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Distinct Mechanisms of Regulation by Ca^{2+}/Calmodulin of Type 1 and 8 Adenylyl Cyclases Support Their Different Physiological Roles*[S]

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Nine membrane-bound mammalian adenylyl cyclases (ACs) have been identified. Type 1 and 8 ACs (AC1 and AC8), which are both expressed in the brain and are stimulated by Ca^{2+}/calmodulin (CaM), have discrete neuronal functions. Although the Ca^{2+} sensitivity of AC1 is higher than that of AC8, precisely how these two ACs are regulated by Ca^{2+}/CaM remains elusive, and the basis for their diverse physiological roles is quite unknown. Distinct localization of the CaM binding domains within the two enzymes may be essential to differential regulation of the ACs by Ca^{2+}/CaM. In this study we compare in detail the regulation of AC1 and AC8 by Ca^{2+}/CaM both *in vivo* and *in vitro* and explore the different role of each Ca^{2+}-binding lobe of CaM in regulating the two enzymes. We also assess the relative dependence of AC1 and AC8 on capacitative Ca^{2+} entry. Finally, in real-time fluorescence resonance energy transfer-based imaging experiments, we examine the effects of dynamic Ca^{2+} events on the production of cAMP in cells expressing AC1 and AC8. Our data demonstrate distinct patterns of regulation and Ca^{2+} dependence of AC1 and AC8, which seems to emanate from their mode of regulation by CaM. Such distinctive properties may contribute significantly to the divergent physiological roles in which these ACs have been implicated.

Nine membrane-bound mammalian adenylyl cyclases (ACs),[2] AC1–AC9, have been identified (1). They possess a common predicted structure (2)[3] and are stimulated by forskolin (FSK; except AC9) and $G_{s\alpha}$, although they are distributed and regulated differently (1, 3, 4). Four ACs are regulated by physiological concentrations of Ca^{2+} and thereby provide a critical link between the Ca^{2+}- and cAMP-signaling pathways (3, 5); AC5 and AC6 are directly inhibited by Ca^{2+}, whereas AC1 and AC8 are stimulated by Ca^{2+} in a calmodulin (CaM)-dependent manner (5). AC3 is also regulated by CaM *in vitro*, although this requires supramicromolar concentration of Ca^{2+} (6), and *in vivo* AC3 is inhibited by Ca^{2+} via CaM kinase II (7), unlike AC1 and AC8.

AC1 is closely related in sequence to the Ca^{2+}/CaM-stimulable *rutabaga* AC from *Drosophila*, which is important in *Drosophila* learning tasks (8–10). AC1 and the other Ca^{2+}/CaM-stimulable mammalian AC, AC8, have also been implicated in learning and memory (11). As a means of establishing their proposed roles, single and/or double AC1 and AC8 knockout mice have been generated. Mouse models have demonstrated that Ca^{2+}/CaM-stimulable ACs are involved in long-term potentiation and long-term memory (12). However, despite the general view that AC1 and AC8 can behave similarly, discrete physiological actions of each isoform are becoming apparent. Recent studies by Zhuo's group demonstrated that AC1 specifically participates in *N*-methyl-D-aspartic acid receptor-induced neuronal excitotoxicity (13) and an increase in GluR1 synthesis induced by blocking AMPA receptors (14). Furthermore, Nicol and colleagues (15, 16) showed a contribution of AC1, but not AC8, in axon terminal refinement in the retina. On the other hand, AC8 specifically was seen to be responsible for retrieval from adaptive presynaptic silencing (17) and the acquiring of new spatial information (18). These differences in physiological roles must reflect not only differences in their distributions but also presumably in their regulatory properties. Both enzymes are expressed in brain; AC1 is neuro-specific, whereas the expression of AC8 is more widespread (1, 12). Within the central nervous system, AC1 is abundant in the hippocampus, the cerebral cortex, and the granule cells of the cerebellum, whereas AC8 has a high expression level in the thalamus and the cerebral cortex (19). Studies of mouse brain revealed that AC1 is distributed pre-synaptically and AC8 post-synaptically (18, 20).

Although physiological differences in the roles of these two enzymes are suggested from the studies outlined above, the regulatory mechanisms that might underlie these differences are not. AC1 is more sensitive to Ca^{2+} than is AC8 *in vitro* (21, 22), yet details on how these two enzymes are regulated by Ca^{2+}/CaM are sparse. In non-excitable cells, a Ca^{2+} elevation

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[2] The abbreviations used are: AC, adenylyl cyclase; FSK, forskolin; CaM, calmodulin; CCE, capacitative Ca^{2+} entry; CaM_{1234}, combined N- and C-lobe mutant of calmodulin; CaM_{WT}, wild-type calmodulin; CaM_{12}, N-lobe mutant of calmodulin; CaM_{34}, C-lobe mutant of calmodulin; TG, thapsigargin; M13, M13 skeletal muscle myosin light chain kinase peptide; Epac1-camps, Epac1-based fluorescent cAMP sensor; CCh, carbachol; IBMX, isobutylmethylxanthine; CMV, cytomegalovirus; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein.

[3] All ACs have the following structure: a variable N terminus is followed by two transmembrane cassettes each of which is followed by highly conserved C1 and C2 domains (2). C1 and C2 domains are subdivided into C1a and C1b, and C2a and C2b. C1a and C2a domains are required for the catalytic activity (3).

caused by capacitative Ca^{2+} entry (CCE), the mode of Ca^{2+} entry triggered by emptying Ca^{2+} from internal stores (23), preferentially stimulates AC1 and AC8 (21). Although stimulation of AC8 by CCE has been shown to be at least partially dependent on its localization at lipid rafts (24), whether AC1 is also targeted to this region of plasma membranes has never been addressed. In addition, CaM regulation of AC1 and AC8 has not been compared in detail, although CaM appears to bind to different domains of the two enzymes. AC8 utilizes two CaM binding domains: a classic amphipathic "1-5-8-14" motif at the N terminus and an IQ-like motif in the C2b domain (25). A recent study indicates that CaM pre-associates with the N terminus of AC8, where it becomes fully saturated upon a Ca^{2+} rise, and activates the enzyme via a C-terminally mediated relief of auto-inhibitory mechanisms (26). By contrast, only residues 495–522 of the C1b region of AC1 have been shown to bind CaM in a Ca^{2+}-dependent manner (27, 28). With the presence of only one CaM binding domain in AC1, a simpler mechanism of CaM regulation might be expected.

CaM mediates the regulation of numerous Ca^{2+}-dependent processes in eukaryotic cells (29). The protein possesses N- and C-terminal lobes, both of which contain two Ca^{2+} binding EF hands (EF1 and EF2 in the N lobe, and EF3 and EF4 in the C lobe (30)). Mutations in the EF hands have been valuable for investigating the interaction of CaM with its targets. Alanine substitutions in the EF12 pair or EF34 pair have generated CaM_{12} and CaM_{34} to investigate the independent function of the C and N lobes of CaM, respectively (31, 32).

Against the background of the distinct physiological roles carried out by AC1 and AC8, we performed a detailed comparison of the two enzymes expressed in HEK 293 cells. Their sensitivity to Ca^{2+}/CaM was compared both *in vitro* and *in vivo*; the possibility that they might be expressed in different domains of the plasma membrane was addressed; and putative lobe-specific CaM regulation was assessed using Ca^{2+}-binding mutants of CaM. Single cell measurements using a FRET-based cAMP sensor were performed to compare the kinetic responses of the enzymes to physiological elevations of $[Ca^{2+}]_i$. The results demonstrate superficial similarities in the regulation of AC1 and AC8 but critical disparities in their mechanism of activation by the lobes of CaM and in the speed and pattern of their responsiveness to $[Ca^{2+}]_i$. These discrete behaviors provide a physiological opportunity for different outcomes to elevation of $[Ca^{2+}]_i$, depending on the AC that is expressed in particular contexts.

EXPERIMENTAL PROCEDURES

Materials—FSK and thapsigargin (TG) were purchased from Merck (Nottingham, UK). [2-^{3}H]Adenine, [2,8-^{3}H]cAMP, ECL Western blotting analysis system, Hyperfilm™, and horseradish peroxidase-conjugated goat anti-rabbit IgG were obtained from GE Healthcare (Little Chalfont, UK). [α-^{32}P]ATP was from PerkinElmer Life Sciences. Lipofectamine™ 2000 transfection reagent, fura-2/AM, fura-2 free acid, fura-FF free acid, and pluronic F-127 were from Invitrogen. Horseradish peroxidase-conjugated goat anti-mouse IgG was from Promega (Madison, WI). Polyclonal caveolin antibody was obtained from BD Transduction Laboratories, and monoclonal FLAG antibody was from Stratagene. M13 skeletal muscle myosin light chain kinase peptide (M13) was from Cambridge Bioscience (Cambridge, UK). CaM mutant constructs were a gift from J. H. Caldwell (University of Colorado Health Sciences Center, Denver, CO), and cDNA encoding the Epac1-based fluorescent cAMP sensor (Epac1-camps) was a gift from M. J. Lohse (Institute of Pharmacology and Toxicology, Würzburg, Germany). All other agents were purchased from Sigma unless stated otherwise.

Construction of AC1 Expression Vectors—A cDNA fragment encoding the full-length AC1 (bovine) protein was excised from pcDNA3-AC1 using the restriction enzymes HindIII and SalI. The AC1 fragment was then subcloned into the HindIII/SalI-cut pCMV-Tag2A vector (Stratagene) to obtain the expression plasmid pCMV-AC1, which encodes the N-terminally FLAG-tagged AC1 protein. A DNA fragment encoding the N-terminal deletion mutant (Δ1–35) AC1M1 was amplified by PCR using Phusion DNA polymerase (Finnzymes) and pcDNA3-AC1 as template DNA (primers: 5′-CCCAAGCTTCTGCGGGCGT-GCGATGAGGAG-3′ and 5′-ACGCGTCGACCTAAGC-CTCCTTCCCAGAG-3′). The AC1M1 fragment was cut with HindIII/SalI, and subcloned into the HindIII/SalI-cut pCMV-Tag2B vector (Stratagene). Transfection of the plasmid pCMV-AC1M1 results in the expression of the N-terminally FLAG-tagged AC1M1 protein. To obtain the FLAG-tagged CaM binding mutant of AC1, AC1F503A, site-directed mutagenesis was performed according to the QuikChange protocol (Stratagene) using pCMV-AC1 as DNA template. The following primers were used: 5′-primer, 5′-CAGGTAGCACACG-GTCTTGGCCTTCATCCTC-3′; 3′-primer, 5′-CCAAGAG-GATGAAGGCCAAGACCGTGTGC-3′.

Purification of Rat His_6 CaM—Wild-type and mutant rat His_6-CaM were produced following cloning of CaM cDNA into the pQE30 vector (Qiagen), and propagation in the XL10 Gold strain of *Escherichia coli*. His_6-CaM was purified using TALON® resin (BD Clontech) immobilized metal ion affinity chromatography.

Cell Culture and Transfection of HEK 293 Cells—HEK 293 cells (European Collection of Cell Cultures, Porton Down, UK) were grown in minimum essential medium with Earle's salts, supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 50 μg/ml penicillin, 50 μg/ml streptomycin, and 100 μg/ml neomycin. The cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. To establish stable cell lines expressing AC8, AC8M1 ($AC8_{\Delta 1-106}$), AC1, AC1M1, AC1F503A, or vector (pcDNA3.1; pcD), HEK 293 cells were transfected with 2 μg of cDNA according to the calcium phosphate method described previously (33). Two days after transfection, cells were selected by growth in medium containing 800 μg/ml G-418 for 4 days, and subsequently maintained in medium containing 400 μg/ml G-418. For transient transfection with Epac1-camps, pcD or various forms of CaM cDNA, cells stably expressing AC1 or AC8 were plated onto 25-mm poly-L-lysine-coated coverslips or 100-mm dishes at 60% confluence 1 day prior to transfection with 1 μg of cDNA using Lipofectamine™ 2000 according to the manufacturer's instructions. Cells were used 2 days after transfection.

Measurement of AC Activity—Determination of adenylyl cyclase activity *in vitro* was performed as described previously (34) with some modifications. Adenylyl cyclase activity of crude membranes isolated from transfected HEK 293 cells (35) was measured in the presence of the following components: 12 mM phosphocreatine, 2.5 units of creatine phosphokinase, 0.1 mM cAMP, 1.4 mM $MgCl_2$, 0.1 mM ATP, 0.04 mM GTP, 0.5 mM IBMX, 0.5 μCi of [α-^{32}P]ATP, and 10 μM FSK. Wild-type or mutant CaM was included where indicated. Free Ca^{2+} concentrations were established from a series of $CaCl_2$ solutions buffered with 200 μM EGTA, based on the BAD4 program (36) and confirmed by spectrofluorometric measurements with fura-2 and fura-FF as described previously (26). Membranes were washed twice with assay buffer (800 μM EGTA, 0.25% bovine serum albumin, 40 mM Tris, pH 7.4) to remove Ca^{2+}-bound CaM before starting the assay. Where indicated, membranes were incubated with 1 μM M13 for 10 min at 4 °C prior to washing with assay buffer. The reaction mixture (final volume, 100 μl) was incubated at 30 °C for 20 min, and the reactions were terminated with 0.5% (w/v) sodium lauryl sulfate. [^{3}H]cAMP (~6000 cpm) was added as a recovery marker, and the [^{32}P]cAMP formed was quantitated using a sequential chromatography technique described previously (37). Data points are presented as mean activities ± S.D. of triplicate determinations. Where indicated, the AC activity of cells transfected with empty vector was subtracted from the data.

$[Ca^{2+}]_i$ Measurements in Cell Populations—$[Ca^{2+}]_i$ was measured in populations of HEK 293 cells as described previously (38). Briefly, cells were loaded with 2 μM fura-2/AM plus 0.02% pluronic F-127 for 40 min at room temperature, washed twice, and then aliquoted into samples containing 4×10^6 cells. The cells were finally resuspended in 3 ml of nominally Ca^{2+}-free Krebs buffer (120 mM NaCl, 4.75 mM KCl, 1.44 mM $MgSO_4$, 11 mM glucose, 25 mM HEPES, pH 7.4), and used for $[Ca^{2+}]_i$ measurements in a PerkinElmer Life Sciences LS50B spectrofluorometer. Fluorescence emission ratios at 340 nm/380 nm were measured and converted to $[Ca^{2+}]_i$ values using the standard formula (39).

Measurement of cAMP Accumulation— cAMP accumulation in intact cells was measured as described previously (40), with some modifications. Transfected HEK 293 cells were incubated in minimum essential medium with [2-^{3}H]adenine (1.5 μCi/well in 24-well plates) at 37 °C for 90 min to label the ATP pool. Assays were carried out in Krebs buffer supplemented with bovine serum albumin (1 mg/ml) at 30 °C and terminated by the addition of ice-cold 5% (w/v final concentration) trichloroacetic acid. Unlabeled cAMP (50 μl, 20 mM), ATP (10 μl, 65 mM), and [α-^{32}P]ATP (~6000 cpm) were added to monitor recovery of cAMP and ATP. The [^{3}H]ATP and [^{3}H]cAMP contents were quantified using the sequential chromatography technique described above (see "Measurement of Adenylyl Cyclase Activity"). Accumulation of cAMP is expressed as the conversion of [^{3}H]ATP into [^{3}H]cAMP. Results are presented as mean ± S.D. of triplicate determinations.

Non-detergent Isolation and Immunoblotting of Raft and Non-raft Membranes—Raft and non-raft membranes were separated by a procedure that exploited their different buoyancies as previously described (41), using sonication with sodium bicarbonate (42). Raft and non-raft membranes were resolved using 7.5 and 12% SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane, as described previously (42). Anti-FLAG antibody (1:1000) or anti-caveolin polyclonal antibody (1:5000) was used as a primary antibody, and goat anti-mouse IgG conjugated to horseradish peroxidase (1:3000) or goat anti-rabbit IgG conjugated to horseradish peroxidase (1:5000) was used as a secondary antibody, respectively. Membranes were then treated with ECL reagent and exposed to Hyperfilm™.

Single Cell Measurement of $[Ca^{2+}]_i$—$[Ca^{2+}]_i$ in single cells was measured as described previously (43). Briefly, cells were plated onto poly-L-lysine-coated coverslips 1 day before experiments and loaded with 2 μM fura-2/AM plus 0.02% Pluronic F-127 for 40 min at room temperature in HBS buffer (140 mM NaCl, 4 mM KCl, 0.2 mM $MgCl_2$, 11 mM D-glucose, 10 mM HEPES, pH 7.4) with 1 mM $CaCl_2$. After loading, cells were washed, further incubated in HBS buffer to de-esterify intracellular AM esters, and then imaged using a CoolSNAP-HQ CCD camera (Photometrics, Tucson, AZ) and monochromator system (Cairn Research, Kent, UK) attached to a Nikon TMD microscope (×40 objective). Emission images (D510/80M) at 340 nm and 380 nm excitation were collected every second with MetaFluor software (Molecular Devices, Downingtown, PA). Data are plotted as 340/380 nm ratio changes (Δ340/380) relative to the fluorescence ratio at 0 min.

Single Cell Measurement of cAMP in AC1 and AC8 Expressing Cells Using Epac1-based Fluorescent cAMP Sensor—$[cAMP]_i$ in single cells expressing AC1 or AC8 was measured by fluorescent imaging of Epac1-camps as described previously (43). Briefly, images were captured using an Andor Ixon+ camera and an Optosplit (505DC) to separate CFP (470 nm) and YFP (535 nm) emission images (Cairn Research). For dual emission-ratio imaging, cells were excited at 436 nm using a monochromator (Cairn Research) and 51017 filter set (Chroma, Rockingham, VT) attached to a Nikon eclipse TE2000-S microscope (×40 objective). Emission images at 470 nm and 535 nm were collected every 3 s (250-ms integration time). Captured images were background-subtracted and analyzed using Metamorph imaging software (Molecular Devices). Fluorescence resonance energy transfer data are plotted as changes in 470 nm *versus* 535 nm (ΔCFP/YFP) emission ratio relative to the fluorescence ratio at 0 min, for each individual cell.

Curve-fitting and Statistical Analysis—Sigmoidal dose-response curves, linear regressions, and kinetic parameters were obtained using GraphPad Prism Version 4 (GraphPad Software Inc., La Jolla, CA). Results are shown as mean ± S.D. of at least three individual experiments, or mean ± S.E. The initial rates of increase in $[cAMP]_i$ in single cell measurements were determined by obtaining slopes of linear regressions fitted over a 20-s period, starting 30 s after the addition of TG or carbachol (CCh). Statistical significance was assessed by using one-way analysis of variance followed by Newman-Keuls multiple comparisons tests, where $p < 0.05$ was considered significant.

RESULTS

AC1 and AC8 Differ in Their Regulation by Ca^{2+}—To establish a baseline for comparing the regulation of AC1 and AC8 by

Ca^{2+}, their responses were first examined *in vitro*. Prior to each assay, membranes prepared from HEK 293 cells stably expressing AC1 or AC8 were washed in EGTA to remove Ca^{2+}-dependent bound CaM. AC1 and AC8 were stimulated by 10 μM FSK and a range of $[Ca^{2+}]_{free}$ in the absence (control) or presence of exogenous CaM (1 μM). Inclusion of 10 μM FSK enhanced the activities of both enzymes, allowing more sensitive assays, although this did not affect the individual kinetics of regulation by Ca^{2+}/CaM (data not shown). Membranes from HEK 293 cells stably expressing empty vector (pcD) showed Ca^{2+} inhibition (supplemental Fig. S1) due to the background of the Ca^{2+}-inhibitable AC6 in these cells (44). To eliminate the inhibitory component, endogenous AC activity was subtracted from activities measured in membranes from cells expressing AC1 or AC8 at each Ca^{2+} concentration (Fig. 1, *A* and *B*). AC1 and AC8 were both robustly stimulated by submicromolar concentrations of Ca^{2+} (Fig. 1, *A* and *B*). (Their sensitivities to Ca^{2+} were unaffected by the background subtraction (data not shown).) A reduced Ca^{2+} stimulation in AC1 and AC8 was seen in the absence of added CaM, indicating that both enzymes can be regulated by CaM that was not removed by the wash with EGTA.

To verify that the observed residual Ca^{2+} stimulation was due to endogenous CaM, the synthetic peptide M13 was used as a "CaM sponge." M13 is a 26-residue peptide derived from a CaM binding domain of skeletal muscle myosin light chain kinase (45), which has a very high affinity for CaM ($K_d \sim 0.2$ nM (46)). When membranes were incubated with 1 μM M13 prior to washing with EGTA, the residual Ca^{2+} stimulation in the absence of added CaM was completely eliminated (Fig. 1, *A* and *B*), showing that endogenous CaM was responsible for Ca^{2+} stimulation in the absence of added CaM.

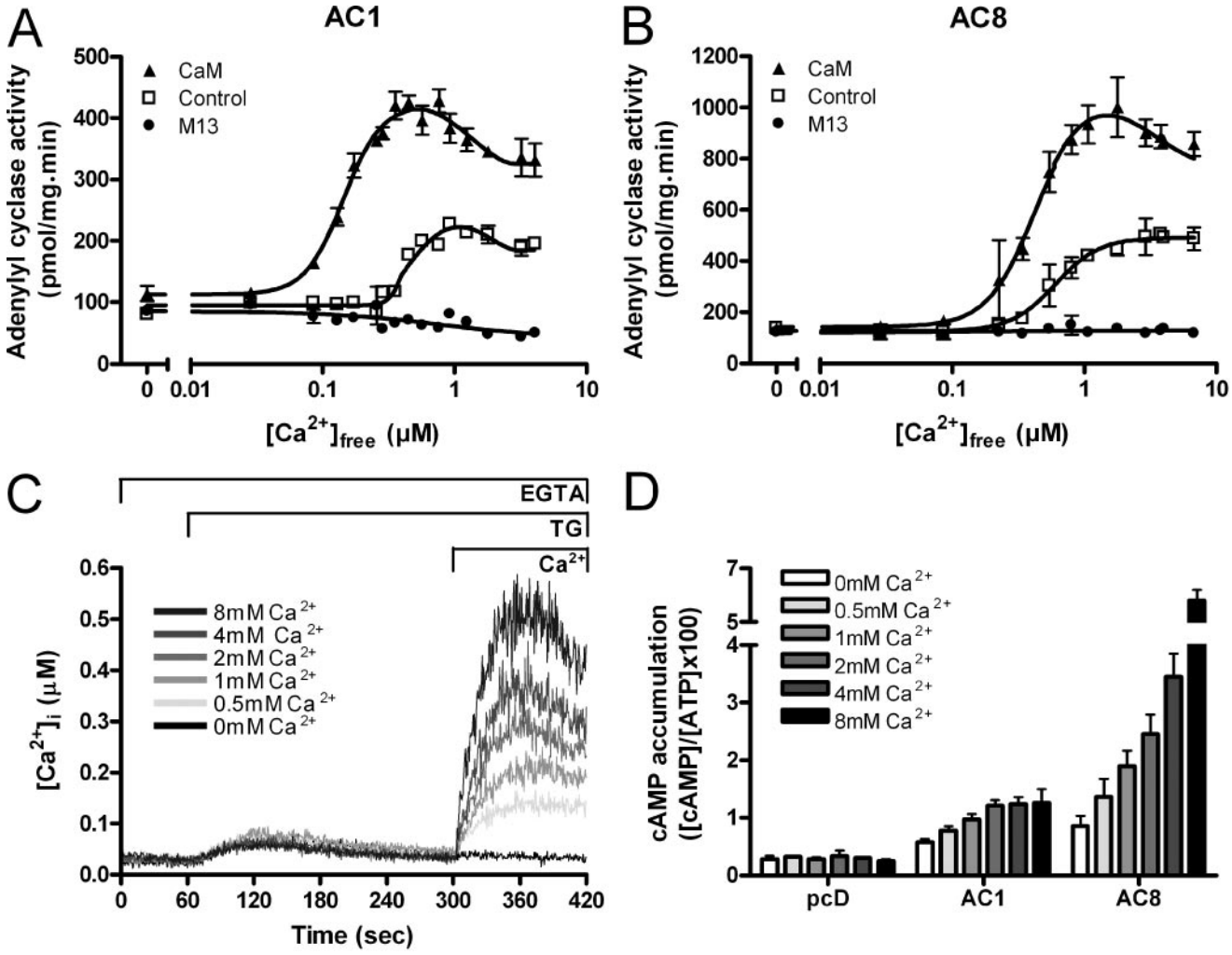
Although basal activities varied depending on expression levels, the differences in Ca^{2+} sensitivity between AC1 and AC8 were consistent. AC8 was stimulated by Ca^{2+} by 5- to 10-fold in the presence of CaM and 2.7- to 4.2-fold in the absence of added CaM ($n = 11$, Fig. 1*B*). AC1, in contrast, was much less stimulated by Ca^{2+}; Ca^{2+} stimulation of AC1 was 1.5- to 3-fold and 1.1- to 1.5-fold, in the presence and absence of added CaM, respectively ($n = 9$, Fig. 1*A*). Although AC1 is less stimulable, AC1 is more sensitive to Ca^{2+} than AC8. AC1 was stimulated by Ca^{2+} with an EC_{50} of 0.15 μM in the presence of added CaM (Fig. 1, *A* and *E*), whereas the equivalent value for AC8 was 0.56 μM (Fig. 1, *B* and *E*).

The elevation of Ca^{2+} in intact cells also regulates Ca^{2+}-sensitive ACs. AC8 is stimulated robustly by CCE, but not by ionophore-mediated Ca^{2+} release or arachidonic acid- or oleylarachidonylglycerol-mediated Ca^{2+} entry (21, 35, 47). To compare the Ca^{2+} regulation of AC1 and AC8 *in vivo*, cAMP accumulation in response to CCE was measured in cell populations expressing either AC1 or AC8. Ca^{2+} was emptied from ER stores by passive depletion with the sarcoplasmic-endoplasmic reticulum calcium ATPase pump inhibitor, TG (100 nM) in Ca^{2+}-free buffer; CCE was subsequently evoked by the addition of extracellular Ca^{2+} ($[Ca^{2+}]_{ex}$) after 4 min (Fig. 1*C*). CCE achieved values of 0.15–0.55 μM $[Ca^{2+}]_i$ (Fig. 1*C*). CCE in cells expressing AC1 or AC8 was not significantly different from that in untransfected cells (data not shown). Under the same conditions, cAMP accumulation in cell populations was measured over a 1-min period beginning with the addition of 10 μM FSK and the indicated $[Ca^{2+}]_{ex}$, following 4-min pretreatment with TG, in the presence of 100 μM EGTA and 100 μM IBMX, phosphodiesterase inhibitor. Cells expressing vector (pcD) did not show Ca^{2+} stimulation (Fig. 1*D*), underscoring the absence of endogenous Ca^{2+}-stimulable ACs in HEK 293 cells. As with the *in vitro* experiments, Ca^{2+} stimulation of AC8 was much greater than that of AC1; AC8 was stimulated by 4- to 8-fold ($n = 25$), whereas AC1 was stimulated only by 1.8- to 3-fold ($n = 11$) by 8 mM $[Ca^{2+}]_{ex}$ (Fig. 1*D*). However, the apparent Ca^{2+} sensitivity of AC1 was greater than AC8. Ca^{2+} stimulation of AC1 was maximal at 2 mM $[Ca^{2+}]_{ex}$ (half-maximum concentration, 0.59 mM, Fig. 1*E*). In contrast, Ca^{2+} stimulation of AC8 did not reach a plateau unless $[Ca^{2+}]_{ex}$ was increased to 8 mM or more (half-maximum concentration, $\geq$3.71 mM, Fig. 1*E*).

Fig. 1*E* summarizes the -fold stimulations and EC_{50} values for AC1 and AC8 *in vitro* and *in vivo*. The effective $[Ca^{2+}]_i$ was estimated from the plot of $[Ca^{2+}]_{ex}$ *versus* $[Ca^{2+}]_i$ at 60 s after the addition of $[Ca^{2+}]_{ex}$ (supplemental Fig. S2). The half-maximally effective Ca^{2+} concentrations of AC1 and AC8 regulation *in vivo* (0.19 μM and $\geq$0.32 μM, respectively) reflected the EC_{50} values obtained *in vitro* (Fig. 1*E*). AC8 was not further stimulated when 10 mM Ca^{2+} was added, possibly due to saturation of CCE (data not shown). Thus, although both AC1 and AC8 are stimulated by Ca^{2+}, they clearly differ in their sensitivity to Ca^{2+} both *in vitro* and *in vivo*.

Ca^{2+} Regulation and Localization of AC1 Mutant—Ca^{2+}-sensitive AC5, AC6, and AC8 are targeted to cholesterol- and sphingolipid-rich regions of the plasma membrane, called lipid rafts (24, 42, 48–50). In contrast, the Ca^{2+}-insensitive AC7 is not targeted to this domain (24, 42). Inhibition of AC6 by CCE was compromised by cholesterol depletion but fully restored upon cholesterol repletion (48). Thus, specific raft localization seems to be a key requirement for the CCE regulation of Ca^{2+}-sensitive ACs. To investigate whether the Ca^{2+}-stimulable AC1 shares this property, the subcellular localization of AC1 and a key mutant was examined. The N terminus of AC8 is not critical for targeting of AC8 to lipid rafts, but it is important for Ca^{2+} stimulation *in vivo* (24). Deletion of the N terminus of AC8 resulted in reduced stimulation by CCE (24). The N terminus of AC8 also interacts with protein phosphatase 2A (51), demonstrating a function other than CaM binding. Whether the N terminus of AC1 has a similar role has not been addressed, although the amino acid sequence is quite dissimilar to that of AC8. Tang and coworkers made an N-terminally truncated AC1, $AC1_{\Delta 1-52}$, which showed only 10% activity compared with the wild-type AC1, despite having a higher expression level (52). In the present experiments, a less extensively truncated AC1 construct, $AC1_{\Delta 1-35}$ (AC1M1), was generated to provide a direct comparison with AC8M1, which also lacks a similar proportion (the first 106 amino acids) of the N terminus.

Caveolar and non-caveolar membranes from HEK 293 cells stably expressing FLAG-tagged AC1 and AC1M1 were separated by density gradient fractionation as a function of their increased buoyancy in sucrose gradients. To avoid any detergent-induced artifacts, membranes were disrupted by sonica-



E

	In vitro		*In vivo*		
	Fold stimulation	EC_{50} (μM)	Fold stimulation	Half maximum $[Ca^{2+}]$	
				$[Ca^{2+}]_{ex}$ (mM)	$[Ca^{2+}]_i$ (μM)
AC1	1.86 ± 0.31	0.15 ± 0.06	2.26 ± 0.26	0.59 ± 0.05	0.19 ± 0.04
AC8	7.56 ± 1.87	0.56 ± 0.58	≥7.36 ± 0.39	≥3.71 ± 0.39	≥0.32 ± 0.06

FIGURE 1. **Ca^{2+} regulation of AC1 and AC8.** Adenylyl cyclase activity was measured in crude membranes from HEK 293 cells expressing AC1 (*A*) or AC8 (*B*), as described under "Experimental Procedures." *In vitro* assays were carried out either in the absence (control (□)) or presence (▲) of 1 μM exogenous CaM, or in the presence of 1 μM M13 peptide (●). Data after subtraction of the endogenous AC activity (see supplemental Fig. S1) are plotted as mean ± S.D. and are representative of at least six separate experiments. *C*, $[Ca^{2+}]_i$ was measured in populations of HEK 293 cells loaded with 2 μM fura-2/AM. CCE was triggered by the addition of 0.5, 1, 2, 4, or 8 mM Ca^{2+} (300 s) after 4-min pre-treatment with 100 nM TG (60 s) in the presence of 100 μM EGTA. *D*, under the same conditions as *C*, the effect of CCE on cAMP accumulation was determined in cell populations expressing vector alone (pcD), AC1, or AC8, as described under "Experimental Procedures." A 1-min assay was started by the addition of Ca^{2+} and 10 μM FSK in the presence of 100 μM IBMX and 100 μM EGTA. Data are plotted as mean ± S.D. and are representative of at least 11 experiments. *E*, AC1 and AC8 activities were plotted using sigmoidal dose-response (variable slope) fits. The half-maximum $[Ca^{2+}]_i$ was calculated by plotting $[Ca^{2+}]_{ex}$ *versus* $[Ca^{2+}]_i$ at 360 s (see supplemental Fig. S2). The -fold stimulation and the kinetic parameters obtained from fitted curves are summarized in the *table*, where results show the mean ± S.D. of at least nine independent experiments.

tion, instead of separating detergent-insoluble membranes using Triton X-100. Ten fractions were collected from top to bottom, and then sucrose and protein concentrations of each fraction were determined (Fig. 2*B*). Following fractionation, Western blot immunoreactivity for FLAG (for AC1) and the lipid raft marker, caveolin of each fraction were examined.

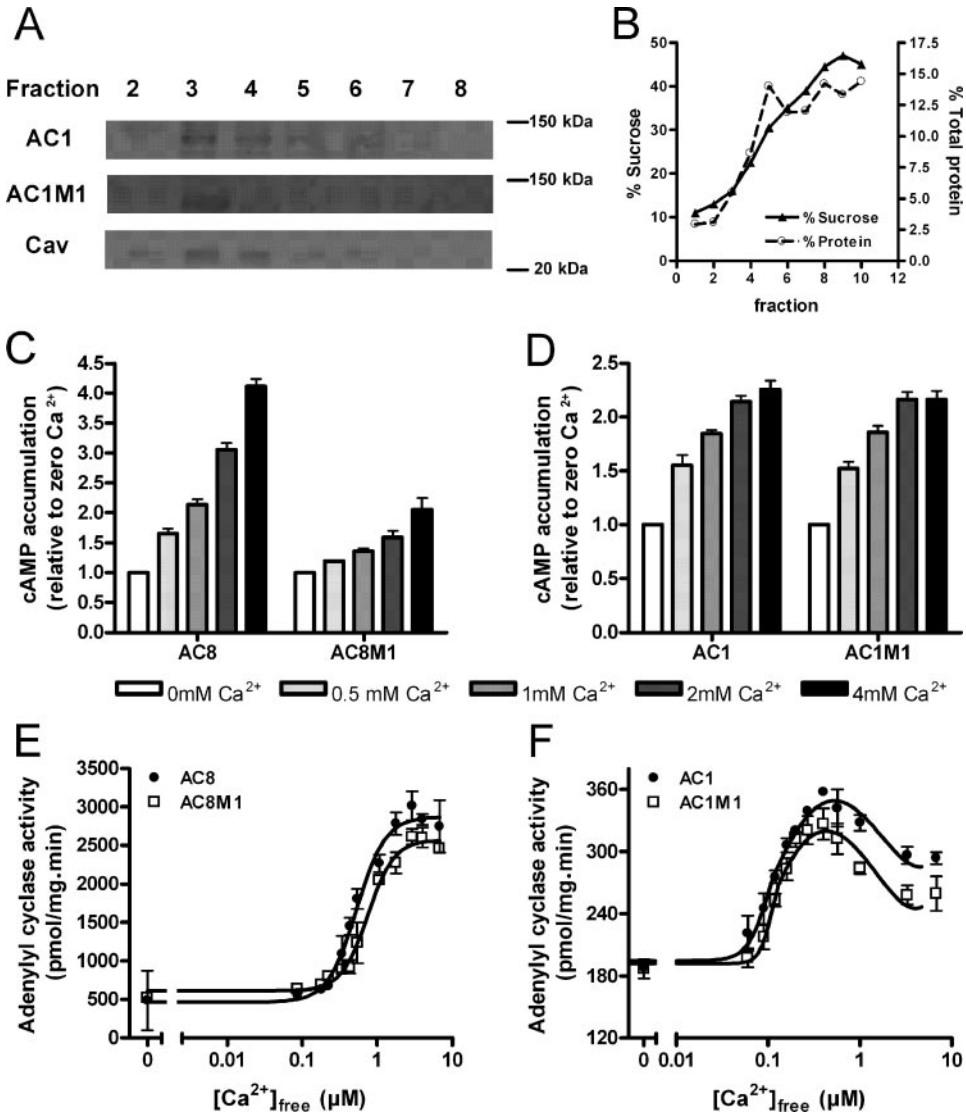


FIGURE 2. **Subcellular localization and Ca^{2+} regulation of AC1 and its mutant.** *A*, fractions (*lanes 2–8*) from HEK 293 cells stably expressing FLAG-tagged AC1 and AC1M1 were separated using a non-detergent method, resolved by SDS-PAGE, and then immunoblotted with antibodies raised against FLAG and the lipid-raft marker, caveolin (*CaV*). *B*, the percentage of protein content as compared with the total recovered protein (○) and percentage of sucrose (▲) from each fraction are indicated. *C* and *D*, *in vivo* cAMP accumulation was measured in cell populations expressing AC8 and AC8M1 (*C*) or AC1 and AC1M1 (*D*) in response to increasing CCE, as described under "Experimental Procedures." Data are normalized to cAMP accumulation at zero Ca^{2+} and plotted as mean ± S.D. of at least six independent experiments. *E* and *F*, *in vitro* adenylyl cyclase activity was measured in crude membranes prepared from cells expressing AC8 and AC8M1 (*D*, ● and □, respectively), or AC1 and AC1M1 (*F*, ● and □, respectively), in the presence of 1 μM exogenous CaM. Data are plotted as mean ± S.D. and are representative of at least five separate experiments.

Most of the FLAG and caveolin immunoreactivities were enriched at fractions 3 and 4 (15–25% sucrose, Fig. 2, *A* and *B*). This result revealed that, like AC8, AC1 (and AC1M1) co-localizes with caveolin, and that the deletion of the early N-terminal domain did not prevent the targeting of AC1 to lipid rafts.

The first part of the N terminus of AC1 was not essential for targeting of AC1, but would it impact on Ca^{2+} stimulation *in vivo*? To address this question, cAMP accumulation in intact cells was measured in response to CCE. The robust stimulation by 4 mM $[Ca^{2+}]_{ex}$ observed in AC8 was greatly reduced in AC8M1 (<2-fold stimulation by the same $[Ca^{2+}]_{ex}$, Fig. 2*C*). However, unlike AC8, deletion of the first part of the N terminus of AC1 did not affect its activity or Ca^{2+} stimulation. Both AC1 and AC1M1 showed 1.8- to 3-fold stimulation by 2 mM $[Ca^{2+}]_{ex}$ with the same half-maximum values (Fig. 2*D*). When membranes were isolated from cells expressing AC8 or AC8M1, they showed robust Ca^{2+} stimulation. Although AC8 has a CaM binding domain at the N terminus, AC8M1 was fully active in the presence of exogenous CaM, and its Ca^{2+} sensitivity was similar to that of AC8 (Fig. 2*E*). Crude membranes from cells expressing AC1M1 also showed stimulation by Ca^{2+}/CaM, which was comparable to AC1 (Fig. 2*F*). Both AC8M1 and AC1M1 showed full activation by Ca^{2+} in the presence of exogenous CaM, demonstrating that the N-terminal region is not essential for Ca^{2+}/CaM stimulation of AC8 or AC1 *in vitro*. However, the N terminus is important for AC8, but not AC1, activity *in vivo*.

Effect of CaM Mutants on Ca^{2+} Stimulation of AC8, AC8M1, AC1, and AC1 F503A—The amphipathic CaM binding domain at the N terminus of AC8, which is absent in AC8M1, plays a key role in the stimulation of AC8 by Ca^{2+} *in vivo*. Although AC8M1 showed full activation by Ca^{2+} in the presence of added CaM *in vitro* (Fig. 2*E*), this was strictly dependent on added CaM, unlike AC8, which showed ~3-fold stimulation without exogenous CaM (Fig. 1*B* (26)). To determine how the CaM binding domain of AC1 affects its Ca^{2+}/CaM stimulation in comparison with AC8M1, another mutant of AC1 was generated. A phenylalanine residue in the C1b domain was previously suggested to be critical for CaM binding based on an F503R mutation (53). Because arginine is a positively charged, basic amino acid, we produced a construct with a milder mutation to neutrally charged alanine at this residue (AC1F503A).

A further facet of the regulation of ACs by Ca^{2+}/CaM is the potentially separate roles of the two lobes of CaM. Ca^{2+} stimulation of AC8 is affected by partially liganded Ca^{2+}/CaM (26). Gao *et al.* showed that AC1 could be activated by CaM mutants, which were occupied by three Ca^{2+} ions due to a single mutation at EF1, EF2, EF3, or EF4 (54). Ca^{2+} binds cooperatively within each lobe of CaM (55); however, the effect on AC1 of CaM with two Ca^{2+} bound at one lobe has never been addressed. Here, we used lobe-specific EF-hand mutants of

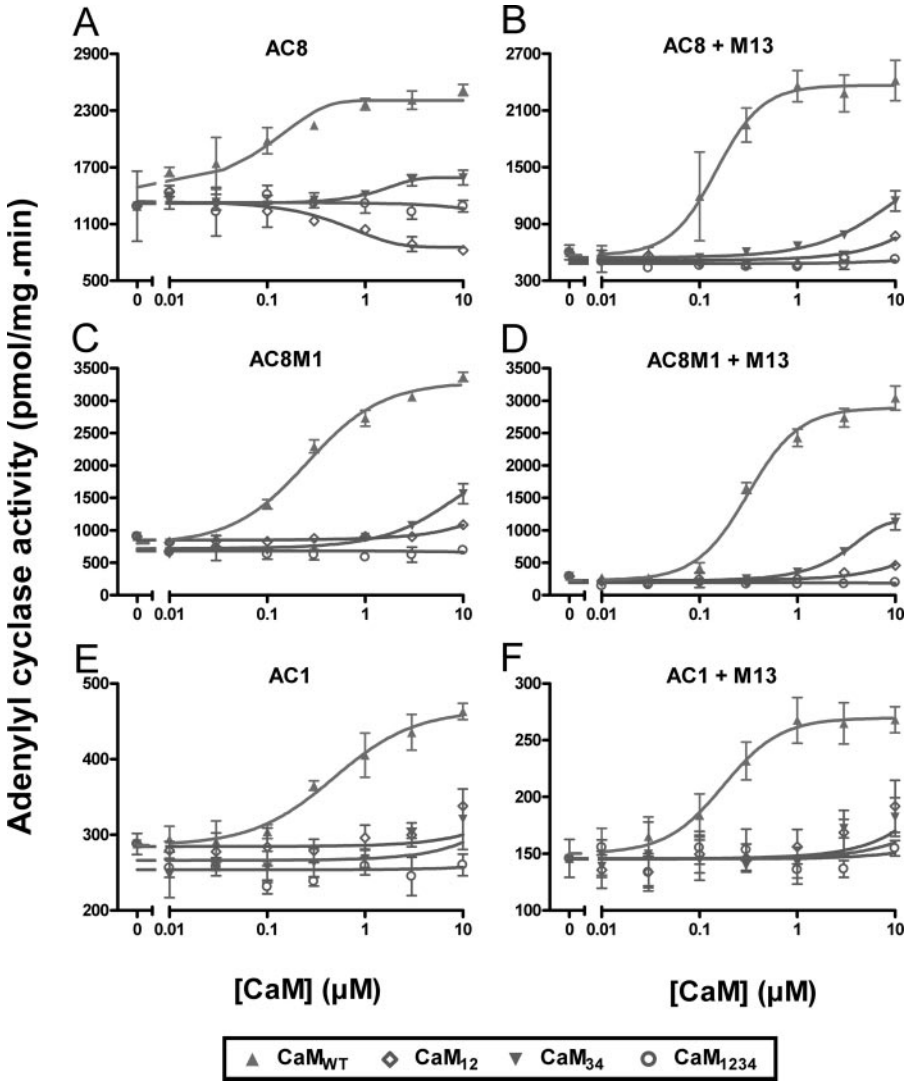


FIGURE 3. **CaM regulation of AC8, AC8M1, and AC1 *in vitro*.** Adenylyl cyclase activity was measured in crude membranes prepared from cells expressing AC8 (*A* and *B*), AC8M1 (*C* and *D*), or AC1 (*E* and *F*). Membranes were preincubated at 4 °C for 10 min with either assay buffer alone (*A*, *C*, and *E*), or assay buffer containing 1 μM M13 peptide (*B*, *D*, and *F*), and subsequently washed again in EGTA-containing buffer. Assays were performed following the protocol given under "Experimental Procedures," with increasing concentrations of CaM_{WT} (▲), CaM_{12} (◇), CaM_{34} (▼), or CaM_{1234} (○), in the presence of 1 μM (*A–D*) or 0.3 μM (*E* and *F*) free Ca^{2+}. Data are plotted as mean ± S.D. and are representative of at least three separate experiments.

CaM to compare the contribution of each CaM lobe in regulating AC1 and AC8. The CaM mutant, CaM_{1234}, does not bind Ca^{2+} due to point mutations of aspartate residues in all four EF hands, whereas CaM_{12} (N-lobe mutant) and CaM_{34} (C-lobe mutant) retain partial Ca^{2+} sensitivity and can bind certain target proteins (32). To observe a clear effect of exogenous CaM, M13 treatment was employed. Activities of AC8, AC8M1, AC1, and AC1F503A were measured in crude membranes washed with EGTA (supplemental Fig. S3, *A*, *C*, *E*, and *G*, respectively), or in crude membranes incubated with M13 prior to the EGTA wash (supplemental Fig. S3, *B*, *D*, *F*, and *H*, respectively), with a range of $[Ca^{2+}]_{free}$ in the absence or presence of different forms of exogenous CaM (1 μM). Although the activities without Ca^{2+} or exogenous CaM were reduced due to the removal of endogenous CaM, incubation of membranes with M13 did not alter their regulation by Ca^{2+}, when 1 μM CaM_{WT} was present. AC activities were also measured with increasing concentrations of CaM in the presence of 1 μM free Ca^{2+} for AC8 and AC8M1 (Fig. 3, *A–D*) or 0.3 μM free Ca^{2+} for AC1 (Fig. 3, *E* and *F*). CaM_{WT} stimulated AC activities in a dose-dependent manner, and CaM_{1234} (even up to 10 μM) did not affect activities.

AC8 was stimulated 1.8-fold with 10 μM CaM_{WT}, and the stimulation was increased to 4-fold with an EC_{50} of 0.12 μM, when membranes were incubated with M13 prior to the assay ($n = 3$, $p < 0.001$, Fig. 3*B* and supplemental Fig. S4*B*). Ca^{2+} stimulation of AC8 after EGTA wash was unchanged in the presence of CaM_{34} or CaM_{1234}, but significantly inhibited when CaM_{12} was present (supplemental Fig. S3*A*), as shown previously (26). This inhibition was concentration-dependent; AC8 showed a 25–30% reduction with 10 μM CaM_{12} in the absence of M13 pretreatment ($n = 4$, $p < 0.001$, Fig. 3*A* and supplemental Fig. S4*A*). However, when endogenous CaM was completely removed from AC8 by M13 incubation, the inhibition by CaM_{12} was no longer observed (supplemental Fig. S3*B*), even when $[CaM_{12}]$ was increased to 10 μM (Fig. 3*B* and supplemental Fig. S4*B*), demonstrating that the inhibition was due to competition with endogenous CaM. Interestingly, AC8 displayed a slight stimulation by Ca^{2+} in the presence of 1 μM CaM_{34} (~1.7-fold), after endogenous CaM was removed (supplemental Fig. S3*B*). To investigate the possibility that CaM_{34} can stimulate AC8, we increased the concentration up to 10 μM and found this further activated AC8 (Fig. 3*A* and supplemental Fig. S4*A*). This stimulation was enhanced from 1.3- to 2.3-fold when endogenous CaM was removed by M13 washing ($n = 3$, $p < 0.001$, Fig. 3*B* and supplemental Fig. S4*B*).

As shown previously, AC8M1 was stimulated by Ca^{2+} only slightly in the absence of CaM (supplemental Fig. S3*C* (26)). AC8M1 did not show Ca^{2+} stimulation to any great extent in the presence of 1 μM CaM mutants (supplemental Fig. S3*C*), which reaffirms the idea that CaM_{12} competes with endogenous CaM at the N terminus in intact AC8. When endogenous CaM was removed by incubation with M13, AC8M1 was regulated by Ca^{2+}/CaM very similarly to AC8; AC8M1 was fully stimulated by Ca^{2+} with CaM_{WT}, stimulated ~1.7-fold with CaM_{34}, and not stimulated by Ca^{2+} in the absence of exogenous CaM, or in the presence of CaM_{12} or CaM_{1234} (supplemental Fig. S3*D*). The CaM regulation of AC8M1 was comparable to

A

	EGTA wash				M13 incubation + EGTA wash			
	CaM_{WT}	CaM_{12}	CaM_{34}	CaM_{1234}	CaM_{WT}	CaM_{12}	CaM_{34}	CaM_{1234}
AC8	↑↑	↓	(↑)	--	↑↑	--	↑	--
AC8M1	↑↑	--	↑	--	↑↑	--	↑	--
AC1	↑↑	--	--	--	↑↑	--	--	--
AC1F503A	(↑)	--	--	--	(↑)	--	--	--

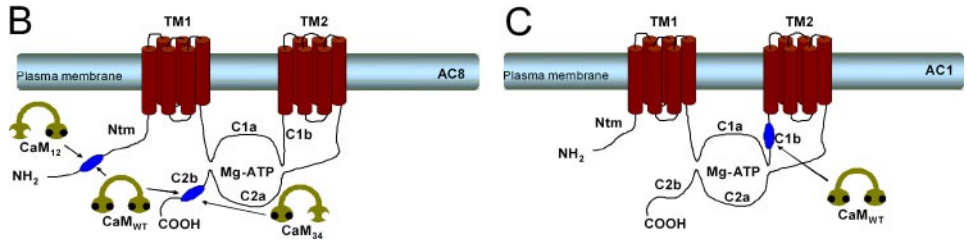


FIGURE 4. **The effect of CaM mutants on Ca^{2+} stimulation of AC8, AC8M1, AC1, and AC1F503A *in vitro*.** *A*, the Ca^{2+} regulation of AC8, AC8M1, AC1, and ACF503A in the presence of CaM_{WT}, CaM_{12}, CaM_{34}, or CaM_{1234} was compared with that in the absence of exogenous CaM, with or without M13 incubation. *B* and *C*, schematic diagram of AC8 (*B*) and AC1 (*C*) based on Fig. 3, showing the interaction of CaM_{WT}, CaM_{12}, or CaM_{34} with CaM binding domains of ACs. Ca^{2+} ions binding to CaM are indicated by black circles.

that of AC8 with M13 pretreatment, although the EC_{50} value for CaM_{WT} was slightly increased (0.27 μM, $n = 3$, Fig. 3*C* and supplemental Fig. S4*C*). These data demonstrate that the endogenous CaM, which is resistant to EGTA washing, is tethered at the N terminus of AC8, and a high concentration of CaM_{34} activates AC8 through the C terminus. Although -fold stimulation and the EC_{50} value for CaM_{WT} were increased, the overall profile of regulation by CaM of AC8M1 activity after M13 pretreatment was similar to that without M13 (Fig. 3*D* and supplemental Fig. S4*D*). The CaM_{34}-mediated Ca^{2+} stimulation of AC8 and AC8M1 suggests that the N lobe of CaM is more important than the C lobe in the binding to and activation of AC8.

The effect of CaM mutants on Ca^{2+} stimulation of AC1 was quite different. None of the CaM mutants affected the Ca^{2+} stimulation of AC1. AC1 was stimulated by Ca^{2+} in the absence of exogenous CaM by ~1.2-fold, and this stimulation was neither enhanced nor reduced by CaM mutants (supplemental Fig. S3*E*). The Ca^{2+} inhibition of AC1 observed in the absence of exogenous CaM after M13 incubation was again unaffected by CaM mutants (supplemental Fig. S3*F*). Unlike AC8, neither CaM_{12} nor CaM_{34} affected AC1 activity compared with CaM_{1234}, even when endogenous CaM was washed away (Fig. 3, *E* and *F*, and supplemental Fig. S4, *E* and *F*). CaM_{WT} stimulated AC1 activity 1.8-fold with an EC_{50} value of 0.27 μM ($n = 3$, Fig. 3*F* and supplemental Fig. S4*F*). The apparent stimulation of AC1 by 10 μM CaM_{12} when membranes were incubated with M13 was not statistically significantly different compared with the activity by 10 μM CaM_{1234} or in the absence of exogenous CaM (Fig. 3*F* and supplemental Fig. S4*F*).

The activity of AC1F503A was neither stimulated nor inhibited by Ca^{2+} in the presence of CaM_{WT}; however, it showed inhibition by Ca^{2+} in the absence of CaM and in the presence of CaM mutants (supplemental Fig. S3*G*). This profile was unchanged by M13 incubation (supplemental Fig. S3*H*). These data establish that, although the residue Phe-503 is important for AC1 to be fully stimulated by Ca^{2+}/CaM, AC1F503A can still interact with and be activated weakly by fully loaded CaM.

To prove that the mutated C1b domain of AC1 could still interact with CaM, pulldown experiments were conducted between GST-AC1C1b, GST-AC1C1b^{F503A}, and CaM. In these experiments both constructs bound CaM, although the interaction was weaker with the point mutation in the C1b domain (supplemental Fig. S5). The weak activation of AC1F503A by CaM_{WT} and no other CaM mutant (supplemental Fig. S3, *G* and *H*) supports the mechanism whereby only fully liganded CaM activates AC1.

Fig. 4*A* summarizes the effect of CaM on Ca^{2+} stimulation of AC8, AC8M1, AC1, and ACF503A, compared with that in the absence of exogenous CaM, and schematic diagrams show interactions between different forms of CaM and AC8 (Fig. 4*B*) and AC1 (Fig. 4*C*). These results demonstrate a distinct mechanism of CaM regulation in AC8 and AC1. Although both AC8 and AC1 require Ca^{2+}-saturated CaM for stimulation, AC8, but not AC1, is partially activated by CaM with two Ca^{2+} bound at the N lobe.

The effect of CaM mutants on Ca^{2+} stimulation of AC8 and AC1 was then compared *in vivo*. An empty vector (pcD), wild-type, or mutant forms of CaM were transfected in cells expressing AC8 or AC1, and cAMP accumulation in response to CCE was monitored in cell populations. CaM_{WT} did not enhance the Ca^{2+} stimulation of AC8 or AC1, presumably because endogenous cellular CaM levels were adequate (56) to stimulate the overexpressed ACs. *In vivo* measurements also showed that unlike AC8, which was strongly inhibited by CaM_{12} (Fig. 5*A*), AC1 activity was not affected by CaM mutants (Fig. 5*B*). Along with the *in vitro* data, these *in vivo* data underscore a clear difference in the regulation by CaM of AC8 and AC1.

Regulation of AC1 and AC8 by Physiological $[Ca^{2+}]_i$ Induced by TG and CCh—The biochemical differences in the regulation of AC1 and AC8 noted up to this point hint that these differences may be exploited in a physiological context. Consequently we compared the temporal responsiveness of the two enzymes to physiological elevations in $[Ca^{2+}]_i$. Inositol 1,4,5-trisphosphate-dependent Ca^{2+} increase caused by the muscarinic receptor agonist, CCh (1 mM), stimulates AC8 by 1.7-fold, which is significant, but much lower than the stimulation by CCE (21). We wished to examine whether AC1 showed any selectivity toward regulation by CCE. In Ca^{2+}-free media, various concentrations of CCh were added to release Ca^{2+} from intracellular stores in HEK 293 cells. Values of 80–300 nM $[Ca^{2+}]_i$ were achieved (Fig. 6*A*). The Ca^{2+} influxes caused by CCh in cells expressing AC1 and AC8 were comparable to those in untransfected cells (data not shown). In the same procedure, cAMP accumulation in HEK 293 cells expressing AC1

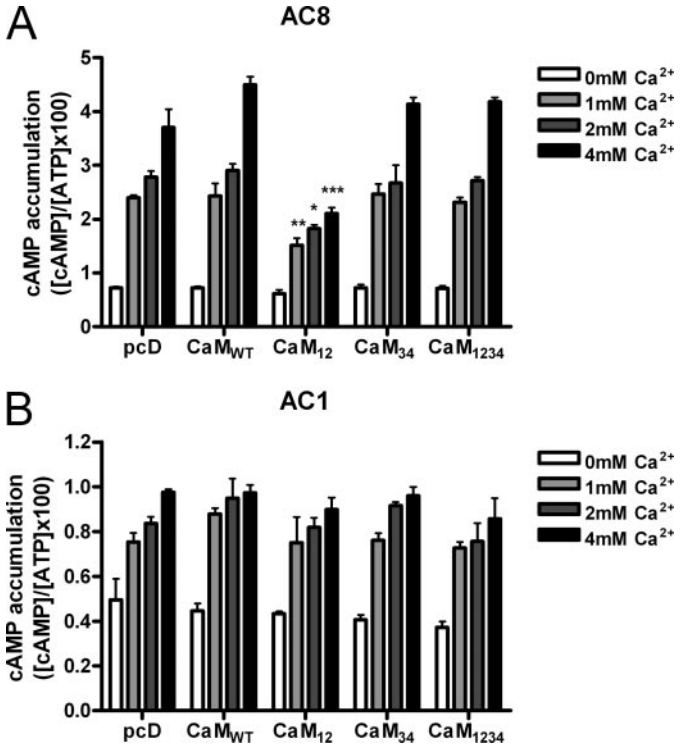


FIGURE 5. **The effect of CaM mutants on Ca^{2+} stimulation of AC1 and AC8 cell populations.** Stable AC8 (*A*) and AC1 (*B*) cells were transiently transfected with vector (pcD), CaM_{WT}, CaM_{12}, CaM_{34}, or CaM_{1234} to determine the effect of CaM mutants in whole cells. cAMP accumulation in cell populations was measured over a 1-min period beginning with the addition of 10 μM FSK and indicated $[Ca^{2+}]_{ex}$ after pre-treatment with 100 nM TG for 4 min. Data are plotted as mean ± S.D. of three separate experiments (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$).

or AC8 was measured over a 1-min period beginning with the addition of 10 μM FSK and various concentration of CCh in the presence of 100 μM EGTA and 100 μM IBMX. Although AC8 was stimulated robustly by CCE (Fig. 1*D*), stimulation by CCh-mediated Ca^{2+} release was only up to 35% of the stimulation brought about by CCE resulting from 4 mM Ca^{2+} (Fig. 6*B*), which gave a comparable increase in $[Ca^{2+}]_i$. (The stimulation caused by CCh was likely due to Ca^{2+} release, because this stimulation was abolished when the Ca^{2+} store was depleted by TG prior to the addition of CCh.) This demonstrates that AC8 is stimulated by CCE much more efficiently than by CCh-mediated Ca^{2+} release. By contrast, AC1 showed a 2-fold stimulation by CCh-mediated Ca^{2+} release, which was the same as the maximal stimulation achieved by CCE (Fig. 6*B*). Thus AC1 shows no particular selectivity for CCE over Ca^{2+} release. The kinetics of cAMP accumulation was very similar in AC1 and AC8; the maximum stimulation by CCh was achieved in both by 20 μM CCh with a half-maximum concentration of ~6 μM CCh (Fig. 6*B*).

AC1 showed a higher Ca^{2+} sensitivity and lower -fold stimulation than AC8, when stimulated by TG-evoked CCE for 1 min (Fig. 1*D*). To compare the kinetics of their regulation at more physiological Ca^{2+} concentrations, we examined the time course of cAMP accumulation in response to a sub-maximal Ca^{2+} rise evoked by TG or CCh. In the presence of 1 mM Ca^{2+}, 100 nM TG and 10 μM CCh gave a comparable Ca^{2+} increase (up to ~200 nM; Fig. 6, *C* and *D*, respectively), which were caused by both Ca^{2+} release from the internal stores and CCE (57). CCh produced a rapid inositol 1,4,5-trisphosphate-dependent Ca^{2+} increase followed by sustained CCE, whereas TG gave a slower, sustained Ca^{2+} rise due to its passive depletion of ER stores and subsequent CCE (Fig. 6, *D* and *C*, respectively). We then applied these conditions to measure cAMP generation in AC1 and AC8 cell populations over 200 s after the addition of TG or CCh. Cells were pretreated with low dose of FSK (100 nM) for 60 s to enhance AC activity. AC1 and AC8 responded similarly to TG-induced Ca^{2+} increase; they were both stimulated about 3-fold and showed a similar slow, gradual increase in cAMP (Fig. 6*E*). By contrast, the $[cAMP]_i$ rise induced by CCh activated AC1 and AC8 differently. AC8 responded much more rapidly than AC1 (Fig. 6*F*), although both responses were faster than those evoked by TG. Ca^{2+} release mediated by CCh stimulated AC1 fully but not AC8 (Fig. 6*B*), demonstrating that the faster response of AC8 was not due to enhanced responsiveness of AC8 to CCh-mediated Ca^{2+} release. This result demonstrated, perhaps paradoxically, that AC1, the more Ca^{2+}-sensitive AC isoform, was activated more slowly than AC8.

To examine the kinetics of the regulation in a continuous and more discerning setting, single cell experiments were performed. First, $[Ca^{2+}]_i$ changes were established in cells loaded with fura-2/AM. In the presence of 1 mM Ca^{2+}, 100 nM TG or 10 μM CCh was added at 2 min to permit an increase in $[Ca^{2+}]_i$; this increase was reduced when the buffer was switched to Ca^{2+}-free at 7 min. Cells treated with TG showed smooth, homogenous $[Ca^{2+}]_i$ responses (data are representative of 10 cells from 6 coverslips ($n = 600$), Fig. 7*A*), whereas $[Ca^{2+}]_i$ responses following CCh addition were heterogeneous (data are representative of 10 cells from 12 coverslips ($n = 1200$), Fig. 7*B*). Addition of CCh caused a rapid increase in $[Ca^{2+}]_i$, which was observed in all cells followed by a plateau phase in ~65% of cells, and ~35% cells displayed Ca^{2+} oscillations, as shown previously (43). The $[Ca^{2+}]_i$ responses in cells expressing AC1 or AC8 did not differ from those in untransfected HEK 293 cells (data not shown). Parallel experiments were carried out to assess cAMP levels in HEK 293 cells expressing AC1 and AC8, using a genetically encoded cAMP sensor, Epac1-camps. Because of its high temporal resolution, the Epac1-camps fluorescent probe has been used as a sensor for rapid changes in intracellular cAMP levels caused by phosphodiesterase activity (58, 59) and AC activity (43, 60). Epac1-camps was transiently transfected into cells stably expressing AC1 or AC8, and the changes in CFP/YFP emission ratio of the probe were measured. As in cell population measurements, AC1 and AC8 were primed by a low dose of FSK (100 nM), which did not change the fluorescent signals in buffer containing 1 mM Ca^{2+}. AC1 and AC8 responded analogously, and slowly, to TG-induced Ca^{2+} rises (Fig. 7, *C* and *E*). The initial rates of increase in cAMP upon the addition of TG did not differ significantly with AC1 and AC8 (0.041 ± 0.003 min^{-1} ($n = 39$) and 0.054 ± 0.010 min^{-1} ($n = 24$), respectively, Fig. 7*G*). Upon addition of 10 μM CCh in 1 mM Ca^{2+} buffer, cAMP levels in both AC1 and AC8 expressing cells increased rapidly, compared with TG. However, AC8 cells showed a heterogeneous, often oscillatory cAMP response upon CCh addition (Fig. 7*F*), whereas by contrast, AC1 cells all

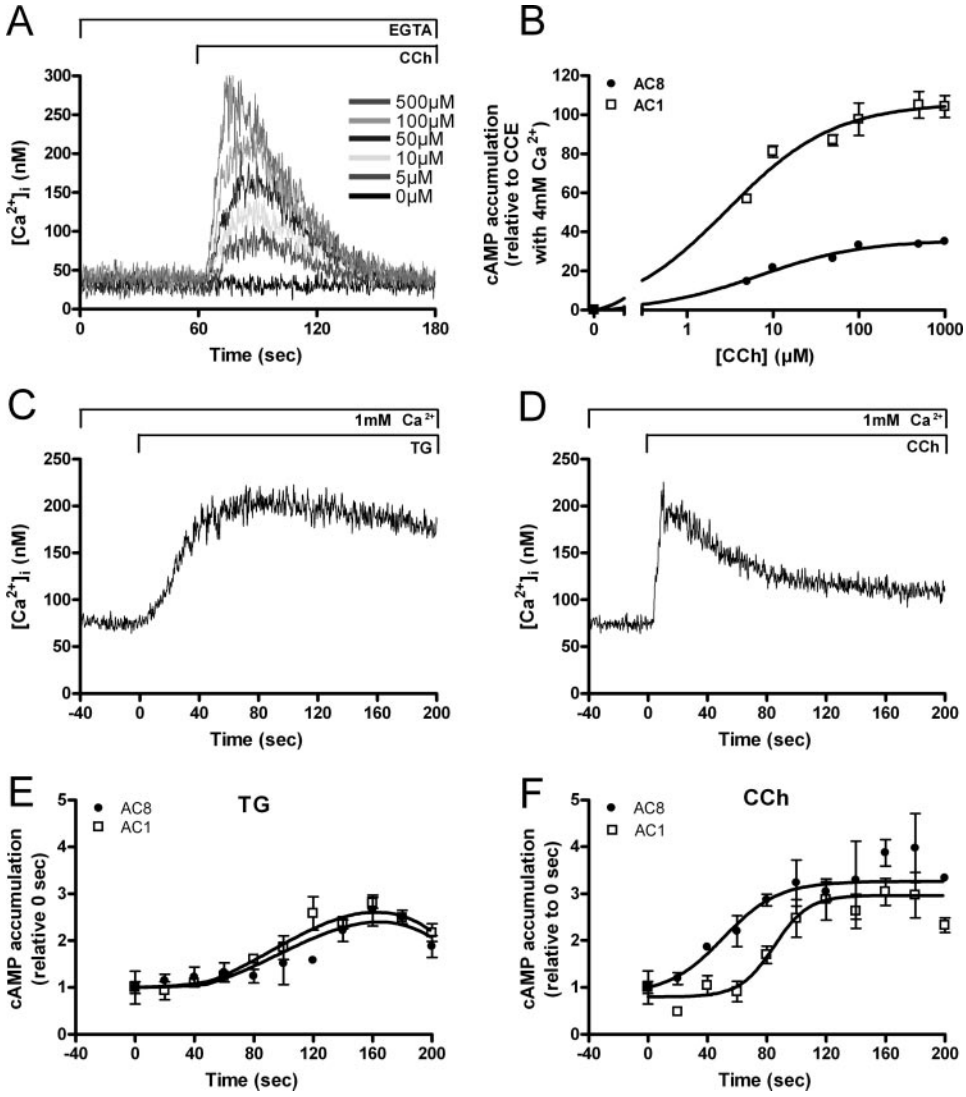


FIGURE 6. **Differential effects of TG- and CCh-mediated Ca^{2+} increase on cAMP generation by AC1 and AC8 in cell populations.** *A*, $[Ca^{2+}]_i$ was measured in populations of HEK 293 cells loaded with 2 μM fura-2/AM. To cause a Ca^{2+} release from the internal stores, 0, 5, 10, 50, 100, or 500 μM CCh was added at 60 s in the presence of 100 μM EGTA. *B*, under the same conditions as *A*, the effect of CCh-mediated Ca^{2+} release on cAMP accumulation was determined in cell populations expressing AC1 (□) or AC8 (●), as described under "Experimental Procedures." A 1-min assay was started by the addition of CCh and 10 μM FSK in the presence of 100 μM IBMX and 100 μM EGTA. Data are normalized to % of each cAMP accumulation obtained by CCE with 4 mM Ca^{2+}, and plotted as mean ± S.D. ($n = 3$). *C* and *D*, $[Ca^{2+}]_i$ was monitored in populations of fura-2/AM-loaded HEK 293 cells. HEK 293 cells were incubated in Krebs buffer with 1 mM Ca^{2+}, and 100 nM TG (*C*) or 10 μM CCh (*D*) was added at 0 s to increase $[Ca^{2+}]_i$. *E* and *F*, cAMP accumulation was measured in cell populations expressing AC1 (□) or AC8 (●) for the time intervals indicated. Cells were incubated with Krebs buffer containing 1 mM Ca^{2+}. 100 nM FSK was added at 60 s prior to the assay, and 100 nM TG (*C*) or 10 μM CCh (*D*) was added at 0 s. Data are normalized to cAMP accumulation at 0 s and plotted as mean ± S.D. of three independent experiments.

exhibited a steady increase in $[cAMP]_i$, which was sustained until the buffer was switched to Ca^{2+}-free buffer (Fig. 7*D*). This kinetics reflects the slower activation of AC1. The initial rate of increase in cAMP of AC1 was 0.082 ± 0.007 min^{-1} ($n = 65$), whereas that of AC8 was 0.313 ± 0.022 min^{-1} ($n = 71$), upon the addition of CCh (Fig. 7*G*). These single cell data confirmed that the rise in cAMP levels caused by CCh-mediated Ca^{2+} rise was significantly faster in cells expressing AC8 compared with AC1.

DISCUSSION

Behavioral, developmental, and electrophysiological studies in mice have clearly established that AC1 and AC8 play distinct physiological roles; however, few biochemical or cell biological insights are available to provide potential mechanisms for these differences. The expression levels of AC1 and AC8 vary depending on the developmental stage and region of the brain (19, 20). AC1 seems to act both pre-synaptically (16, 61, 62) and post-synaptically (63, 64). In mouse brain, AC1 is primarily localized at the postsynaptic density and AC8 at the presynaptic active zone (18, 20). Conti *et al.* suggested that the presynaptic role of AC1 and postsynaptic role of AC8 might arise in extrasynaptic membranes, where they are both expressed (20). Furthermore, the expression level of AC8 in cerebellum decreased significantly from postnatal day 7 to 14, whereas that of AC1 was constant (20). The different localization and expression pattern may reflect the discrete roles of these enzymes, but their individual regulatory properties might also explain how they can be utilized in particular contexts. Consequently, we used HEK 293 cells as a model system to compare and assess the properties of these enzymes against a constant background.

AC1 and AC8 are both stimulated by Ca^{2+}/CaM *in vitro* and CCE *in vivo*, but AC1 is significantly more sensitive to Ca^{2+}; thus AC1 could reach maximum stimulation with much lower concentrations of $[Ca^{2+}]_i$, or lesser activation of Ca^{2+} channels. In intact non-excitable cells, both AC1 and AC8 were not stimulated by Ca^{2+} influx caused by ionophores, but they were stimulated robustly by CCE, due to an apparent co-localization with CCE channels (21). A component of the dependence of AC8 on CCE is its residence in lipid raft domains of the plasma membrane. The present study finds that AC1 is also targeted to lipid rafts, adding to the sense that all Ca^{2+}-sensitive ACs are concentrated in such domains (65) and underscoring the regulatory importance of their localization in such environments. Another difference between AC1 and AC8 is that AC1 but not AC8 is maximally stimulated by CCh-mediated Ca^{2+} release. This difference could reflect the higher sensitivity to Ca^{2+} of AC1, which might in turn allow AC1 to be localized somewhat more distally from CCE channels than AC8. In this regard it is also notable that the N terminus of AC8 is required for its sensitivity

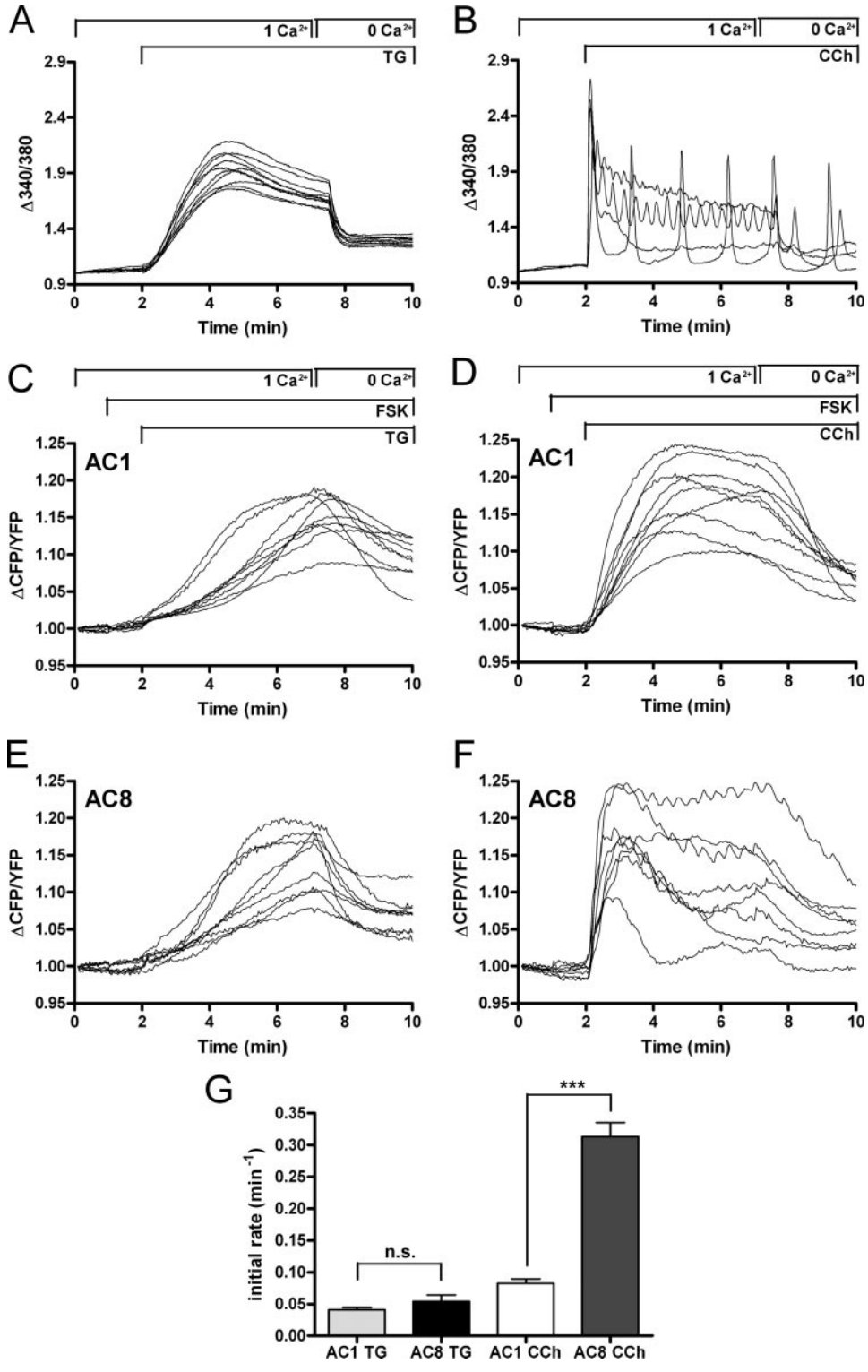


FIGURE 7. **Stimulation of AC1 and AC8 by TG- or CCh-evoked CCE, measured in single cells via a genetically encoded cAMP sensor.** *A* and *B*, $[Ca^{2+}]_i$ was monitored in single cells loaded with 2 μM fura-2/AM. HEK 293 cells were incubated in HBS buffer with 1 mM Ca^{2+}, and 100 nM TG (*A*) or 10 μM CCh (*B*) was added at 2 min to cause an increase in $[Ca^{2+}]_i$, which dropped when Ca^{2+} was replaced by 100 μM EGTA at 7 min. Data are plotted as 340/380 nm ratio changes (Δ340/380) relative to the fluorescence ratio at 0 min, and 10 representative traces from at least 600 cells are shown. *C–F*, parallel experiments were carried out to measure cAMP levels in AC1 (*C* and *D*) and AC8 (*E* and *F*) using Epac1-camps. 100 nM FSK was added at 1 min to prime ACs, and 100 nM TG (*C* and *E*) or 10 μM CCh (*D* and *F*) was added at 2 min. 1 mM Ca^{2+} was replaced by 100 μM EGTA at 7 min. Data are plotted as CFP/YFP ratio changes (ΔCFP/YFP) relative to the fluorescence ratio at 0 min, and 10 representative traces from at least 30 cells are shown. *G*, the initial rates of increase in cAMP upon the addition of TG and CCh at 2 min were calculated as detailed under "Experimental Procedures." Data are plotted as mean ± S.E. with *n* values ranging from 24 to 78 cells (***, $p < 0.001$ and *n.s.*, $p > 0.05$).

to CCE, whereas this is not the case for AC1. Could this reflect a potential direct interaction between the N terminus of AC8, but not of AC1, and the CCE apparatus?

The CaM binding domains of AC1 and AC8, and their interactions with CaM are clearly distinct. AC8 has CaM binding domains at the N terminus (an amphipathic helix) and C2b domain (an IQ-like motif), whereas AC1 apparently has only one CaM binding domain (an amphipathic helix) at the C1b domain. The present study has clarified differences in the mechanism of activation by CaM of the two ACs and added to the literature on differential roles of the N and C lobes of CaM in regulating different CaM targets. CaM pre-associates with the N terminus of AC8 (26). Mutant CaM that could not bind Ca^{2+} exerted no effect on wild-type AC8 activity. However, activity was decreased in the presence of an N-lobe mutant of CaM both *in vitro* and *in vivo*, which suggested an interaction between the C lobe of CaM and AC8. When endogenous CaM was removed by M13 incubation or deletion of the N terminus, inhibition was no longer observed, illustrating that CaM with two Ca^{2+} bound at the C lobe competes with endogenous CaM at the N terminus of AC8. In elegant studies, Yue's group has shown that Ca^{2+}-dependent facilitation of the P/Q Ca^{2+}-channel opening is initiated by Ca^{2+} binding to the C lobe of CaM, which detected primarily a local Ca^{2+} rise; Ca^{2+} inactivation was induced by the N lobe of CaM, which sensed a global Ca^{2+} rise (32). The present study showed a distinct effect of the N lobe and the C lobe of CaM on Ca^{2+} stimulation of AC8. Unlike the N-lobe mutant, a C-lobe mutant of CaM stimulated AC8 and AC8M1 activities, and the stimulation was increased when membranes were washed with M13, indicating that N-lobe Ca^{2+}-occupied CaM interacts more weakly than fully liganded CaM, but it can bind at the C2b domain of AC8. In contrast to AC8, neither N-lobe nor

C-lobe mutants of CaM affected AC1 activity. Thus, none of these partially Ca^{2+}-occupied CaM states regulates AC1; therefore, unlike AC8, only fully loaded CaM regulates AC1. Thus, a fundamentally different mode of activation by Ca^{2+}/CaM is suggested for the two enzymes.

Not only did the sensitivity of AC1 and AC8 to Ca^{2+} differ, the kinetics of this regulation also diverged. Unexpectedly (given that AC1 was more sensitive to Ca^{2+}), the response of AC1 was significantly slower both in population and in single cell measurements. This kinetics might reflect the different mechanism of regulation by CaM of AC1 and AC8 noted above. Although the affinity of CaM for Ca^{2+} is higher in the C lobe than the N lobe (55), Ca^{2+} may bind the N lobe of CaM first due to its higher on-rate (and off-rate) for Ca^{2+} binding (66, 67), in response to an initial fast $[Ca^{2+}]_i$ rise, as would be observed nearer to a Ca^{2+}-entry site. Indeed, a study by Johnson *et al.* showed that the N lobe, but not the C lobe of CaM is occupied maximally during a fast Ca^{2+} transient (68). An interaction between partially liganded CaM and a target protein then increases the affinity of the other lobe for Ca^{2+} and may decrease off-rates (69). Thus, because AC8 can interact with partially loaded CaM giving rise to such a cooperative mechanism, its speed of activation by Ca^{2+} influx may be faster than AC1.

Single cell studies revealed further differences between AC8 and AC1. AC8 gives rise to oscillations in cAMP in response to Ca^{2+} transients (43), but quite surprisingly AC1 activity yielded only a steady cAMP increase in response to CCh-evoked Ca^{2+} oscillation. Two possible reasons for non-oscillatory response of AC1 are suggested; first, the "sub-maximal" Ca^{2+} rise induced by 10 μM CCh in the presence of 1 mM Ca^{2+} is sufficient to activate maximally the more Ca^{2+}-sensitive AC1 and, second, the frequency of Ca^{2+} oscillations under this condition was too fast for AC1 to respond to, possibly reflecting a slower on/off-rate for Ca^{2+} by CaM bound to AC1. Biophysical measurements of Ca^{2+} dissociation rates from CaM in the absence or presence of peptides representing the CaM binding domains of AC1 or AC8 could clarify these possibilities. Whether the oscillatory behavior of AC8 or its faster response to rapid $[Ca^{2+}]_i$ rises would be of more significance in its neuronal setting remains to be addressed.

These real-time observations anticipate different functional roles for the two ACs. Earlier studies had shown that Ca^{2+} transients yielded distinct transcriptional consequences in B lymphocytes than did equivalent but steady Ca^{2+} rises (70). Later these authors showed that oscillations are more efficient at activating the nuclear transcription factor of activated T-cells than a steady Ca^{2+} rise (71). They also showed that different transcription factors are activated depending on the frequency of Ca^{2+} oscillations (71). The possibility that equivalent Ca^{2+} transients can give rise to either cAMP transients or a steady rise in cAMP depending on the AC target, combined with the role of cAMP in mediating stimulation of CREB transcription factors (72), might anticipate different transcriptional consequences of Ca^{2+} activation of the two enzymes.

Overall, a series of major differences arise between what might be considered AC homologues. The dissimilarity in sensitivity to Ca^{2+} both *in vitro* and *in vivo* was a first observation; this coupled with a distinct relative sensitivity to Ca^{2+} release *versus* Ca^{2+} entry hinted at more significant mechanistic differences. This was confirmed when obviously disparate mechanisms of activation by Ca^{2+}/CaM were observed, both in terms of where CaM binds to the ACs and in the different roles played by the two lobes of CaM. Finally, strikingly dissimilar kinetics in individual cell analysis in response to physiological elevation of $[Ca^{2+}]_i$ underscore how these regulatory differences can be translated into diverse outcomes for cellular cAMP, which in turn might have quite distinct results for cellular cAMP targets. One might also speculate that, because of their dissimilar amino acid sequences, especially at the N and C termini, the complement of putative binding partners of AC1 and AC8 could also vary, such that the creation and regulation of their immediate environment would be distinct. Thus this relatively simple regulatory motif of stimulation by Ca^{2+}/CaM can be seen to provide the potential range of physiological roles for these ACs that are inferred from knockout studies.

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REFERENCES

1. Defer, N., Best-Belpomme, M., and Hanoune, J. (2000) *Am. J. Physiol.* **279,** F400–F416
2. Krupinski, J., Coussen, F., Bakalyar, H. A., Tang, W. J., Feinstein, P. G., Orth, K., Slaughter, C., Reed, R. R., and Gilman, A. G. (1989) *Science* **244,** 1558–1564
3. Sunahara, R. K., Dessauer, C. W., and Gilman, A. G. (1996) *Ann. Rev. Pharmacol. Toxicol.* **36,** 461–480
4. Willoughby, D., and Cooper, D. M. F. (2007) *Physiol. Rev.* **87,** 965–1010
5. Cooper, D. M. F., Mons, N., and Karpen, J. W. (1995) *Nature* **374,** 421–424
6. Choi, E. J., Xia, Z., and Storm, D. R. (1992) *Biochemistry* **31,** 6492–6498
7. Wayman, G. A., Impey, S., and Storm, D. R. (1995) *J. Biol. Chem.* **270,** 21480–21486
8. Levin, L. R., Han, P. L., Hwang, P. M., Feinstein, P. G., Davis, R. L., and Reed, R. R. (1992) *Cell* **68,** 479–489
9. Livingstone, M. S. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 5992–5996
10. Livingstone, M. S., Sziber, P. P., and Quinn, W. G. (1984) *Cell* **37,** 205–215
11. Mons, N., Guillou, J. L., and Jaffard, R. (1999) *Cell Mol. Life Sci.* **55,** 525–533
12. Ferguson, G. D., and Storm, D. R. (2004) *Physiology* (*Bethesda*) **19,** 271–276
13. Wang, H., Gong, B., Vadakkan, K. I., Toyoda, H., Kaang, B. K., and Zhuo, M. (2007) *J. Biol. Chem.* **282,** 1507–1517
14. Gong, B., Wang, H., Gu, S., Heximer, S. P., and Zhuo, M. (2007) *Eur. J. Neurosci.* **26,** 275–288
15. Nicol, X., Bennis, M., Ishikawa, Y., Chan, G. C. K., Reperant, J., Storm, D. R., and Gaspar, P. (2006) *Eur. J. Neurosci.* **24,** 3401–3414
16. Nicol, X., Muzerelle, A., Rio, J. P., Metin, C., and Gaspar, P. (2006) *J. Neurosci.* **26,** 862–872
17. Moulder, K. L., Jiang, X., Chang, C., Taylor, A. A., Benz, A. M., Conti, A. C., Muglia, L. J., and Mennerick, S. (2008) *J. Neurosci.* **28,** 5159–5168
18. Zhang, M., Moon, C., Chan, G. C. K., Yang, L., Zheng, F., Conti, A. C., Muglia, L., Muglia, L. J., Storm, D. R., and Wang, H. (2008) *J. Neurosci.* **28,** 4736–4744
19. Nicol, X., Muzerelle, A., Bachy, I., Ravary, A., and Gaspar, P. (2005) *J. Comp. Neurol.* **486,** 281–294
20. Conti, A. C., Maas, J. W., Jr., Muglia, L. M., Dave, B. A., Vogt, S. K., Tran, T. T., Rayhel, E. J., and Muglia, L. J. (2007) *Neuroscience* **146,** 713–729

21. Fagan, K. A., Mahey, R., and Cooper, D. M. F. (1996) *J. Biol. Chem.* **271,** 12438–12444
22. Villacres, E. C., Wu, Z., Hua, W., Nielsen, M. D., Watters, J. J., Yan, C., Beavo, J., and Storm, D. R. (1995) *J. Biol. Chem.* **270,** 14352–14357
23. Parekh, A. B., and Putney, J. W., Jr. (2005) *Physiol. Rev.* **85,** 757–810
24. Smith, K. E., Gu, C., Fagan, K. A., Hu, B., and Cooper, D. M. F. (2002) *J. Biol. Chem.* **277,** 6025–6031
25. Gu, C., and Cooper, D. M. F. (1999) *J. Biol. Chem.* **274,** 8012–8021
26. Simpson, R. E., Ciruela, A., and Cooper, D. M. F. (2006) *J. Biol. Chem.* **281,** 17379–17389
27. Levin, L. R., and Reed, R. R. (1995) *J. Biol. Chem.* **270,** 7573–7579
28. Vorherr, T., Knopfel, L., Hofmann, F., Mollner, S., Pfeuffer, T., and Carafoli, E. (1993) *Biochemistry* **32,** 6081–6088
29. Hoeflich, K. P., and Ikura, M. (2002) *Cell* **108,** 739–742
30. Babu, Y. S., Sack, J. S., Greenhough, T. J., Bugg, C. E., Means, A. R., and Cook, W. J. (1985) *Nature* **315,** 37–40
31. 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
32. DeMaria, C. D., Soong, T. W., Alseikhan, B. A., Alvania, R. S., and Yue, D. T. (2001) *Nature* **411,** 484–489
33. Chen, C., and Okayama, H. (1987) *Mol. Cell. Biol.* **7,** 2745–2752
34. Boyajian, C. L., Garritsen, A., and Cooper, D. M. F. (1991) *J. Biol. Chem.* **266,** 4995–5003
35. Martin, A. C., and Cooper, D. M. F. (2006) *Mol. Pharmacol.* **70,** 769–777
36. Brooks, S. P., and Storey, K. B. (1992) *Anal. Biochem.* **201,** 119–126
37. Salomon, Y., Londos, C., and Rodbell, M. (1974) *Anal. Biochem.* **58,** 541–548
38. Chiono, M., Mahey, R., Tate, G., and Cooper, D. M. F. (1995) *J. Biol. Chem.* **270,** 1149–1155
39. Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260,** 3440–3450
40. Evans, T., Smith, M. M., Tanner, L. I., and Harden, T. K. (1984) *Mol. Pharmacol.* **26,** 395–404
41. Yamamoto, M., Toya, Y., Schwencke, C., Lisanti, M. P., Myers, M. G., Jr., and Ishikawa, Y. (1998) *J. Biol. Chem.* **273,** 26962–26968
42. Crossthwaite, A. J., Seebacher, T., Masada, N., Ciruela, A., Dufraux, K., Schultz, J. E., and Cooper, D. M. F. (2005) *J. Biol. Chem.* **280,** 6380–6391
43. Willoughby, D., and Cooper, D. M. F. (2006) *J. Cell Sci.* **119,** 828–836
44. Hellevuo, K., Yoshimura, M., Kao, M., Hoffman, P. L., Cooper, D. M. F., and Tabakoff, B. (1993) *Biochem. Biophys. Res. Commun.* **192,** 311–318
45. Ikura, M., Clore, G. M., Gronenborn, A. M., Zhu, G., Klee, C. B., and Bax, A. (1992) *Science* **256,** 632–638
46. Findlay, W. A., Martin, S. R., Beckingham, K., and Bayley, P. M. (1995) *Biochemistry* **34,** 2087–2094
47. Shuttleworth, T. J., and Thompson, J. L. (1999) *J. Biol. Chem.* **274,** 31174–31178
48. Fagan, K. A., Smith, K. E., and Cooper, D. M. F. (2000) *J. Biol. Chem.* **275,** 26530–26537
49. Ostrom, R. S., Liu, X., Head, B. P., Gregorian, C., Seasholtz, T. M., and Insel, P. A. (2002) *Mol. Pharmacol.* **62,** 983–992
50. Rybin, V. O., Xu, X., Lisanti, M. P., and Steinberg, S. F. (2000) *J. Biol. Chem.* **275,** 41447–41457
51. Crossthwaite, A. J., Ciruela, A., Rayner, T. F., and Cooper, D. M. F. (2006) *Mol. Pharmacol.* **69,** 608–617
52. Tang, W. J., Krupinski, J., and Gilman, A. G. (1991) *J. Biol. Chem.* **266,** 8595–8603
53. Wu, Z., Wong, S. T., and Storm, D. R. (1993) *J. Biol. Chem.* **268,** 23766–23768
54. Gao, Z. H., Krebs, J., VanBerkum, M. F., Tang, W. J., Maune, J. F., Means, A. R., Stull, J. T., and Beckingham, K. (1993) *J. Biol. Chem.* **268,** 20096–20104
55. Linse, S., Helmersson, A., and Forsen, S. (1991) *J. Biol. Chem.* **266,** 8050–8054
56. Persechini, A., and Cronk, B. (1999) *J. Biol. Chem.* **274,** 6827–6830
57. Bird, G. S., and Putney, J. W., Jr. (2005) *J. Physiol.* **562,** 697–706
58. Goraya, T. A., Masada, N., Ciruela, A., Willoughby, D., Clynes, M. A., and Cooper, D. M. F. (2008) *Cell Signal.* **20,** 359–374
59. Landa, L. R., Jr., Harbeck, M., Kaihara, K., Chepurny, O., Kitiphongspattana, K., Graf, O., Nikolaev, V. O., Lohse, M. J., Holz, G. G., and Roe, M. W. (2005) *J. Biol. Chem.* **280,** 31294–31302
60. Nikolaev, V. O., Bunemann, M., Hein, L., Hannawacker, A., and Lohse, M. J. (2004) *J. Biol. Chem.* **279,** 37215–37218
61. Lu, H. C., Butts, D. A., Kaeser, P. S., She, W. C., Janz, R., and Crair, M. C. (2006) *J. Neurosci.* **26,** 2692–2703
62. Ravary, A., Muzerelle, A., Herve, D., Pascoli, V., Ba-Charvet, K. N., Girault, J. A., Welker, E., and Gaspar, P. (2003) *J. Neurosci.* **23,** 2228–2238
63. Lu, H. C., She, W. C., Plas, D. T., Neumann, P. E., Janz, R., and Crair, M. C. (2003) *Nat. Neurosci.* **6,** 939–947
64. Plas, D. T., Visel, A., Gonzalez, E., She, W. C., and Crair, M. C. (2004) *Vision Res.* **44,** 3357–3364
65. Cooper, D. M. F., and Crossthwaite, A. J. (2006) *Trends Pharmacol. Sci.* **27,** 426–431
66. Bayley, P., Ahlstrom, P., Martin, S. R., and Forsen, S. (1984) *Biochem. Biophys. Res. Commun.* **120,** 185–191
67. Persechini, A., White, H. D., and Gansz, K. J. (1996) *J. Biol. Chem.* **271,** 62–67
68. Johnson, J. D., Snyder, C., Walsh, M., and Flynn, M. (1996) *J. Biol. Chem.* **271,** 761–767
69. Olwin, B. B., Edelman, A. M., Krebs, E. G., and Storm, D. R. (1984) *J. Biol. Chem.* **259,** 10949–10955
70. Dolmetsch, R. E., Lewis, R. S., Goodnow, C. C., and Healy, J. I. (1997) *Nature* **386,** 855–858
71. Dolmetsch, R. E., Xu, K., and Lewis, R. S. (1998) *Nature* **392,** 933–936
72. Mayr, B., and Montminy, M. (2001) *Nat. Rev. Mol. Cell. Biol.* **2,** 599–609