Stimulation of Mammalian G-protein-responsive Adenylyl Cyclases by Carbon Dioxide

Received for publication, September 18, 2008, and in revised form, October 30, 2008
Published, JBC Papers in Press, November 13, 2008, DOI 10.1074/jbc.M807239200

Philip D. Townsend1,1, Philip M. Holliday1,1, Stepan Fenyk‡, Kenneth C. Hess‡, Michael A. Gray‡, David R. W. Hodgson1, and Martin J. Cann1

From the 1School of Biological and Biomedical Sciences, the 4Department of Chemistry, Durham University, South Rd., Durham, DH1 3LE, United Kingdom, the 5Department of Pharmacology, Joan and Sanford I. Weill Medical College of Cornell University, New York, New York 10065, and the 6Institute for Cell and Molecular Biosciences, The Medical School, Newcastle University, Framlington Place, Newcastle upon Tyne, NE2 4HH, United Kingdom

Carbon dioxide is fundamental to the physiology of all organisms. There is considerable interest in the precise molecular mechanisms that organisms use to directly sense CO2. Here we demonstrate that a mammalian recombinant G-protein-activated adenylyl cyclase and the related Rv1625c adenylyl cyclase of Mycobacterium tuberculosis are specifically stimulated by CO2. Stimulation occurred at physiological concentrations of CO2 through increased Vmax. CO2 increased the affinity of enzyme for metal cofactor, but contact with metal was not necessary as CO2 interacted directly with apoenzyme. CO2 stimulated the activity of both G-protein-regulated adenylyl cyclases and Rv1625c in vivo. Activation of G-protein regulated adenylyl cyclases by CO2 gave a corresponding increase in cAMP-response element-binding protein (CREB) phosphorylation. Comparison of the responses of the G-protein regulated adenylyl cyclases and the molecularly, and biochemically distinct mammalian soluble adenylyl cyclase revealed that whereas G-protein-regulated enzymes are responsive to CO2, the soluble adenylyl cyclase is responsive to both CO2 and bicarbonate ion. We have, thus, identified a signaling enzyme by which eukaryotes can directly detect and respond to fluctuating CO2.

Inorganic carbon (Ci) is central to prokaryotic and eukaryotic physiology. The predominant biologically active forms of Ci are CO2 and HCO3- and their relative contributions to the total Ci pool are pH-dependent. The biological roles for CO2 and HCO3- include photosynthetic carbon fixation (1), pH homeostasis (2), carbon metabolism (3), activation of virulence in pathogenic organisms (4), sperm maturation (5), and as an alarmone in Drosophila (6, 7).

1 This work was supported by the Wellcome Trust, the Leverhulme Trust, and the Biotechnology and Biological Sciences Research Council. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

1 These authors contributed equally to this work.

1 To whom correspondence should be addressed: School of Biological and Biomedical Sciences, Durham University, South Rd., Durham, DH1 3LE, UK. Tel: 44-191-3341313; Fax: 44-191-3341201; E-mail: m.j.cann@durham.ac.uk.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

1 These authors contributed equally to this work.

1 To whom correspondence should be addressed: School of Biological and Biomedical Sciences, Durham University, South Rd., Durham, DH1 3LE, UK. Tel: 44-191-3341313; Fax: 44-191-3341201; E-mail: m.j.cann@durham.ac.uk.

The abbreviations used are: Ci, inorganic carbon; AC, adenylyl cyclase; sAC, soluble AC; CREB, cAMP-response element-binding protein; Mes, 2-[N-morpholino]ethanesulfonic acid; Mops, 3-[N-morpholino]propanesulfonic acid; GTP/S, guanosine 5’-3-O-(thio)triphosphate.

Given its importance in biology, the identification of CO2 responsive signaling pathways is key to understanding how organisms cope with fluctuating CO2. Two seven transmembrane receptors, Gr21a and Gr63a, have been shown to confer CO2 responsiveness in Drosophila neurons (6, 7). Guanylyl cyclase D expressing olfactory neurons also mediate sensitivity to CO2 in mice (8). A role for cGMP-activated channels in CO2 sensing has been observed in CO2 avoidance behavior in Caenorhabditis (9, 10). Despite these impressive advances, no eukaryotic signaling enzymes unequivocally demonstrated to respond to CO2 have been identified.

The mammalian soluble adenylyl cyclase (sAC) synthesizes the second messenger 3’,5’-cAMP and is directly stimulated by HCO3- (11–13). Stimulation of sAC by HCO3- has an unequivocal role in sperm maturation (5, 14–16). sAC is a member of the Class III family of adenylyl cyclases (ACs), a family that also includes the G-protein-regulated ACs and many examples from prokaryotic genomes (17, 18). The Class III ACs can be divided into four subclasses (a–d) based upon polymorphisms within the active site (19). sAC is a member of Class IIIb, a subclass characterized partly by replacement of a substrate binding Asp with Thr. The Class IIIa ACs include the mammalian G-protein-stimulated ACs and numerous prokaryotic examples. These have been previously assumed to be non-responsive to Ci (12).

All prokaryotic Class IIIb ACs examined to date respond to Ci including enzymes from organisms as diverse as Anabaena PCC 7120, Mycobacterium tuberculosis, Stigmatella aurantiaca, and Chloroflexus aurantiacus (20, 21). Two Class IIIb ACs, Ssr1991 of Synechocystis PCC 6803 and CyaB1 of Anabaena PCC 7120, have been proven to respond to CO2 and not HCO3- giving rise to the idea of AC as a true gas-sensing molecule (22, 23). The finding that Class IIIb ACs respond to CO2 and not HCO3- necessitates an examination of the assumption that G-protein-regulated ACs and related prokaryotic enzymes do not respond to Ci.

Here we demonstrate, contrary to previous work, that a recombinant G-protein-regulated AC and the Class IIIa Rv1625c AC of M. tuberculosis H37Rv show a pH-dependent response to Ci due to specific stimulation by CO2 at physiologically relevant concentrations. CO2 interacted directly with the apoprotein and modulated the activity of both the prokaryotic enzyme and G-protein-regulated AC in vivo. Finally, we contrasted the responses of sAC- and G-protein-regulated ACs to different species of Ci.
and propose that the mammalian cAMP signaling pathway is able to discriminate between CO₂ and HCO₃⁻ in vivo.

**EXPERIMENTAL PROCEDURES**

**Recombinant Proteins**—Rv1625c<sup>204–443</sup> wild type and mutant proteins, Str1991<sup>120–337</sup> wild type and mutant proteins, recombinant protein corresponding to amino acids 1–469 of human sAC (truncated splice variant (13); sAC<sub>T</sub>), recombinant protein corresponding to the first catalytic domain (amino acids 263–476; 7C<sub>T</sub>) of human AC type 7, and recombinant protein corresponding to the second catalytic domain (amino acids 821–1090; 2C<sub>T</sub>) of rat AC type 2 were expressed and purified as previously described (22, 24–27). A mixture of 7C<sub>T</sub> with an excess of 2C<sub>T</sub> (7C<sub>T</sub>:2C<sub>T</sub>) represents a catalytically active G-protein responsive AC without the transmembrane domains of the native molecule. Recombinant protein representing the short splice variant of bovine G<sub>α</sub> was purified and activated with GTP·γS·Mg<sup>2+</sup> as previously described (28). Single amino acid mutations were introduced by site-directed mutagenesis using appropriate primers and the appropriate wild type construct as template. Double amino acid mutations were introduced by site-directed mutagenesis using appropriate primers and the appropriate single amino acid mutant construct as template. All constructs were confirmed by double-stranded sequencing. Mutagentic primer sequences are provided in Table S1. Plasmids encoding Rv1625c<sup>204–443</sup> K296A and D256A mutagenic proteins were a kind gift of Joachim Schultz (25).

**Adenylyl Cyclase Assays**—AC assays were performed at 37 °C (Rv1625c<sup>204–443</sup>) or 30 °C (7C<sub>T</sub>:2C<sub>T</sub>) in a final volume of 100 µl and contained 50 mM buffer, 2 mM [2,8-<sup>3</sup>H]cAMP (150 Bq), and [α-<sup>32</sup>P]ATP (25 kBq) if not stated otherwise (29). Protein concentrations were adjusted to maintain substrate utilization at <10%. The following buffers were used at pH 6.5 (Mes), pH 7.0–7.5 (Mops), and pH 8.0–8.5 (Tris-hydrochloride). Enzyme, buffer, and substrate were prepared at the appropriate pH. CO₂ was quantified by titration against NaOH. Assay pH was stable over a period of at least 40 min. For dose-response experiments, NaHCO₃ was added to the assay, and the CO₂ concentration was calculated using the Henderson-Hasselbalch equation, and the total salt concentration was adjusted with NaCl. All errors correspond to the S.E. If absent, errors were smaller than the symbol used to depict the data point. Adenylyl Cyclase Assays at Ci Disequilibrium—For Ci disequilibrium assays, dissolved CO₂ was prepared by bubbling into double-distilled H₂O at 0 °C to saturation and quantified by titration against NaOH. NaHCO₃ and NaCl were prepared in double-distilled H₂O at 0 °C. CO₂, HCO₃⁻, or Cl⁻ were subsequently added to the assay at 0 °C simultaneous with substrate to the required concentration. Buffer and substrate for assays were prepared at the appropriate pH and temperature for the experiment. pH changes in assays were monitored using a pH electrode (Biotrode; Hamilton) connected to a computer with a PC card (Orion Sensorlink). The pH was measured in a time-driven acquisition mode in assays identical to those used for biochemistry. All pH measurements were accurate to ±0.02 pH units (manufacturers specifications). All errors correspond to the S.E.

CO₂, Activation of AC in Vivo—pCTXLacZ, a plasmid with lacZ expression driven from a cAMP-responsive promoter, and pQE30-Rv1625c<sup>204–443</sup> (25) were transformed into *Escherichia coli* M15 (pREP4). Cells were grown in Luria broth with 100 µg ml⁻¹ ampicillin, 50 µg ml⁻¹ kanamycin, and 5 µg ml⁻¹ tetracycline at 30 °C until an A<sub>600</sub> of 0.6. Rv1625c<sup>204–443</sup> protein production was induced with 30 µM isopropyl 1-thio-β-d-galactopyranoside for 3 h. Cells were pelleted at 4000 × g for 10 min and resuspended in Luria broth containing 50 mM Tris, pH 7.1. Cell suspensions were bubbled with either 10% (v/v) CO₂ in air or in air for 30 min at 30 °C. Cells were disrupted with 0.1 mg of sodium deoxycholate and 1% (v/v) toluene and mixed for 10 min at 30 °C. The lysate was made up to 50 mM sodium phosphate, pH 7.0, 0.5 mM ortho-nitrophenol-β-d-galactopyranoside and incubated for 15 min at 30 °C. Reactions were stopped with 2 mM sodium carbonate, and absorbance was read at 420 nm. A standard curve was generated using 0–250 µM ortho-nitrophenol.

CO₂, Binding Assays—1 ml of 50% (v/v) Sephadex G50 in 50 mM Mes, pH 6.5 (bed volume 0.5 ml), was pre-spun at 1500 × g for 30 s. A freshly prepared binding reaction of 23 nmol of protein, 30 mM NaH<sub>14</sub>CO₃, pH 6.5, and 50 mM Mes, pH 6.5, (total volume 50 µl) was immediately added and centrifuged at 1500 × g for 30 s, and the flow-through collected into 50 µl of 2 M NaOH. Scintillation counting was used to measure 14C counts in the flow-through.

**Measurement of Intracellular pH—**HEK 293T cells attached to a 24-mm diameter glass coverslip were loaded with the pH-sensitive fluorescent dye 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF) through exposure to 1 µM BCECF-AM (an acetoxymethyl ester derivative) for 30 min. Intracellular pH was measured by exciting a small patch of cells at 490 and 440 nm using a microspectrofluorometric system and measuring emission at 535 nm. pH was calibrated using the high potassium nigericin method (30).

**cAMP Accumulation in Vivo—**HEK 293T cells were cultured in 12-well plates and labeled overnight with 1.5 µCi of [³H]adenine at 80–90% confluence. Cells were washed with phosphate-buffered saline solution and incubated at the required CO₂ concentration in preincubation media (10 mM HEPES-NaOH, 117 mM NaCl, 4.5 mM KCl, 1 mM MgCl₂, 11 mM glucose, 10 mM sucrose, and 2.5 mM CaCl₂) containing 1 mM isobutylmethylxanthine. Preincubation mixes were gassed with the desired CO₂ concentration and adjusted to pH 7.0. The assay was initiated after 30 min by the addition of agonist and incubated at the required CO₂ concentration. Assays were stopped with 5% (w/v) trichloroacetic acid containing 1 mM ATP and 1 mM cAMP. Products were quantified by twin column chromatography (29). For immunoblotting, samples were harvested after treatment as above except in the absence of [³H]adenine and isobutylmethylxanthine. Immunoblotting was performed using standard methodologies with anti-phospho-CREB (Set<sup>133</sup>) and anti-α-tubulin as load control.

**RESULTS AND DISCUSSION**

The *M. tuberculosis* H37Rv genome contains at least 15 putative ACs and one cAMP phosphodiesterase, suggesting an important role for cAMP in the physiology of *Mycobacterium*
(31–34). cAMP is implicated in the pathogenesis of mycobacteria, and CO₂ has been suggested as a signal to enable Mycobacterium to avoid phagosomal acidification (35, 36). The Rv1625c gene of M. tuberculosis encodes an enzyme consisting of six putative transmembrane helices and a single Class IIIa AC catalytic domain (25, 37). The predicted topology, therefore, resembles one-half of a mammalian G-protein-regulated AC enzyme. A further similarity arises in the active site where six key catalytic residues distributed among the two catalytic domains of the G-protein-regulated ACs are present in Rv1625c to generate a homodimeric enzyme with two active sites (Fig. 1a).

The Class IIIa Rv1625c AC was reported to be insensitive to Ci under experimental conditions where HCO₃⁻/H₂CO₃ was the predominant form of Ci. We expressed the AC domain of Rv1625c as a recombinant protein (Rv1625c204–443) and investigated the response of enzyme to constant Ci at varying pH (Fig. 1b). Relative stimulation (Ci:Cl⁻/H₂CO₃) varied from less than 1 at pH 8.5 (0.1 mM CO₂, 19.6 mM HCO₃⁻/H₂CO₃, 0.3 mM CO₃²⁻) to 6.3 at pH 6.5 (7.7 mM CO₂, 12.3 mM HCO₃⁻/H₂CO₃). Stimulation of Rv1625c specific activity was most evident below pH 7.5, explaining a failure to previously observe a stimulation with Ci (20). A requirement for low pH to observe a response to Ci is consistent with a role for CO₂ as the activating species but may also be due to the altered protonation status of Rv1625c204–443 limiting the ability of the enzyme to respond to HCO₃⁻ at higher pH. We, therefore, assayed Rv1625c204–443 specific activity (n = 6) was plotted against increasing CO₂. The assay mixture contained 433 nM protein and 200 μM Mn²⁺-ATP, pH 6.5. The total salt concentration was adjusted to 30 mM for all data points.
equilibrium by following the acquisition of the CO$_2$/HCO$_3^-$ equilibrium through measuring the pH of a weakly buffered (5 mm) Mes solution on the addition of 20 mM CO$_2$ or NaHCO$_3$ in the presence or absence of carbonic anhydrase at 0 °C (data not shown). In this manner we defined conditions for assaying AC under conditions of disequilibrium using 20 mM CO$_2$ or HCO$_3^-$ as a 10-s assay period at 0 °C after the addition of Ci. Under these conditions, Ci is predominantly in the form added to the assay (CO$_2$ or HCO$_3^-$) and has not significantly advanced toward the equilibrium determined by assay pH (clamped with 100 mM Mes in the actual AC assays). Control experiments demonstrated that under the conditions used for the assay final pH was equivalent when either CO$_2$, HCO$_3^-$, or Cl$^-$ were added, demonstrating that any observed stimulation was due to addition of Ci and not a change in assay pH (Fig. 1c; inset). Ci disequilibrium assays proved that Rv1625c$^{204-443}$ responded to CO$_2$ and not HCO$_3^-$ (Fig. 1c). This demonstrates that a Class IIIa AC is able to respond to Ci and confirms that the response is to CO$_2$, as with Class IIIb ACs.

Given the similarity in response to CO$_2$ seen in Rv1625c$^{204-443}$ and Class IIIb ACs, we examined the kinetic parameters for Rv1625c and compared them to the Class IIIb ACs (Table 1). CO$_2$ stimulated Rv1625c$^{204-443}$ specific activity through an increase in $k_{cat}$, similar to findings with Class IIIb ACs, supporting the idea that the two subclasses share a similar mechanism of response to CO$_2$ (20, 22). A dose-response curve with increasing Ci revealed a 5-fold stimulation at 11.6 mM CO$_2$ (Fig. 1c). Concentrations over 12 mm caused a gradual decrease in specific activity from this peak, making an EC$_{50}$ impossible to unambiguously calculate. Stimulation was significant to a 95% confidence interval at 1.9 mM CO$_2$.

Given the clear relationship between Rv1625c and the Class IIIb ACs with respect to the kinetics of activation in response to CO$_2$, we investigated the activation mechanism. Mutation of a key substrate determining lysine (Lys-646) in the Class IIIb CyaB1 AC of *Anabaena* ablated the response of the enzyme to CO$_2$ (20). We generated recombinant protein for the corresponding mutation in Rv1625c (K296A) and assessed its response to CO$_2$. Surprisingly, Rv1625c$^{204-443}$ K296A retained responsiveness to CO$_2$. This finding was not unique to Rv1625c as the corresponding mutation in the Class IIIb Slr1991 AC of *Synechocystis* (K177A) was also responsive to CO$_2$. It is plausible that the substrate determining lysine is not actually a direct site of action for CO$_2$, and we sought evidence for an alternative binding site. Ci has been proposed to help recruit the second metal ion to the active site of the Class IIIb CyaC AC of *Spirulina platensis* (39). Assay of Rv1625c$^{204-443}$ at varying Mn$^{2+}$ concentrations revealed that CO$_2$ increased the slope of the dose response (6.6) compared with NaCl (3.0), indicating an increase in cooperativity between binding sites (Fig. 2a). On the basis of their findings in CyaC, Steegborn et al. (39) suggested that Ci interacted directly with an active site metal ion. Given our findings on Mn$^{2+}$ recruitment for Rv1625c, we further investigated this hypothesis. Attempts to identify the metal co-factor as a site of CO$_2$ interaction through enzyme assay proved uninformative, and we, therefore, developed an alternative methodology.

Radiolabeled CO$_2$ bound to protein has been previously recovered after mixing and rapid gel filtration (40). We, therefore, performed a binding analysis to examine the requirements for CO$_2$ binding to enzyme. CO$_2$ bound Rv1625c$^{204-443}$ with no requirement for metal or substrate (Fig. 2b). Identical results were obtained for the Class IIIb ACs Slr1991 and CyaB1. Control proteins including bovine serum albumin and an alternative hexahistidine-tagged protein showed recovery indistinguishable from buffer alone, indicating an absence of any specific CO$_2$ binding. These data would appear to eliminate a requirement for metal in the active site for CO$_2$ binding, but it is possible that metal co-purified with protein and remained bound to enzyme. We, therefore, investigated CO$_2$ binding in a mutant protein in which both metal binding aspartate residues were mutated to alanine (39, 41). The low yield of protein for Rv1625c$^{204-443}$ D256A/D300A made this experiment impossi-

### Table 1

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rv1625c$^{204-443}$</th>
<th>7C, 2C$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$ (nmol of cAMP mg$^{-1}$ min$^{-1}$)</td>
<td>30.4 ± 0.8</td>
<td>76.0 ± 2.8</td>
</tr>
<tr>
<td>$K_{cat}$ (s$^{-1}$)</td>
<td>0.54 ± 0.02</td>
<td>1.72 ± 0.09</td>
</tr>
<tr>
<td>$k_{cat}/k_{on}$ (s$^{-1}$)</td>
<td>5.9</td>
<td>14.6</td>
</tr>
</tbody>
</table>

**Adenylyl Cyclase and Carbon Dioxide**

**FIGURE 2.** CO$_2$ binds Rv1625c in vitro and activates in vivo. a, Rv1625c$^{204-443}$ specific activity (*n* = 6) was plotted against increasing Mn$^{2+}$. The assay mixture contained 1.8 μM protein and 200 μM Mn$^{2+}$-ATP, pH 6.5, and 20 mM NaCl (triangles) or 20 mM NaHCO$_3$ (7.7 mM CO$_2$, squares). b, recovered CO$_2$ from a binding assay in the presence of Rv1625c$^{204-443}$, bovine serum albumin (BSA), or buffer alone. c, recovered CO$_2$ from a binding assay in the presence of Slr1991$^{200-337}$ wild type (wt), Slr1991$^{200-337}$ D137A D181A (Δmetal), BSA, or buffer alone. d, cAMP-dependent lacZ activity in E. coli under control (vector) conditions or in the presence of Rv1625c$^{204-443}$ in samples treated with air or 10% (v/v) CO$_2$ in air (*n* = 9; *, *p* < 0.05). The y axis denotes the concentration of ortho-nitrophenol (ONP) in the lacZ assays performed.

---

4 P. D. Townsend, P. M. Holliday, D. R. W. Hodgson, and M. J. Cann, unpublished observations.
endogenous Cya AC (47). This demonstrates that a prokaryotic AC can be stimulated by CO₂ in an intact bacterium and, thus, fulfills a key criterion for AC as a functional CO₂ sensor in bacteria.

Building on our findings with Rv1625c, we investigated CO₂ as a stimulating ligand for a related mammalian G-protein regulated AC, an experiment of some importance as CO₂-stimulated signaling enzymes are not known in eukaryotes (7C1/2C2; Fig. 1a). We investigated the response of 7C1/2C2 to 20 mM total Cl or NaCl (n = 4). The figure shows specific activity in the presence of 20 mM NaCl (triangles; right-hand axis) and relative stimulation with Ci (squares; left-hand axis). b, 5 μg of sAC was assayed at 0 °C for 10 s at pH 6.4 with 0.8 mM ATP, 5 mM MgCl₂, and 5 mM CaCl₂ with either 20 mM CO₂, NaHCO₃, or NaCl (n = 15; *, p < 0.05).

As sAC is proposed but not proven to respond to HCO₃⁻, we investigated any overlap in specificity for Ci between sAC and the G-protein regulated ACs. sAC_C relative stimulation (Cᵢ/Cl⁻) varied from 2.0 at pH 8.5 to 3.1 at pH 6.5 (Fig. 4a). The
result at pH 8.5 is consistent with a role for HCO₃⁻ as an activating ligand; however, the slight increase in fold stimulation as pH is lowered suggests a response to CO₂. Under conditions of Ci disequilibrium, both CO₂ and HCO₃⁻ stimulated sAC₃ (Fig. 4b).

We next investigated whether CO₂ stimulated G-protein activated cAMP signaling in vivo. As CO₂/HCO₃⁻ is a potent biological buffer, we defined conditions under which changes in internal pH (pHᵢ) were minimized on changing CO₂. Moving from a lower to a higher CO₂ concentration gave a transient cellular acidification and vice versa (Fig. 5a). Assays were, therefore, performed after allowing pH homeostasis to occur, although it is pertinent to note that G-protein-regulated ACs have been demonstrated to be offered some protection from severe respiratory acidosis or alkalosis. Some tissues are, however, able to act as a sensing mechanism for CO₂ in vivo through the action of Na⁺/H⁺ antiporters (48).

Stimulation of G-protein-activated ACs with the β-adrenergic receptor agonist isoproterenol gave an increase in cAMP accumulation in 5% (v/v) CO₂ in air versus air (0.03% CO₂; open bars) or 5% (v/v) CO₂ (filled bars) with 5 μM forskolin and 1 μM KH7 (n = 12; *p < 0.05). d, lower panel shows immunoblot of HEK 293T cell material after treatment with and without isoproterenol at varying CO₂. The upper panel shows the ratio of phospho-CREB:α-tubulin bands from the quantified bands.

Our findings demonstrate that the G-protein-activated ACs are specifically CO₂-activated signaling enzymes, and this is supported by similar data in a related prokaryotic enzyme. It is possible that our findings are specific only for the 7C1/2C2 protein used in this study, as any amino acid residue(s) required for CO₂ binding may not be conserved among G-protein-regulated ACs in general. We hypothesize, however, that CO₂ regulation will be a general feature of most if not all G-protein-activated AC isoforms. A diverse range of Class IIIa, -b, and -d ACs have been demonstrated to respond to Cᵢ. Given the extent of sequence diversity between these AC subclasses, it is unlikely that the relatively closely related G-protein-regulated ACs of Class IIIa will differ significantly in their responses to CO₂, but an examination of individual isoforms will be required to formally prove this.

Importantly, our findings overturn previous assumptions about these enzymes as non-responsive to Ci. Furthermore, we demonstrate that CO₂ interacts directly with apoprotein to stimulate metal recruitment and not through metal contact as previously proposed. We demonstrate that sAC is not the sole Ci-sensitive AC in mammals as thought and that sAC and G-protein-regulated ACs show differential sensitivity to Ci species with G-protein-regulated ACs responsive to CO₂ and sAC responsive to HCO₃⁻ and CO₂. Not only is the cAMP signaling pathway in its entirety, therefore, able to act as a sensing system for Ci, but different aspects of this pathway are able to discriminate between CO₂ and HCO₃⁻. An interesting facet of this differential regulation is that sAC detection of Ci may be entirely independent of intracellular pH, whereas the G-protein-responsive AC signaling in response to Ci may occur predominantly under conditions of pathophysiology, e.g. severe respiratory acidosis or alkalosis. Some tissues are,
Adenylyl Cyclase and Carbon Dioxide

however, exposed to large variations in pCO₂ and have G-protein-activated cAMP signaling central to their physiology. The duodenum is exposed to a pCO₂ of up to 400 mm Hg, and the cAMP activated cystic fibrosis transmembrane conductance regulator is key to HCO₃⁻ secretion in this tissue (51). Acidification of the epididymal lumen with an associated low HCO₃⁻ concentration is essential to maintain stored spermatozoa in a quiescent state (52). It might be envisaged that these conditions would be sufficient to maintain sAC in an inactive state, but our data demonstrating that sAC is able to respond to CO₂ suggest that this cannot be the sole mechanism for keeping sAC activity switched off. A potential specific role for CO₂ signaling through G-protein-regulated ACs is evident in respiratory alkalosis. A key finding should assess the role of G-protein-regulated ACs in this tissue is also conserved in a prokaryotic counterpart of the mammalian Class IIIa enzyme. Future research from these key findings should assess the role of G-protein-regulated ACs in response to fluctuating CO₂ as discussed. The cAMP signaling pathway, therefore, represents a novel signaling pathway able to directly respond to CO₂ in eukaryotes.

Acknowledgments—We thank Roger Sunahara, Matthew Wolkang, Joachim Schultz, Lonny Levin, and Jochen Buck for the kind gift of reagents used in this work and Lonny Levin for comments on the manuscript.

REFERENCES