AC8Ct | GST-AC8Ct | GST-AC8Ct |
| High Ca⁺ | High Ca⁺ | Low Ca⁺ |

**FIGURE 3. Binding of mutant and wild-type CaM to the two CaMBDs of AC8.** Equal volumes of GST fusion protein-bound beads, of either A, the N-terminal (GST-AC8Nt) or B, the C-terminal (GST-AC8Ct) CaMBD of AC8 were used to pull down CaM from solution, as an indication of whether CaMwt or mutant CaM could bind. All binding events were compared with nonspecific binding between CaM and the GST tag alone. Experiments were performed in high Ca²⁺ (20 μM CaCl₂) or low Ca²⁺ (200 μM EGTA) and were all in the presence of the individual CaM species (0.5 μM). Samples were analyzed by Western blotting and probed for CaM. The band intensity was quantified using GeneGnome software. CaM input was measured to ensure an equal concentration of CaM was added to each pulldown experiment (quantification not shown).

...of AC8 in either high or low Ca²⁺ compared with the GST alone control (Fig. 3A), suggesting that CaM with Ca²⁺ at only the N lobe is not sufficient for CaM to bind to the N terminus.

The C terminus of AC8 contains an IQ-like motif, which differs from a classic IQ motif, because it contains only the first half of the motif, namely (F/L/V/I)QXXX(R/K) (29). Whereas IQ motifs are commonly Ca²⁺-independent CaMBDs, IQ-like motifs can display Ca²⁺ dependence. CaMwt bound to the C terminus of AC8 in both high and low Ca²⁺ with apparently equal affinity (Fig. 3B), whereas CaM12 bound strongly to the C terminus in a Ca²⁺-enhanced manner. CaM34 could bind only very weakly, in high Ca²⁺ conditions, whereas CaM1234 did not bind under either condition. CaM with Ca²⁺ at only the C lobe of CaM can clearly bind to the C terminus of AC8, whereas CaM with Ca²⁺ at only the N lobe binds very weakly.

In summary, the N terminus of AC8 only binds to CaM that has Ca²⁺ loaded at the C lobe or both lobes of CaM. Similarly, the C terminus of AC8 also only binds strongly to CaM with Ca²⁺ loaded at the C lobe or both lobes, but in addition, the C terminus of AC8 binds weakly to CaM with Ca²⁺ loaded only at the N lobe.

**Only Fully Liganded Ca²⁺/CaM Stimulates AC8—Partially liganded Ca²⁺/CaM can mediate Ca²⁺-dependent effects of some target proteins.** For example, the gating mechanism of Ca²⁺-activated K⁺ channels is regulated by CaM with Ca²⁺ bound at only the N lobe (27). Similarly, Bordetella pertussis AC exotoxin only requires CaM with Ca²⁺ bound to the C lobe for activation (30). After discovering that partially liganded CaM can bind to AC8, we wondered whether Ca²⁺ stimulation of AC8 could also be mediated by CaM that only has Ca²⁺ bound to a single lobe. To address this issue, adenylyl cyclase activity was measured in vitro, in the presence of mutant CaM.

As described above, the presence of Ca²⁺ stimulation in AC8-transfected cells indicates that AC8 is being expressed. The -fold stimulation of AC8 activity over basal (and not the maximum value of activity) indicates the degree of Ca²⁺/CaM stimulation. Full stimulation of AC8 elicits a 5- to 11-fold increase in activity (mean = 7.9 ± 2; n = 7). In the absence of exogenous CaM, AC8 shows a 2.3- to 4-fold increase in activity over basal (mean = 3.1 ± 0.7; n = 7), mediated by endogenous (pre-bound) HEK293 cell CaM.

CaM1234, which cannot effectively bind Ca²⁺ at either lobe, did not permit Ca²⁺ stimulation of AC8 in vitro (Fig. 4A). AC8 activity was only increased 2.4-fold in the presence of CaM1234, compared with 7-fold in the presence of CaMwt. The 2.4-fold increase is consistent with stimulation mediated by endogenous CaM, bound to the N terminus of AC8, as seen in Fig. 1B. To confirm that this small stimulation was the action of endogenous CaM and not CaM1234, CaM81 activity was measured in vitro, in the presence of CaMwt or CaM1234. CaMwt mediated full activation of AC8M1 in response to Ca²⁺ (8-fold stimulation), whereas AC8M1 activity remained at basal levels in the presence of CaM1234 (Fig. 4B), thus confirming that CaM1234 was not active.

The C lobe of CaM34 is mutated so that only the N lobe can bind Ca²⁺. In the presence of CaM34, Ca²⁺ only increased AC8 activity by 3-fold (Fig. 4C). Once again, this weak stimulation of AC8 can be accounted for by endogenous CaM retained by AC8, which was confirmed by the inability of CaM12 to stimulate AC8M1 activity above basal, in response to Ca²⁺ (Fig. 4D).

Finally, CaM12 was used to mediate Ca²⁺ stimulation of AC8. Only the C lobe of CaM12, effectively binds to Ca²⁺, and with this mutant AC8 activity was only very weakly stimulated by Ca²⁺. In fact, AC8 activity increased by <50% above basal (Fig. 4C). This value is lower than the average 3-fold increase produced by endogenous CaM, suggesting that CaM12 inhibits the action of endogenous CaM. To confirm the inhibitory action of CaM12, on
endogenous CaM, AC8 was stimulated by 2.5 μM Ca2+ in the presence of CaM12 or CaM1234. In the presence of CaM12, AC8 activity was reduced in a concentration-dependent manner, whereas CaM1234 had no effect on AC8 activity (Fig. 4E). Furthermore, CaM12 did not mediate Ca2+ stimulation of AC8M1 in vitro (Fig. 4D).

The data show that only CaMwt is functional in the in vitro assay. As none of the CaM mutants were able to mediate Ca2+ stimulation of AC8 in vitro, we conclude that AC8 is only regulated by fully liganded Ca2+/CaM, where both the N and C lobes of CaM are bound to Ca2+. Furthermore, because AC8M1, which lacks the N terminus, is only fully stimulated by CaMwt, it suggests that ultimately both lobes of CaM must be loaded with Ca2+ and bind the C terminus of AC8 to mediate activation of AC8.

CaM12 Inhibits Ca2+ Stimulation of AC8 in Vivo—These results described above imply an early association between AC8 and CaM, well before the enzyme is stimulated by Ca2+. This pre-association of target protein with CaM has already been described with voltage-gated Ca2+ channels (VGCCs). For example, when P/Q-type VGCCs are co-transfected with CaM12 or CaM34, the CaM mutant represses Ca2+-dependent inactivation or facilitation of the channel, respectively (31). These mutants pre-associate with the channel prior to Ca2+ entry, so that, upon Ca2+ influx through the channel, the Ca2+ signal is not effectively transduced (13). We have shown that partially liganded CaM can bind to AC8, even though only fully liganded CaM can mediate Ca2+ stimulation of AC8. Therefore, if AC8 does pre-associate with CaM, we might expect one or more of the CaM mutants to have a similar repressive effect on AC8 activity. As seen for VGCCs in vivo. To determine whether AC8 can pre-associate with CaM, AC8 was co-transfected with CaMwt, CaM12, CaM34, or CaM1234. Whole cell cAMP accumulation was measured in response to CCE stimulation. As before, AC8 was stimulated by Ca2+ in a concentration-dependent manner (Fig. 5A). Co-expressing AC8 with CaMwt, CaM12, or CaM1234 did not alter the response of AC8 to Ca2+. Under these three conditions, AC8 activity, in the presence of CaM, was comparable to control AC8 activity (co-transfected with empty vector). However, when AC8 was co-expressed with CaM12, Ca2+ stimulation of AC8 was clearly reduced. AC8 was still stimulated by Ca2+ in a concentration-dependent manner, but AC8 activity was ~50% lower in the presence of CaM12 compared with con-
Calmodulin Regulation of AC8

FIGURE 5. Effect of CaMwt and CaM mutants on CCE stimulation of AC8 in vivo. The effect of CCE on cAMP accumulation, measured over a 1 min assay, using HEK293 cells transiently transfected with AC8 and either pDNA3.1, CaMwt, CaM12, CaM34, or CaM1234 as indicated (A). Data are plotted as mean ± S.D. and are representative of three separate experiments, with similar results. Data have been analyzed using one-way analysis of variance followed by Newman-Keuls multiple comparison test. *, data are statistically significant (p < 0.05). **, data are statistically significant (p < 0.01). Extracts from the same batch of cells assayed in A were loaded on SDS-polyacrylamide gel (25 μg/lane) and probed and quantified as described under “Experimental Procedures.” B, CaM antibody against cytosol fraction. C, as in A, except cells expressing AC8M1 instead of AC8. D, cells from the same batch as those assayed in C were probed to determine CaM overexpression.

discussion

Ca2+/CaM stimulation of AC8 during Ca2+ entry is eliminated by deleting the first 106 amino acids from the N terminus of AC8 (7). Subsequent work revealed that the loss of sensitivity was not due to alterations in targeting of AC8 to lipid rafts, which is essential for regulation by CCE (12). Recent work has shown that the cytosolic Ca1 and Ca2a domains are in fact responsible for targeting Ca2+-stimulated ACs to caveolae (32). Thus, our study aimed to elucidate the role of the N terminus in Ca2+/CaM stimulation of AC8.

We have found that the N terminus of AC8 is associated with CaM, because Ca2+/CaM stimulation could only be ablated by the removal of the N terminus of AC8 and not by severe depletion of membrane CaM by EGTA, which suggests that the N terminus of AC8 can function as a CaM-tethering site. Furthermore, we have shown that mutating the N-terminal CaMBD prevents CaM from binding, which delimits the tethering site to the CaMBD alone. Interestingly, the N-terminal CaMBD of AC8 is analogous to the CaM-tethering site identified in the α1C subunit of L-type VGCCs. Both are classified as type A CaMBDs with conserved hydrophobic residues at positions 1, 8, and 14 within the motif (5, 14).

When CaM was not included in vitro, Ca2+ stimulation of AC8 was absolutely dependent on the presence of the N-terminal CaMBD, which strongly supports the hypothesis that the N terminus of AC8 pre-associates with CaM for the activation of AC8. Pre-association with CaM is highly beneficial, if not essential, considering that in the intact cell, the free CaM concentration is constrained, with CaM-binding proteins outnumbering free CaM by a factor of 2. The global free CaM concentration is ~45 nm, compared with a total CaM concentration of 10 μM, compelling evidence that the majority of CaM is sequestered, tethered, or compartmentalized and not freely available to target proteins (23).

In vivo, we found that AC8M1 was insensitive to CCE triggered by addition of Ca2+ up to 4 mM. This effect had been observed previously but not understood (12). AC8M1 may simply be CaM-deficient in vivo because it lacks the N terminus to recruit CaM. However, increasing the free CaM concentration by expression of additional CaM did not rescue AC8M1 and restore Ca2+ stimulation. One explanation for this observation is that AC8M1 is blind to the increase in CaM concentration, possibly due its localization to caveolar microdomains (12). However, in opposition to this argument, the response of AC8 to CCE was reduced by 50% when CaM12 was overexpressed, suggesting that AC8 is exposed to the additional CaM that is expressed. Consequently, an alternative explanation for the poor stimulation of AC8M1 by Ca2+ in vivo, regardless of the CaM concentration, is that AC8 recruits CaM before being localized to caveolae. The N terminus of AC8 must recruit CaM from overexpression. CaM expression levels were measured to confirm that transfection of each CaM species did enhance the cellular immunoreactive CaM concentration above basal (Fig. 5B). As before, overexpression of CaM did not alter AC8 expression (data not shown). The inhibitory effect of CaM12 on Ca2+ stimulation of AC8 in vivo suggests that AC8 can pre-associate with CaM12, and, therefore, AC8 does pre-associate with endogenous CaM.

An analogous experiment with AC8M1 was performed to determine any affects of the CaM mutants on AC8M1 activity in vivo. AC8M1 was not stimulated by physiological concentrations of Ca2+ from CCE produced by addition of up to 4 mM Ca2+ (Fig. 5C). Co-expressing AC8M1 with wild-type or mutant CaM did not change the response of AC8M1 to Ca2+, and AC8M1 activity remained at basal levels. Once again, overexpression of the various CaM species was confirmed by Western blot analysis (Fig. 5D). AC8M1 expression again remained consistent in the presence of additional CaM (data not shown).
the endogenous-free pool, priming AC8 to respond to an increase in \([\text{Ca}^{2+}]_i\). AC8M1, which lacks the N terminus, cannot recruit CaM, regardless of the CaM concentration. Therefore, considering the \textit{in vivo} and \textit{in vitro} data together, the N terminus could be a tool for recruiting CaM that is necessary for activation of AC8. Another possibility is that the N terminus of AC8 has an indirect effect and modulates the interaction of CaM with the C-terminal CaMBD. If so, when the N terminus is removed, the C terminus cannot effectively bind to CaM, and AC8 is therefore not activated. However, this proposal ignores the fact that the N terminus contains a seemingly high affinity CaMBD, which is more likely to bind CaM directly, rather than have a secondary, modulating role. Taken together, the proposed model of CaM recruitment is the most likely. As shown in Fig. 3, there is a direct interaction between CaM and the N (and C) terminal CaMBD of AC8, which consolidates preliminary binding studies between expressed parts of AC8 and CaM.

Our approach examinesCaM and the isolated CaMBDs of AC8, but we do not wish to rule out the possibility that other domains of AC8, or indeed other proteins help mediate the interaction between CaM and AC8. Further \textit{in vitro} protein-protein binding studies are needed to confirm whether other domains of AC8 and/or other proteins may participate in, or stabilize, the AC8-CaM complex.

CaM recruitment may be an essential mechanism to ensure that AC8 always has a supply of CaM, particularly because AC8 is finally located to caveolae, where the available CaM concentration may be very low. The CaM initially recruited by AC8 may never fully dissociate from the enzyme, which is advantageous, considering the number of other proteins that compete for CaM. For example, proteins with a high affinity for CaM, such as the caveolar protein endothelial nitric-oxide synthase, use CaM at the expense of other, lower affinity CaM-binding proteins (33). Pre-recruitment of CaM by the N terminus of AC8 would be a highly efficient device to permit rapid activation of the enzyme following an increase in \([\text{Ca}^{2+}]_i\).

We were interested to know how CaM recruited at the N terminus, impacts on the regulation of \([\text{Ca}^{2+}]_i\) stimulation at the C terminus. \textit{In vivo} binding studies reveal how CaM interacts with each CaMBD, and we found that CaM binds to the C lobe of CaM is sufficient for CaM to bind to both CaMBDs of AC8. The C lobe of CaM may, therefore, be the principal, or at least the initial site on CaM that associates with AC8. The association of AC8 with partially liganded CaM supports a mechanism where AC8 can pre-associate with CaM prior to \([\text{Ca}^{2+}]_i\) entry. The C lobe of CaM has a higher affinity for \([\text{Ca}^{2+}]_i\) than the N lobe (\(K_D = 0.2 \mu M\) and 2 \(\mu M\), respectively) so the C lobe may be CaM-loaded while the N lobe is \([\text{Ca}^{2+}]_i\)-free. However, these values are obtained from unbound CaM, and binding of CaM to AC8 will almost certainly alter the affinity of CaM for \([\text{Ca}^{2+}]_i\), as has been reported previously for other CaM-regulated proteins (34). Determining the effect of the N- and C-terminal CaMBD on the affinity of CaM for \([\text{Ca}^{2+}]_i\) by means of stopped flow analysis could be an insightful strategy in resolving this issue (35). Pre-association of AC8 with CaM prior to \([\text{Ca}^{2+}]_i\) stimulation would prime AC8 to respond rapidly to an increase in \([\text{Ca}^{2+}]_i\).

Although partially liganded \([\text{Ca}^{2+}]_i\)/CaM can bind to AC8, our \textit{in vitro} functional assays clearly demonstrate that only fully liganded \([\text{Ca}^{2+}]_i\)/CaM can mediate activation of AC8. Therefore, \([\text{Ca}^{2+}]_i\) is ultimately essential at both lobes of CaM so that a distinction can be made between partially liganded CaM prior to activation and fully liganded CaM during AC8 activity. AC8M1, which lacks the N-terminal CaMBD, is also only stimulated by fully liganded \([\text{Ca}^{2+}]_i\)/CaM, which indicates that it is specifically the C-terminal CaMBD of AC8 that requires fully liganded \([\text{Ca}^{2+}]_i\)/CaM. Such information, coupled with the poor response of AC8M1 to \([\text{Ca}^{2+}]_i\) \textit{in vivo}, supports a mechanism whereby CaM at the N terminus of AC8 is passed over to the C terminus. In this scenario, only a single CaM molecule is needed, a proposal that is in agreement with regulation of L-type VGCCs, which also only require a single CaM (36).

Work from the Yue group (31) has shown that CaM12 inhibits \([\text{Ca}^{2+}]_i\)-dependent inactivation of P/Q type VGCCs, when channel and CaM12 are co-transfected. Co-expressing CaM12 with the L-type VGCC also ablates \([\text{Ca}^{2+}]_i\)-dependent inactivation (28). These findings led to the hypothesis that CaM could pre-associate with VGCCs. Similarly, the ability of CaM12 to bind to and inhibit AC8 activity is evidence that CaM can pre-associate with AC8. It was not surprising that CaM12 was the only CaM mutant to affect \([\text{Ca}^{2+}]_i\) stimulation of AC8 \textit{in vitro}, because CaM12 was the only CaM mutant that bound strongly to AC8 and subsequently, the only mutant that was able to pre-associate with AC8. Interestingly, CaM12 also acted as an inhibitor \textit{in vitro}, possibly by replacing endogenous CaM bound to the N terminus or via direct access to the C-terminal CaMBD, which could occur \textit{in vitro}, allowing CaM12 to act as a competitive antagonist to endogenous CaM tethered at the N terminus.

Based on the present findings and previously published data, we can present a model for the activation of AC8 by \([\text{Ca}^{2+}]_i$/CaM. Previous work showed that when both CaMBDs are deleted, basal activity of AC8 is greatly increased and cannot be stimulated further \textit{in vivo} (7). This observation suggests that the N and C termini prevent binding of ATP or synthesis of cAMP by steric hindrance, so that removal of these domains produces a constitutively active enzyme. The C terminus
Calmodulin Regulation of AC8

appeared to be the main cause of the steric hindrance (7). AC8 would therefore be auto-inhibited in the basal state. CaM may regulate AC8 activity by triggering a conformational change within the N and C termini of AC8 that relaxes the auto-inhibition. Our data suggests that the contribution the N terminus makes to the obstruction of ATP binding is minimal. If the N terminus was involved in sterically hindering ATP binding, we would expect AC8M34 to have a significantly lower sensitivity to Ca2+ than AC8, but this was not observed. Furthermore, AC8M1 is not active in the absence of CaM. If the N terminus prevented catalysis, then removal of the N terminus should partially relieve auto-inhibition to produce some activity in the absence of CaM, but this was not evident.

Relief of auto-inhibition is considered one of the main mechanisms of CaM mediated regulation of CaM-dependent enzymes, such as CaM kinase II, myosin light chain kinase (37), certain PDE1A splice variants and CaM mediated regulation of CaM-dependent enzymes, such as CaM kinase II, myosin light chain kinase (37), certain PDE1A splice variants. The exact structure that AC8 adopts is unknown, so Fig. 6 may serve to explore the intramolecular interactions of AC8 and consider whether auxiliary proteins might be involved in mediating the interaction between AC8 and CaM.

The current model adds to a growing number of examples where CaM pre-associates with the target protein prior to an increase in [Ca2+]i. As well as VGCCs (13), voltage-gated Na+ channels (39), small conductance Ca2+ -activated K+ channels (40), and cyclic nucleotide-gated channels are all permanently associated with CaM (41). The previously unknown mechanism of Ca2+ stimulation of AC8 is now being resolved and is emerging as an elegant example of how CaM can integrate Ca2+ and CaM signaling.

Acknowledgments—We are grateful to Nanako Masada for her contribution on the Ca2+ measurements and Dr. D. Willoughby for her careful reading and suggestions on the manuscript.

REFERENCES

1. Fodceich, K. P., and Ikura, M. (2002) Cell 108, 739–742
2. Persechini, A., and Kretsinger, R. H. (1988) J. Biol. Chem. 263, 12175–12178
3. Jarud, I. A., Chockalingam, P. S., and Jarrett, H. W. (1999) Physiol. Rev. 79, 661–682
4. Vetter, S. W., and Leclerc, E. (2003) Eur. J. Biochem. 270, 404–414
5. Rhoads, A. R., and Friedman, F. (1997) FASEB J 11, 331–340
6. Cooper, D. M. (2003) Biochem. J. 375, 517–529
7. Gu, C., and Cooper, D. M. F. (1999) J. Biol. Chem. 274, 8012–8021
8. Putney, J. W. (1986) Cell Calcium 7, 1–12
9. Fagan, K. A., Smith, K. E., and Cooper, D. M. F. (2000) J. Biol. Chem. 275, 26530–26537
10. Ionen, E., and Simons, K. (1998) Sem. Cell Dev. Biol. 9, 503–509
11. Feron, O., Saldana, F., Michel, J. B., and Michel, T. (1998) J. Biol. Chem. 273, 3125–3128
12. Smith, K. E., Gu, C., Fagan, K. A., Hu, B., and Cooper, D. M. F. (2002) J. Biol. Chem. 277, 6025–6031
13. Erickson, M. G., Alseikhan, B. A., Peterson, B. Z., and Yue, D. T. (2001) Neuron 31, 973–985
14. Pitt, G. S., Zuliffe, R. D., Hudson, A., Schulman, H., Reuter, H., and Tsien, R. W. (2001) J. Biol. Chem. 276, 30794–30802
15. Cali, J. J., Parekh, R. S., and Krupinski, J. (1996) J. Biol. Chem. 271, 1089–1095
16. Chen, C., and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
17. Smith, P. K., Krohn, R. L., Herrmann, G. T., Maika, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1995) Anal. Biochem. 150, 76–85
18. Boyajian, C. L., Garrisen, A., and Cooper, D. M. F. (1991) J. Biol. Chem. 266, 4995–5003
19. Salomon, Y., Londos, C., and Rodbell, M. (1974) J Biol. Chem 249, 8830–8838
20. Xiong, L., Kleerekoper, Q. K., He, R., Putkey, J. A., and Hamilton, S. L. (2005) J. Biol. Chem. 280, 7070–7079
21. Persechini, A., and Cronk, B. (1999) J. Biol. Chem. 274, 6827–6830
22. Babu, Y. S., Sack, J. S., Greenhough, T. J., Bugg, C. E., Means, A. R., and Cook, W. J. (1985) Nature 315, 37–40
23. Geiser, J. R., van Tuiinen, D., Brockerhoff, S. E., Neff, M. M., and Davis, T. N. (1991) Cell 65, 949–959
24. Xia, X. M., Fakler, B., Rivard, A., Wayman, G., Johnson-Pais, T., Keen, J. E., Ishii, T., Hirschberg, B., Bond, C. T., Lutsenko, S., Maylie, J., and Adelman, J. P. (1998) Nature 395, 503–507
25. Keen, J. E., Khawaled, R., Farrens, D. L., Neelands, T., Rivard, A., Bond, C. T., Janowsky, A., Fakler, B., Adelman, J. P., and Maylie, J. (1999) J. Neurosci. 19, 8830–8838
26. Peterson, B. Z., DeMaria, C. D., Adelman, J. P., and Yue, D. T. (1999) Neuron 22, 549–558
27. Munshi, H. G., Burks, D. J., Joyal, L. J., White, M. F., and Sacks, D. B. (1996) Biochemistry 35, 15883–15889
28. Drum, C. L., Yan, S. Z., Sarac, R., Mabuchi, Y., Beckingham, K., Bohn, A., Grabarek, Z., and Tang, W. J. (2000) J. Biol. Chem. 275, 36334–36340
29. DeMaria, C. D., Soong, T. W., Alseikhan, B. A., Alvania, R. S., and Yue, D. T. (2001) Nature 411, 481–489
30. Crosswhite, A. J., Seebacher, T., Masada, N., Ciruela, A., Dufrax, K., Schultz, J. E., and Cooper, D. M. (2005) J. Biol. Chem. 280, 6380–6391
31. Tran, Q. K., Black, D. J., and Persechini, A. (2003) J. Biol. Chem 278, 24247–24250
32. Brown, S. E., Martin, S. R., and Bayley, P. M. (1997) J. Biol. Chem. 272, 3383–3397

2 R. E. Simpson, A. Ciruela, T. Rayner, and D. M. F. Cooper, unpublished observation.
35. Gaertner, T. R., Putkey, J. A., and Waxham, M. N. (2004) *J. Biol. Chem.* **279**, 39374–39382
36. Mori, M. X., Erickson, M. G., and Yue, D. T. (2004) *Science* **304**, 432–435
37. Krueger, J. K., Gallagher, S. C., Zhi, G., Geguchadze, R., Persechini, A., Stull, J. T., and Trewhella, J. (2001) *J. Biol. Chem.* **276**, 4535–4538
38. Sonnenburg, W. K., Seger, D., Kwak, K. S., Huang, J., Charbonneau, H., and Beavo, J. A. (1995) *J. Biol. Chem.* **270**, 30989–31000
39. Kim, J., Ghosh, S., Liu, H., Tateyama, M., Kass, R. S., and Pitt, G. S. (2004) *J. Biol. Chem.* **279**, 45004–45012
40. Schumacher, M. A., Rivard, A. F., Bachinger, H. P., and Adelman, J. P. (2001) *Nature* **410**, 1120–1124
41. Bradley, J., Bonigk, W., Yau, K. W., and Frings, S. (2004) *Nat. Neurosci.* **7**, 705–710