Phosphorylation and Inhibition of Type III Adenylyl Cyclase by Calmodulin-dependent Protein Kinase II in Vivo*

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Inhibition of type III adenylyl cyclase (III-AC) by intracellular Ca\textsuperscript{2+} in vivo provides a mechanism for attenuation of hormone-stimulated cAMP signals in olfactory epithelium, heart, and other tissues (Wayman, G. A., Impey, S., and Storm, D. R. (1995) J. Biol. Chem. 270, 21480–21486). Although the mechanism for Ca\textsuperscript{2+} inhibition of III-AC in vivo has not been defined, inhibition is not mediated by G\textsubscript{i}, cAMP-dependent protein kinase, or protein kinase C. However, Ca\textsuperscript{2+} inhibition of III-AC is antagonized by KN-62, a CaM-dependent kinase inhibitor. In addition, constitutively activated CaM kinase II inhibits the enzyme. These data suggest that CaM kinase II regulates the activity of III-AC by direct phosphorylation or by an indirect mechanism involving phosphorylation of a protein that inhibits III-AC. Here we report that III-AC is phosphorylated in vivo when intracellular Ca\textsuperscript{2+} is increased and that phosphorylation is prevented by CaM-dependent kinase inhibitors. Site-directed mutagenesis of a CaM kinase II consensus site (Ser-1076 to Ala-1076) in III-AC greatly reduced Ca\textsuperscript{2+}-stimulated phosphorylation and inhibition of III-AC in vivo. These data support the hypothesis that Ca\textsuperscript{2+} inhibition of III-AC is due to direct phosphorylation of the enzyme by CaM kinase II in vivo.

Adenylyl cyclases exhibit diverse regulatory properties that provide interesting mechanisms for regulation of cAMP by extracellular and intracellular signals (2, 3). These enzymes are regulated by intracellular Ca\textsuperscript{2+}, G\textsubscript{i}- and G\textsubscript{q}-coupled receptors, PKA, PKC, and membrane potential (for a general review, see Ref. 2). Regulation of adenylyl cyclases by various protein kinases generates cross-talk between the cAMP regulatory system and other signal transduction systems as well as mechanisms for feedback inhibition or amplification of cAMP signals. Because most cells express distinct combinations of adenylyl cyclases, phosphodiesterases, and protein kinases, the patterns of cross-talk between signal transduction systems are cell specific.

III-AC is expressed in several tissues including brain, heart, and retina (4), but it is particularly abundant in olfactory tissue, where it may play a major role in coupling olfactory receptors to cAMP and ion channel regulation (5). Although the enzyme is synergistically stimulated by Ca\textsuperscript{2+} and G\textsubscript{i}-coupled receptors in vitro (6), it is inhibited by Ca\textsuperscript{2+} in vivo (1). Ca\textsuperscript{2+} inhibition of III-AC may contribute to cAMP transients and provide a novel mechanism for generation of Ca\textsuperscript{2+} and cAMP oscillations (7). Although the mechanism for Ca\textsuperscript{2+} inhibition of III-AC in vivo has not been established, preliminary evidence suggests that the enzyme may be directly or indirectly regulated by CaM kinase II in vivo (1). To address this issue, we examined Ca\textsuperscript{2+} inhibition and phosphorylation of III-AC in vivo using an antibody specific to III-AC. The data indicate that III-AC is directly phosphorylated by CaM kinase II in vivo.

EXPERIMENTAL PROCEDURES

Cell Culture—Human embryonic kidney 293 (HEK-293) cells were grown at 37 °C in DMEM supplemented with 10% fetal bovine serum in a humidified 95% air/5% CO\textsubscript{2} incubator. Unless otherwise noted, components for cell culture were from Life Technologies, Inc.

Expression of III-AC in HEK-293 Cells—The III-AC cDNA clone (5) was generously provided by R. R. Reed (The John Hopkins University, Baltimore, MD). The coding sequence of III-AC was ligated into pCDM-8 for expression in HEK-293 cells. HEK-293 cells stably expressing III-AC have been described previously (1, 6).

Site-directed Mutagenesis and cDNA Transient Transfection in HEK-293 Cells—Mutagenesis of III-AC cDNA was performed using a Strategene kit (Chameleon™ double-stranded site-directed mutagenesis kit) according to the manufacturer’s recommendations (8). Mutant cDNA was cloned into the pCDM-8 expression vector, and mutations were confirmed by sequencing using a DNA sequencing kit from U. S. Biochemical Corp. The wild type III-AC and the mutant III-AC in which Ser-1076 was converted to Ala-1076 (m-III) were transiently transfected into HEK-293 cells. For transfection, HEK-293 cells were plated at a density of 3 × 10\textsuperscript{6} cells/100-mm plate and were maintained in DMEM, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin 18–24 h before transfection. On the day of transfection, the medium was aspirated, the cells were rinsed with serum-free DMEM, and the medium was replaced with 6.4 ml of serum-free DMEM. Eight μg of DNA (either control CDM-8 alone, CDMIII-AC, or CDMIII-AC(S1076A)) in 800 μl of serum-free DMEM and 64 μl of Lipofectamine (Life Technologies, Inc.) in 800 μl of serum-free DMEM were mixed, and the DNA-lipid complex was allowed to incubate for 30 min. The DNA-lipid mixture was added to each plate to be transfected and cells were then incubated at 37 °C, 5% CO\textsubscript{2} for 6 h. The cells were then split into 6-well plates containing DMEM and 10% fetal bovine serum. On day 2, the cells were labeled with DMEM containing [\textsuperscript{3}H]adenosine (2.0 μCi/ml; ICN) for 16–20 h. On day 3, the cells were assayed for cAMP accumulation as described below.

**AMP Accumulation**—Changes in intracellular cAMP were measured by determining the ratio of [\textsuperscript{3}H]cAMP to a total ATP, ADP, and AMP pool in [\textsuperscript{3}H]adenosine-loaded cells as described by Wong et al. (9). This assay system allows rapid and sensitive measurements of relative changes in intracellular cAMP levels in response to various effectors. Although absolute numbers for cAMP accumulation generally show some variation between experiments using different sets of cells (10), relative changes in cAMP were consistent between experiments. Confuent cells in 6-well plates were initially incubated in DMEM containing [\textsuperscript{3}H]adenosine (2.0 μCi/ml; ICN) for 16–20 h, washed once with 150 mM NaCl, and incubated at 37 °C for 30 min in serum-free DMEM.
containing 1.0 mM isobutylmethylxanthine and various effectors as indicated. Reactions were terminated by aspiration, washing cells once with 150 mM NaCl and adding 1.0 mM of ice-cold 5% trichloroacetic acid containing 1.0 μM CAMP. Culture dishes were maintained at 4°C for 1–4 h, and acid-soluble nucleotides were separated by ion-exchange chromatography as described previously (10). Reported data are the average of triplicate determinations ± S.D.

Membrane Preparation and Immunoprecipitation—The anti-III-AC antibody (Santa Cruz Biotechnology) used for immunoprecipitation of III-AC was a peptide-specific antibody raised against the C-terminal amino acid sequence (amino acids 1125–1144, PAAFPNGSSVTLPHQVVDNP). For [35S]methionine labeling of proteins, the cells were starved in cysteine/methionine-free medium for 2 h and labeled with [35S]methionine (200 μCi/ml for stably transfected cells or 500 μCi/ml for transiently transfected cells; DuPont NEN) for 4 h in the same medium. For [32P]labeling, the cells were starved in phosphate-free medium for 45 min and labeled with [32P]orthophosphate (200 μCi/ml for stably transfected cells or 500 μCi/ml for transiently transfected cells; DuPont NEN) for 3 h in the same medium. After metabolic labeling, cells were washed with cold PBS and harvested in ice-cold homogenization buffer (50 mM Tris, 250 mM sucrose, 5 mM MgCl2, 1 mM EDTA, and 1 mM dithiothreitol) supplemented with protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 10 μg/ml pepstatin). After homogenization in a Dounce homogenizer, cells were centrifuged in a CO2rex tubes at 2,500 rpm for 5 min. The supernatants were collected and centrifuged at 25,000 rpm for 30 min. The pellet was resuspended in solubilization buffer (PBS, 1% Nonidet P-40, 0.1% SDS, 1 mM EDTA and EGTA, 50 mM NaF, 1 mM Na3VO4, and 10 mM sodium pyrophosphate) with a protease inhibitor mixture to a final concentration of 3–5 mg of protein/ml. The suspension was gently shaken at 4°C for 3 h and centrifuged at 40,000 rpm for 30 min. Supernatants were incubated overnight with affinity-purified rabbit polyclonal antibodies directed against the C-terminal sequence of rat III-AC protein (Santa Cruz). Protein A-agarose beads (Pierce) were then added, and the incubation was continued for 3 h. The Protein A-agarose beads, separated by brief centrifugation, were washed five times with solubilization buffer. For peptide-N-glycosidase F (Boehringer Mannheim) treatment, the beads were incubated with peptide-N-glycosidase F in an incubation buffer made according to the manufacturer’s instructions for 1 h at 37°C. Antibody-III-AC complexes were eluted from the beads by heating in SDS-PAGE sample buffer according to the method of Laemmli (11). Immunoprecipitates were resolved by SDS-PAGE (7.5% acrylamide) and subjected to autoradiography.

Immunoblotting—The immunoprecipitation of III-AC from unlabeled cells was performed as described above. After SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore) by electroblotting at 50 V for 3 h at room temperature. Blots were blocked overnight in TBS with 0.05% Tween 20, 3% gelatin, and 3% bovine serum albumin at 4°C and then incubated with anti-III-AC antibody (1:100) for 1.5 h at room temperature in TBS with 1% gelatin and 1% bovine serum albumin. After washing with TBS three times, the blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:1,000, Cappel) for 1 h at room temperature. After several washes with Tris-buffered saline with 0.05% Tween 20, immunoreactive proteins were detected by an alkaline phosphatase substrate kit (Bio-Rad).

Other Procedures—Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard (12).

RESULTS

Immunoadsorption and Western Analysis of III-AC Expressed in HEK-293 Cells—In vivo phosphorylation was monitored by immunoadsorption of III-AC stably expressed in HEK-293 cells. Cells were prelabeled with [35S]methionine (200 μCi/ml) for 3 h and incubated with [35S]methionine (200 μCi/ml) for 3 h and then incubated with anti-III-AC antibody (1:100) for 1.5 h at room temperature in TBS with 1% gelatin and 1% bovine serum albumin. After washing with TBS three times, the blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:1,000, Cappel) for 1 h at room temperature. After several washes with Tris-buffered saline with 0.05% Tween 20, immunoreactive proteins were detected by an alkaline phosphatase substrate kit (Bio-Rad).

Western signal with the III-AC antibody (Fig. 1, lanes I and 2). The levels of III-AC expressed in control cells were too low to be detected by the anti-III-AC antibody. The antibody immunoadsorbed two polypeptides with molecular masses of 125 and 195 kDa from III-AC-transfected cells that were detected by Western analysis. Immunoadsorption of both polypeptides was blocked by the control peptide used to generate the anti-III-AC antibody (Fig. 1, lane 5). The intensity of the polypeptide at 125 kDa was increased, III-AC-expressing cells did not give a positive signal, and the polypeptide at 195 kDa was increased. Nonglycosylated III-AC may not be accessible to CaM kinase II because it is present in the Golgi or endoplasmic reticulum. Alternatively, the conformation of the nonglycosylated III-AC may not be accessible to CaM kinase II because it is present in the Golgi or endoplasmic reticulum. Alternatively, the conformation of the nonglycosylated III-AC may not be accessible to CaM kinase II because it is present in the Golgi or endoplasmic reticulum. Alternatively, the conformation of the nonglycosylated III-AC may not be accessible to CaM kinase II because it is present in the Golgi or endoplasmic reticulum. Alternatively, the conformation of the nonglycosylated III-AC may not be accessible to CaM kinase II because it is present in the Golgi or endoplasmic reticulum.
isoproterenol or forskolin.

III-AC was stimulated 11.02-fold by isoproterenol or forskolin, respectively. The residual level of Ca\(^{2+}\) stimulated phosphorylation seen in vivo for Ca\(^{2+}\) had no effect on forskolin stimulation of m-III (Fig. 4B). These data indicate that inhibition of III-AC by CaM kinase II is very likely due to direct phosphorylation at Ser-1076.

We also coexpressed III-AC and m-III with constitutively active CaM kinase II (CaMKII290) in HEK-293 cells to determine if mutation of Ser-1076 to Ala-1076 affected inhibition of III-AC by exogenously expressed CaM kinase II. CaMKII290 is a truncated form of the kinase that is constitutively active even in the absence of increased Ca\(^{2+}\) (14). Stable transfectants expressing CaMKII290 under the control of a metallothionein promoter were made, and these cells were then transiently transfected with constructs encoding III-AC or m-III. The response of III-AC to CaMKII290 was determined by inducing the expression of the kinase with Zn\(^{2+}\), Zn\(^{2+}\) treatment of cells not expressing CaMKII290 had no effect on basal, isoproterenol-stimulated, or forskolin-stimulated III-AC activities (data not shown). Induction of CaMKII290 in cells expressing III-AC inhibited isoproterenol- or forskolin-stimulated activities (data not shown). Induction of CaMKII290 in cells expressing m-III did not significantly inhibit isoproterenol or forskolin stimulation of m-III (5 ± 3 and 10 ± 4%). These data strongly suggest that direct phosphorylation of III-AC by CaM kinase II at Ser-1076 inhibits stimulation by β-adrenergic agonists or forskolin in vivo.

**Mutagenesis of Ser-1076 Ablates Ca\(^{2+}\) Inhibition of III-AC in Vivo**—If CaM kinase II directly phosphorylates III-AC in vivo, then mutagenesis of CaM-dependent kinase consensus phosphorylation sites within III-AC should prevent Ca\(^{2+}\) -stimulated inhibition and phosphorylation of the enzyme. The most likely CaM-dependent kinase phosphorylation site within an intracellular domain of III-AC is Ser-1076. This putative CaM-dependent kinase phosphorylation domain contains an Arg 3 residues N-terminal of a Ser-(Arg-Met-Asp-Ser) -termin. To determine if Ser-1076 is a regulatory phosphorylation site, it was mutated to Ala by site-directed mutagenesis. Wild type III-AC and m-III were transiently transfected into HEK-293 cells. The mutation did not affect isoproterenol or forskolin stimulation of the enzyme (Fig. 4A and B); m-III was stimulated 10.4 ± 0.51- and 102.6 ± 2.41-fold by isoproterenol or forskolin, respectively. III-AC was stimulated 11.02 ± 1.52- and 126.5 ± 10.66-fold by isoproterenol or forskolin.

Increases in intracellular Ca\(^{2+}\) inhibited isoproterenol stimulation of III-AC 47.8 ± 6.0% (Fig. 4A). In contrast, increased Ca\(^{2+}\) had no significant effect on isoproterenol stimulation of m-III (10.4 ± 0.51-fold versus 9.2 ± 0.8-fold). Furthermore, Ca\(^{2+}\) inhibited forskolin stimulation of III-AC 56 ± 8.7% but had no effect on forskolin stimulation of m-III (Fig. 4B). These data indicate that inhibition of III-AC by CaM kinase II is very likely due to direct phosphorylation at Ser-1076.

**DISCUSSION**

Inhibition of adenylyl cyclase activity by submicromolar Ca\(^{2+}\) has been reported for a number of tissues and cell lines...
data are the mean ± S.D. of triplicate assays and are expressed as the ratio cAMP/(AMP + ADP + ATP) × 100. The basal, isoproterenol, and forskolin activities of III-AC before induction of CaMKII290 expression were 0.23, 2.44, and 4.80, respectively. The basal, isoproterenol, and forskolin activities of m-III before induction of CaMKII290 were 0.24, 2.40, and 4.70, respectively. When present, isoproterenol and forskolin were 10 and 50 μM, respectively. The data are the mean ± S.E. of triplicate assays and are presented as the percentage inhibition of cAMP accumulation caused by induction of CaMKII290 expression. Induction of CaMKII290 expression inhibited isoproterenol and forskolin stimulation of III-AC by 43 and 74%, respectively. CaMKII290 had very little effect on isoproterenol or forskolin stimulation of the mutant enzyme.

between the Ca²⁺ and cAMP signal transduction systems and provides another mechanism, in addition to G₁-coupled receptors, for attenuation of hormone-stimulated cAMP increases. Ca²⁺-inhibitable adenylyl cyclases also provide a novel mechanism for the generation of Ca²⁺ oscillations in animal cells (7).

All of the mammalian adenylyl cyclases are inhibited by high concentrations of Ca²⁺ (>100 μM free Ca²⁺). This has been attributed to Ca²⁺ competition with Mg²⁺ for binding to ATP or a divalent metal ion regulatory site on adenylyl cyclases (24). Although it has been reported that III-AC, type V adenylyl cyclase, and type VI adenylyl cyclase are inhibited by submicromolar intracellular Ca²⁺ (1, 25, 26), mechanisms for Ca²⁺ inhibition have not been defined. The objectives of this study were to determine if III-AC is directly phosphorylated in vitro when intracellular Ca²⁺ is raised and to identify the primary site of phosphorylation. The data indicate that increases in intracellular Ca²⁺ cause phosphorylation of III-AC, that the phosphorylation is blocked by CaM-dependent kinase inhibitors, and that Ser-1076 is the major site of phosphorylation. Because Ser-1076 is within a putative CaM-dependent kinase phosphorylation domain, we conclude that III-AC is directly phosphorylated at this site by CaMKII290 in vitro. The glycosylated form of III-AC (but not the nonglycosylated form) was phosphorylated in vivo, suggesting that only the fully processed enzyme is a physiological substrate for CaM kinase II.

III-AC is synergistically stimulated by G₁-stimulated receptors and Ca²⁺ in vitro but inhibited in vivo through the action

including heart (15, 16), pituitary (17), somatotrophs (18), platelets (19), GH3 cells (20), C6 glioma cells (21), neuroblastoma cells (22), and cardiac myocytes (23). CaM-dependent kinase inhibition of adenylyl cyclases generates cross-talk be-
of CaM kinase II. It might be argued that inhibition of III-AC by CaM kinase II masks Ca\(^{2+}\)/CaM stimulation of the adenylyl cyclase \textit{in vivo}. However, the mutant enzyme lacking the CaM-dependent kinase inhibitory site (m-III) was not inhibited or stimulated by intracellular Ca\(^{2+}\). CaM apparently does not directly modulate the activity of III-AC in vivo.

What is the physiological importance of CaM kinase II inhibition of III-AC? III-AC is expressed in several tissues, including olfactory sensory neurons, brain, retina, and heart (4, 5). Furthermore, CaM kinase II is expressed in most mammalian tissues, including heart (27) and olfactory tissue (28). Although CaM kinase II inhibition of III-AC is relatively modest (40–50% inhibition of hormone-stimulated activity), it is comparable to G\(_i\)-mediated inhibition. These levels of adenylyl cyclase inhibition are physiologically relevant, and cAMP changes of this magnitude can have significant effects on physiological functions (29). The presence of Ca\(^{2+}\)-inhibitable adenylyl cyclases in heart may provide mechanisms for negative-feedback inhibition of cAMP-stimulated Ca\(^{2+}\) increases and the generation of cAMP and Ca\(^{2+}\) oscillations.

In olfactory sensory neurons, odorants stimulate rapid cAMP increases that rise and fall within milliseconds to seconds (30). These increases in cAMP are likely due to stimulation of III-AC and other adenylyl cyclases through G\(_i\) or G\(_s\)-coupled olfactory receptors. There are several possible mechanisms for the subsequent decreases in cAMP, including the actions of cyclic nucleotide phosphodiesterases (31). Because intracellular Ca\(^{2+}\) is elevated during odorant exposure (32, 33), Ca\(^{2+}\) inhibition of III-AC and stimulation of CaM-sensitive phosphodiesterases may both contribute to the transient cAMP response.

In summary, CaM kinase II phosphorylation of III-AC \textit{in vivo} is a mechanism for attenuation of hormone-stimulated cAMP increases that generates unique patterns of cross-talk between the Ca\(^{2+}\) and cAMP signal transduction systems. This is the only documented mechanism for Ca\(^{2+}\) inhibition of adenyl cyclases, and it is possible that Ca\(^{2+}\) inhibition of other adenylyl cyclases \textit{in vivo} may be mediated by the CaM-dependent kinases.

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