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

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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 k₄₅. CO₂ increased the affinity of enzyme for metal co-factor, 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. 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).

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 aurantia, 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**—Rv1625c²⁰⁴–⁴⁴³ wild type and mutant proteins, Sf919911²⁰⁰–³³³ wild type and mutant proteins, recombinant protein corresponding to amino acids 1–469 of human sAC (truncated splice variant (13); sAC₁), recombinant protein corresponding to the first catalytic domain (amino acids 263–476; 7C₁) of human AC type 7, and recombinant protein corresponding to the second catalytic domain (amino acids 821–1090; 2C₂) of rat AC type 2 were expressed and purified as previously described (22, 24–27). A mixture of 7C₁ with an excess of 2C₂ (7C₁:2C₂) 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 Gα₁ was purified and activated with GTPγS·Mg²⁺ 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. Mutagenic primer sequences are provided in Table S1. Plasmids encoding Rv1625c²⁰⁴–⁴⁴³ K296A and D256A mutagenic proteins were a kind gift of Joachim Schultz (25).

**Adenylyl Cyclase Assays**—Assays were performed at 37 °C (Rv1625c²⁰⁴–⁴⁴³) or 30 °C (7C₁:2C₂) in a final volume of 100 µl and contained 50 mM buffer, 2 mM [2,8-³H]cAMP (150 Bq), and [α-³²P]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 C_i 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-Rv1625c²⁰⁴–⁴⁴³ (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 A₆₀₀ of 0.6. Rv1625c²⁰⁴–⁴⁴³ 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) tolune 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 5 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 NaH¹⁴CO₃, 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)-carboxy-fluorescein (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 succrose, and 2.5 mM CaCl₂) containing 1 mM isobutylmethylxanthine. Preincubation mixes were pre-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 (Set¹³³) 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*
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 IIa Rv1625c AC was reported to be insensitive to Ci under experimental conditions where HCO₃⁻/H₁₁₀₀₂ 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⁻/H₁₁₀₀₂) varied from less than 1 at pH 8.5 (0.1 mM CO₂, 19.6 mM HCO₃⁻/H₁₁₀₀₂, 0.3 mM CO₃²⁻/H₁₁₀₀₂) to 6.3 at pH 6.5 (7.7 mM CO₂, 12.3 mM HCO₃⁻/H₁₁₀₀₂). 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.

FIGURE 1. Rv1625c is stimulated by CO₂. a, alignment of the catalytic domains of Rv1625c, human AC type 7 C1 domain, rat AC type 2 C2 domain, Slr1991 of Synechocystis, and CyaB1 of Anabaena. Numbers denote the amino acid sequence number. Arrows indicate conserved metal binding aspartate residues. The triangle indicates the substrate binding lysine residue, and the circle is the polymorphic D/T of Class IIIa/b ACs. b, ratio of the specific activities of Rv1625c²₀₄–₄₄₃ when assayed in the presence of 30 mM total Ci or NaCl at various pH values (1.8 μm protein, 200 μM Mn²⁺-ATP, n = 8). The inset shows the percentage of total Ci made up by CO₂ and HCO₃⁻ over the pH range tested. 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). c, cAMP produced by Rv1625c²₀₄–₄₄₃ under conditions of Ci disequilibrium (36 μM Rv1625c²₀₄–₄₄₃, 0 °C, 10 s, 20 mM CO₂, 20 mM NaHCO₃, 20 mM NaCl, 100 mM Mes, pH 6.5, 200 μM Mn²⁺-ATP, n = 20; *, p < 0.05). The inset shows a representative control experiment demonstrating that the pH was identical in all assays (circles, NaCl; triangles, NaHCO₃; squares, CO₂; arrow, assay start point). d, 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 CO₂/HCO₃ equilibrium through measuring the pH of a weakly buffered (5 mM) Mes solution on the addition of 20 mM CO₂ or NaHCO₃ 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 CO₂ or HCO₃ as a 10-s assay period at 0 °C after the addition of Cₗ. Under these conditions, Cₗ is predominantly in the form added to the assay (CO₂ or HCO₃) 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 CO₂, HCO₃, or Cl⁻ were added, demonstrating that any observed stimulation was due to addition of Cₗ and not a change in assay pH (Fig. 1c; inset). Cₗ disequilibrium assays proved that Rv1625c₂₀₄–₄₄₃ responded to CO₂ and not HCO₃ (Fig. 1c). This demonstrates that a Class IIIa AC is able to respond to Cₗ and confirms that the response is to CO₂, as with Class IIIb ACs.

Given the similarity in response to CO₂ seen in Rv1625c²₀₄–₄₄₃ and Class IIIb ACs, we examined the kinetic parameters for Rv1625c and compared them to the Class IIIb ACs (Table 1). CO₂ stimulated Rv1625c²₀₄–₄₄₃ specific activity through an increase in kₘ₀, similar to findings with Class IIIb ACs, supporting the idea that the two subclasses share a similar mechanism of response to CO₂ (20, 22). A dose-response curve with increasing Cₗ revealed a 5-fold stimulation at 11.6 mM CO₂ (Fig. 1d). Concentrations over 12 mM caused a gradual decrease in specific activity from this peak, making an EC₅₀ impossible to unambiguously calculate. Stimulation was significant to a 95% confidence interval at 1.9 mM CO₂.

Given the clear relationship between Rv1625c and the Class IIIb ACs with respect to the kinetics of activation in response to CO₂, we investigated the activation mechanism. Mutation of a key substrate determining lysine (Lys-646) in the Class IIIb Synechocystis (K177A) was also responsive to CO₂. This finding was not unique to Rv1625c as the corresponding mutation in the Class IIIb Slr1991 AC of Synechocystis (K177A) was also responsive to CO₂. It is plausible that the substrate determining lysine is not actually a direct site of action for CO₂, and we sought evidence for an alternative binding site. Cₗ 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 Rv1625c²₀₄–₄₄₃ at varying Mn²⁺ concentrations revealed that CO₂ 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 Cₗ interacted directly with an active site metal ion. Given our findings on Mn²⁺ recruitment for Rv1625c, we further investigated this hypothesis. Attempts to identify the metal co-factor as a site of CO₂ interaction through enzyme assay proved uninformative, and we, therefore, developed an alternative methodology.

Radiolabeled CO₂ 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 CO₂ binding to enzyme. CO₂ bound Rv1625c²₀₄–₄₄₃ 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 CO₂ binding. These data would appear to eliminate a requirement for metal in the active site for CO₂ binding, but it is possible that metal co-purified with protein and remained bound to enzyme. We, therefore, investigated CO₂ binding in a mutant protein in which both metal binding aspartate residues were mutated to alanine (39, 41). The low yield of protein for Rv1625c²₀₄–₄₄₃ D256A/D300A made this experiment impossi-

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**TABLE 1**

**Kinetic parameters for Rv1625c²₀₄–₄₄₃ and 7C₁–₂C₂**

| Parameter | Rv1625c²₀₄–₄₄₃ | 7C₁–₂C₂ |
|-----------|----------------|--------|
| Vₘₐₓ (nmol of cAMP mg⁻¹ min⁻¹) | 30.4 ± 0.8 | 76.0 ± 2.8 |
| Kₙₙₐₜ (mM) (S.D.) | 5.04 ± 0.02 | 1.72 ± 0.09 |
| kₖₑₜ (s⁻¹) | 1.89 ± 0.25 | 2.04 ± 0.23 |

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**Adenylyl Cyclase and Carbon Dioxide**

**FIGURE 2.** CO₂ binds Rv1625c in vitro and activates in vivo. (a) Rv1625c²₀₄–₄₄₃ specific activity (n = 6) was plotted against increasing Mn²⁺. The assay mixture contained 1.8 μM protein and 200 μM Mn²⁺ ATP, pH 6.5, and 20 mM NaCl (triangles) or 20 mM NaHCO₃ (7.7 mM CO₂, squares). b, recovered CO₂ from a binding assay in the presence of Rv1625c²₀₄–₄₄₃, bovine serum albumin (BSA), or buffer alone. c, recovered CO₂ from a binding assay in the presence of Slr1991¹²₀–₃₃₇ wild type (wt), Slr1991¹²₀–₃₃₇ D17A D181A (Δmetal), BSA, or buffer alone. d, cAMP-dependent lacZ activity in *E. coli* under control (vector) conditions or in the presence of Rv1625c²₀₄–₄₄₃ in samples treated with air or 10% (v/v) CO₂ in air (n = 9; *, p < 0.05). The y axis denotes the concentration of ortho
tnitrophenol (ONP) in the lacZ assays performed.

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P. D. Townsend, P. M. Holliday, D. R. W. Hodgson, and M. J. Cann, unpublished observations.
increased in Rv1625c 204–443 activity at elevated CO2 (Fig. 2). Driven expression of lacZ as readout, we observed a consistent lacZ reporter construct as a suitable alternative. Using cAMP-induced expression of Rv1625c 204–443 likely reduced endogenous cAMP production and eliminated the possibility that our observations were due to the 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 example 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 Ci 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).

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FIGURE 4. sAC is activated by CO2 and HCO3−. a, 300 ng of sAC was assayed at 30°C for 30 min with 0.8 mM ATP, 5 mM MgCl2, and 5 mM CaCl2 and either 20 mM total Ci 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).

FIGURE 3. Stimulation of a G-protein regulated AC by CO2 in vitro. a, ratio of the specific activities of 1.1 µM 7C1, and 5.8 µM 2C2 when assayed in the presence of 20 mM total Ci or NaCl at various pH values (500 µM Mg2+ -ATP, 7 µM GTPyS, n = 6). 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, cAMP produced by 7C1, 2C2, under conditions of Ci disequilibrium (20 µM 7C1, 3.2 µM 2C2, 0°C, 10 s, 20 mM CO2/NaHCO3/NaCl, 100 mM Mes, pH 6.5, 1 mM Mg2+-ATP, 100 µM forskolin, n = 6, *, p < 0.05). Control experiments demonstrated that the pH was identical in all assays. c, 7C1, 2C2 specific activity (n = 9) was plotted against increasing CO2 at pH 6.5. The assay mixture contained 1.1 µM 7C1, 5.8 µM 2C2, 7 µM GTPyS, and 500 µM Mg2+-ATP. The total salt concentration was adjusted to 30 mM for all data points. d, 7C1, 2C2 specific activity (n = 6) was plotted against increasing Mg2+. The assay mixture contained 1.1 µM 7C1, 5.8 µM 2C2, 7 µM GTPyS, and 500 µM Mg2+-ATP, pH 6.5, and 20 mM NaCl (triangles) or 20 mM NaHCO3 (7.7 mM CO2; squares).
result at pH 8.5 is consistent with a role for HCO$_3^-$ as an activating ligand; however, the slight increase in-fold stimulation as pH is lowered suggests a response to CO$_2$. Under conditions ofCi disequilibrium, both CO$_2$ and HCO$_3^-$ stimulated sAC$_{12}$ (Fig. 4b).

We next investigated whether CO$_2$ stimulated G-protein activated cAMP signaling in vivo. As CO$_2$/HCO$_3^-$ is a potent biological buffer, we defined conditions under which changes in internal pH (pH$_i$) were minimized on changing CO$_2$. Moving from a lower to a higher CO$_2$ 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 changes in pH$_i$ 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) CO$_2$ in air versus air (0.03% CO$_2$); atmospheric concentrations of CO$_2$ in solution are $\sim$0.015 μM, although the true cellular concentration is likely to be higher due to the continual production of metabolic CO$_2$ (Fig. 5b). The magnitude of this response was similar to that observed when sAC was challenged with Ci in vivo (12, 13). No further stimulation was observed at 10% (v/v) CO$_2$ as it is likely that full CO$_2$ activation in a precise physiological setting requires an associated carbonic anhydrase to maintain CO$_2$ flux (49, 50). Similar results were obtained when cAMP production was stimulated with forskolin, indicating that the stimulating effect of CO$_2$ does not occur upstream of AC (Fig. 5c). The lack of stimulation by CO$_2$ in the absence of agonist for G-protein-regulated AC confirmed that sAC was not the source of cAMP. The inclusion of the anti-sAC inhibitor KH7 confirmed this finding (Fig. 5c). We assessed downstream activation of cAMP signaling by immunoblotting using an antibody against the phosphorylated form of the cAMP-dependent protein kinase target protein cAMP-response element-binding protein (CREB). A small but significant and independently repeatable increase in phosphorylation on serine 133 of CREB in the presence of agonist was observed at 5% (v/v) CO$_2$ compared with 0.03% (v/v) CO$_2$ (Fig. 5d).

Our findings demonstrate that the G-protein-activated ACs are specifically CO$_2$-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 7C$_1$2C$_2$ protein used in this study, as any amino acid residue(s) required for CO$_2$ binding may not be conserved among G-protein-regulated ACs in general. We hypothesize, however, that CO$_2$ 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 CO$_2$, 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 CO$_2$ 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 CO$_2$ and sAC responsive to HCO$_3^-$ and CO$_2$. 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 CO$_2$ and HCO$_3^-$. 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,
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however, exposed to large variations in \( pCO_2 \) and have G-protein-activated cAMP signaling central to their physiology. The duodenum is exposed to a \( pCO_2 \) of up to 400 mm Hg, and the cAMP activated cystic fibrosis transmembrane conductance regulator is key to \( HCO_3^- \) secretion in this tissue (51). Acidification of the epididymal lumen with an associated low \( HCO_3^- \) 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 \( CO_2 \) suggest that this cannot be the sole mechanism for keeping sAC activity switched off. A potential specific role for \( CO_2 \) signaling through G-protein-regulated ACs is evident in respiratory alkalosis. A key marker of this systemic hypocapnia is a blunted phosphatase-mediated cAMP production in the carotid body, a peripheral chemosensor, independent of pH (54). Although a role for sAC versus G-protein-regulated ACs in this tissue remains to be investigated, the clear role for adenosine-mediated cAMP production in the carotid body is supportive of the latter (55).

As is the case with sAC, G-protein-regulated ACs respond to \( CO_2 \) with activation of downstream signaling molecules. Changes in cAMP concentrations and CREB activation are unlikely to be an artifact of the cell culture conditions used or the composition of the assay buffer as the results obtained are clearly corroborated by data using recombinant protein. Although the percentage activation of CREB detailed in this study is far below the increase in cAMP concentrations observed, it is important to consider that HEK 293T cells may not be representative of cAMP signaling systems that are responsive to \( CO_2 \) in the organism (for example, see the hypothesized roles for cAMP signaling through \( CO_2 \) discussed). Despite these drawbacks, the system used here has successfully demonstrated that \( CO_2 \)-regulated changes in hormone-activated cAMP concentrations can activate CREB phosphorylation. This is an important proof of principle even if the activation is not functional within the context of the HEK 293T cells used in this study. In vivo activation of AC 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 \( CO_2 \) as discussed. The cAMP signaling pathway, therefore, represents a novel signaling pathway able to directly respond to \( CO_2 \) in eukaryotes.

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