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Bicarbonate-responsive "soluble" adenylyl cyclase defines a nuclear cAMP microdomain

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Bicarbonate-responsive "soluble" adenylyl cyclase resides, in part, inside the mammalian cell nucleus where it stimulates the activity of nuclear protein kinase A to phosphorylate the cAMP response element binding protein (CREB). The existence of this complete and functional, nuclear-localized cAMP pathway establishes that cAMP signals in intracellular microdomains and identifies an alternate pathway leading to CREB activation.

Introduction

cAMP is a nearly ubiquitous second messenger molecule that affects a multitude of cellular functions. In mammalian cells, two classes of adenylyl cyclase generate cAMP. Transmembrane adenylyl cyclases (tmACs) are tethered to the plasma membrane and regulated by heterotrimeric G proteins in response to hormonal stimuli (for review see Hanoune and Defer, 2001). A second source of cAMP, the more recently described "soluble" adenylyl cyclase (sAC), resides in discrete compartments throughout the cell (Zippin et al., 2003) and is regulated by the intracellular signaling molecules bicarbonate (Chen et al., 2000) and calcium (Jaiswal and Conti, 2003; Litvin et al., 2003).

cAMP elicits its cellular effects by activation of three known classes of effector proteins: exchange proteins activated by cAMP (EPAC), cyclic nucleotide gated ion channels, and protein kinase A (PKA). A subset of these targets resides at the plasma membrane, where they exist in macromolecular signaling complexes that also include a G protein coupled receptor, its transducing G protein, and the source of cAMP, a tmAC isoform (Davare et al., 2001). The cAMP generated by tmACs acts locally (Rich et al., 2000, 2001; Zaccolo and Pozzan, 2002), most likely restricted by phosphodiesterase "firewalls" (Zaccolo and Pozzan, 2002), which define the limits of these cAMP signaling microdomains. However, targets of cAMP do not solely reside at the plasma membrane. EPAC is localized to the nuclear membrane and mitochondria (Qiao et al., 2002), and PKA is tethered throughout the cell by a class of proteins called AKAP (A-kinase–anchoring proteins; Michel and Scott, 2002). The observation that cAMP does not diffuse far from tmACs (Bacskai et al., 1993; Zaccolo and Pozzan, 2002) reveals that there must be another source of cAMP modulating the activity of these distally localized targets.

sAC (Buck et al., 1999) is widely expressed in mammalian cells (Sinclair et al., 2000). Unlike tmACs, sAC is G protein insensitive (Buck et al., 1999), and among mammalian cyclases, it is uniquely responsive to intracellular levels of bicarbonate (Chen et al., 2000). The ubiquitous presence of carbonic anhydrases ensures that the intracellular bicarbonate concentration (and sAC activity) will reflect changes in pH (Pastor-Soler et al., 2003) and/or CO_2. Because CO_2 is the end product of energy-producing metabolic processes, sAC is poised to function as a cell's intrinsic sensor of metabolic activity (Zippin et al., 2001). sAC possesses no transmembrane spanning domains (Buck et al., 1999) and is distributed to subcellular compartments containing cAMP targets (Zippin et al., 2003) that are distant from the plasma membrane. sAC was also found localized inside the mammalian cell nucleus (Zippin et al., 2003).

To evaluate how sAC-generated cAMP might differ from the second messenger generated by tmACs, we explored a prototypical cAMP-dependent pathway, PKA-dependent phosphorylation of cAMP response element binding protein (CREB; De Cesare and Sassone-Corsi, 2000). In a widely

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Abbreviations used in this paper: CREB, cAMP response element binding protein; PKA, protein kinase A; sAC, soluble adenylyl cyclase; tmAC, transmembrane adenylyl cyclase.

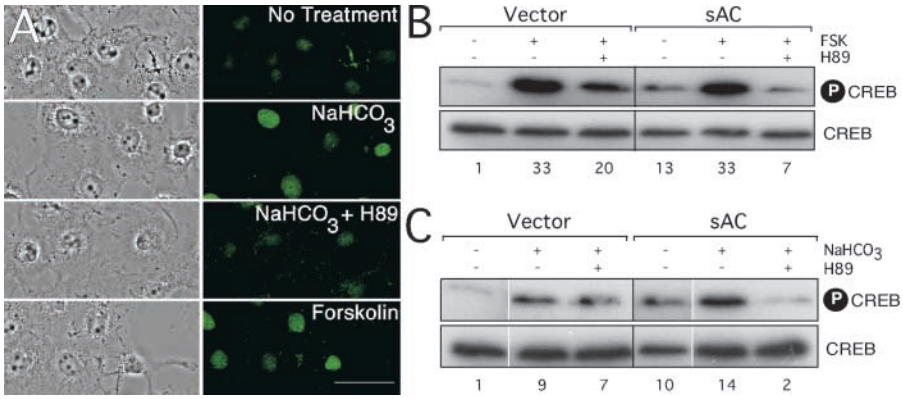

Figure 1. **Bicarbonate induces CREB phosphorylation via sAC activation in a PKA-dependent manner.** (A) COS7 cells were starved for bicarbonate 60 min and were either incubated in the same bicarbonate starvation media for an additional 30 min (No Treatment), incubated in normal, bicarbonate-containing DME in 5% CO_2 for 30 min ($NaHCO_3$), or preincubated with 10 μM H89 for 10 min followed by incubation in normal, bicarbonate-containing DME in 5% CO_2 for 30 min ($NaHCO_3$ + H89). As a control, COS7 cells grown in normal DME in 5% CO_2 were incubated with 10 μM of forskolin for 30 min (Forskolin). Cells were immunostained with phospho-CREB antisera (green). Left, phase images of cells on right. Bar, 50 μm. (B) COS7 cells were transfected with vector control or a 48-kD isoform of sAC, and cells were assayed 36 h after transfection. Cells were treated with vehicle control (DMSO) or 10 μM H89 for 10 min and stimulated with 10 μM of forskolin or given vehicle control (DMSO) for an additional 30 min. (C) Transfected cells were starved for bicarbonate and were either incubated in the same bicarbonate starvation media for an additional 30 min; incubated in normal, bicarbonate-containing DME in 5% CO_2 for 30 min; or preincubated with 10 μM H89 for 10 min followed by incubation in normal, bicarbonate-containing DME in 5% CO_2 for 30 min. (B and C) Top, Western blot using anti–phospho-CREB antisera with phosphorylated CREB (P-CREB) protein indicated; bottom, Western blot using CREB-specific antisera with total CREB protein indicated. Shown below are the intensities of phospho-CREB relative to CREB normalized to vector control no treatment (first lane).

accepted signal transduction paradigm, extracellular signals (i.e., hormones and neurotransmitters) affect CREB family phosphorylation by stimulation of plasma membrane–bound tmACs. The generated cAMP activates nearby PKA, and the liberated catalytic subunit then appears to translocate through the cytoplasm to phosphorylate and activate CREB proteins residing inside the nucleus (Riabowol et al., 1988b; Hagiwara et al., 1993). Intracellular signals, such as metabolic activity, also modulate CREB phosphorylation in a cAMP-dependent manner (Daniel et al., 1998; Singh et al., 2001; Trumper et al., 2002), but the mechanism has yet to be established. Localization of sAC inside the nucleus, in close proximity to the CREB family proteins, and its regulation by calcium and bicarbonate suggested that sAC might be responsible for modulating CREB activity in response to intracellular signals.

In this paper, we demonstrate the existence of a nuclear cAMP signaling microdomain that mediates bicarbonate-dependent activation of the transcription factor CREB. Bicarbonate activation of CREB represents an example of a mammalian cAMP-dependent pathway solely modulated by intrinsic cellular signals. This nuclear cAMP signaling cascade functions independently from the classically defined mechanisms leading to CREB activation, demonstrating that cAMP is a locally acting second messenger that can work autonomously in different compartments within a single cell.

Results

Bicarbonate induces CREB phosphorylation

Bicarbonate treatment of cells uniquely activates sAC (Chen et al., 2000), whereas activation by G proteins or forskolin only stimulates tmACs; therefore, these agents can be used to differentially stimulate the two classes of mammalian adenylyl cyclase. To determine whether sAC activation would elicit PKA activation of CREB, a well-characterized target of tmAC-generated cAMP, we treated cells with bicarbonate and measured PKA-dependent phosphorylation of CREB using antisera specific for the PKA (Ser133) phosphorylated form of CREB. Hormonal stimulation of CREB transcription factors, acting through tmACs, reaches its peak in 30 min (Hagiwara et al., 1992). Treatment of COS7 cells with forskolin, which will activate the total cellular pool of tmACs, stimulated nuclear immunofluorescent staining and Western blot immunoreactivity using the phosphospecific antisera (Fig. 1, A and B). Treatment of COS7 cells for the same amount of time (30 min) with bicarbonate also resulted in CREB phosphorylation (Fig. 1, A and C). These increases in phospho-CREB immunostaining were inhibited by pretreatment with H89, confirming the involvement of PKA (Fig. 1, A–C). Overexpression of sAC led to an increase in basal CREB phosphorylation (Fig. 1, B and C, fourth lane) suggesting that sAC-generated cAMP was sufficient to activate CREB. Consistent with its bicarbonate responsiveness (Chen et al., 2000), sAC overexpressing COS7 cells displayed enhanced bicarbonate-dependent CREB phosphorylation (Fig. 1 C, second and fifth lanes), which was also blocked by H89 (Fig. 1 C). The ability of either bicarbonate or forskolin to induce CREB phosphorylation reveals that CREB represents a downstream target of both tmAC- and sAC-generated cAMP.

Time course of bicarbonate-induced CREB phosphorylation

We directly compared the time course of CREB activation in response to a hormonal activator of tmACs, PGE_2, versus the specific sAC activator bicarbonate in a liver cell line (Hagiwara et al., 1992). Phosphorylation of CREB in response to bicarbonate occurred rapidly; increases in phospho-CREB were detected within 2 min, the earliest time tested (Fig. 2 A). In contrast, PGE_2 (Fig. 2 B) or forskolin (not depicted) stimulation of CREB phosphorylation was detectable only after 5 min, consistent with published papers (Hagiwara et al., 1992). The longer activation kinetics after PGE_2 or forskolin stimulation is thought to reflect the time

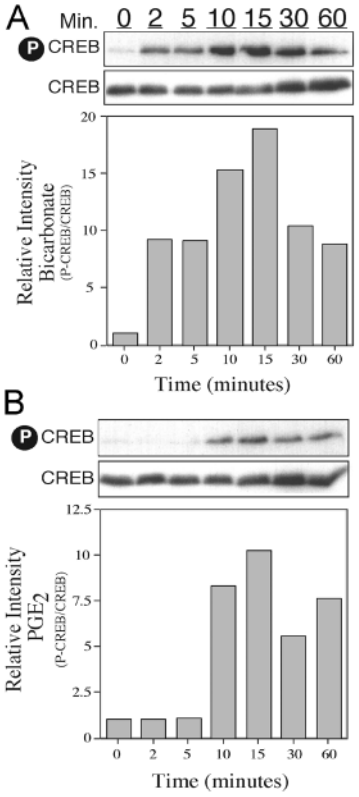

Figure 2. **Time course of CREB phosphorylation by sAC and tmAC.** (A) Huh7 cells were starved for 1 h for bicarbonate and CO_2 and incubated in 44 mM of normal, bicarbonate-containing DME in 5% CO_2 for the time indicated or (B) kept in normal media and treated with 1 μM PGE2 for the time indicated. (A and B) Top, Western blot using anti–phospho-CREB antisera with phosphorylated CREB (P-CREB) protein indicated; bottom, Western blot using CREB-specific antisera with total CREB (CREB) protein indicated. Shown below are graphical representations of the intensities of phospho-CREB relative to CREB normalized to the 0 min time point (first lane).

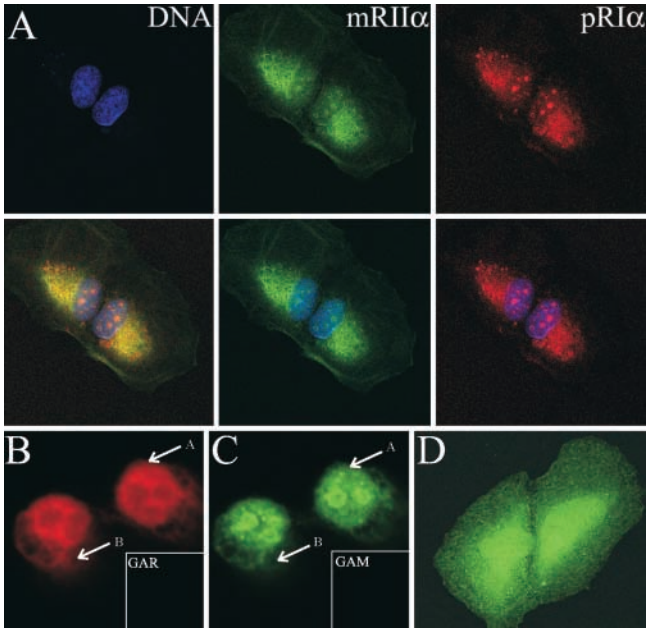

Figure 3. **Immunocytochemistry detects both sAC and PKA in the mammalian cell nucleus.** (A) Confocal immunocytochemistry of Huh7 cells with monoclonal RIIα (top middle, green) and polyclonal RIα antibody (top right, red). Top left, To-Pro 3. Overlay of To-Pro 3 with both RIIα and RIα (bottom left), RIIα (bottom middle), and RIα (bottom right). Secondary controls were negative (not depicted). (B and C) Confocal images of suspension HeLa cells immunostained with (B) PKA regulatory subunit RIIα polyclonal antisera and (C) PKA regulatory subunit RIα mAb. A and B arrows indicate suspension HeLa cytoplasm. Secondary controls were negative (insets). (D) Confocal immunocytochemistry of Huh7 cells stained with R41 mAb against sAC.

required for translocation of PKA catalytic subunit into the nucleus from the plasma membrane where it was activated by a hormonally modulated tmAC (Hagiwara et al., 1993). In addition to being more rapid, the peak intensity of phosphorylation was higher with bicarbonate treatment. The different kinetics and intensity of CREB activation by bicarbonate and PGE_2 reveal that whereas sAC and tmACs may affect overlapping substrates, they may participate in distinct signal transduction cascades.

CREB, sAC, and PKA coexist in the nucleus

Because CREB family members and sAC (Zippin et al., 2003) reside inside the nucleus, we reasoned the accelerated kinetics and intensity of bicarbonate-induced CREB activation could occur if sAC and CREB coexisted in a signal transducing complex. A complete nuclear cAMP signaling cascade capable of phosphorylating CREB family proteins requires the presence of the cAMP-responsive PKA holoenzyme. Both catalytic and regulatory subunits of PKA have been immunologically (Kuettel et al., 1985; Jungmann et al., 1988; Yang et al., 1998) and biochemically (Byus and Fletcher, 1982; Murray et al., 1985; Zhang et al., 1996; Constantinescu et al., 1999) detected inside the nucleus. Nuclear localization of the PKA holoenzyme has been described in lower eukaryotes (Griffioen et al., 2000), but the nuclear presence of the PKA regulatory subunit, and the cAMP-responsive holoenzyme, has been questioned. We repeated and extended the immunological examination of regulatory subunit localization and confirmed that PKA resides inside the nucleus of the human liver cell line Huh7 (Fig. 3 A), in suspension HeLa cells (Fig. 3, B and C), and in a subset of cells within sectioned liver tissue (Fig. 4 A). Confocal microscopy of Huh7 and HeLa cells using polyclonal and monoclonal antibodies, recognizing PKA regulatory subunit isoforms (RIα and RIIα), revealed distinctive cytoplasmic staining in accordance with accepted dogma (Alto et al., 2002; Fig. 3, A–C), but these regulatory subunit isoforms were also detected inside the nucleus (Fig. 3, A–C; and Fig. 4 A). In the case of suspension HeLa cells, it should be stressed that these optical slices were selected to illustrate the intranuclear staining of PKA. Slide preparation and imaging constraints cause PKA cytoplasmic staining to appear as a thin layer surrounding the nucleus (Fig. 3, B and C, A arrows) and within the expanse of cytoplasm stretching out as these suspension cells adhere to the coverslip (Fig. 3, B and C, B arrows).

Nuclear staining of each isoform was distinct. RIIα was present in a diffuse pattern throughout the nucleus with small areas of enrichment (Fig. 3 A, mRIIα; and Fig. 3 B), whereas RIα was distributed in the nucleoplasm but more

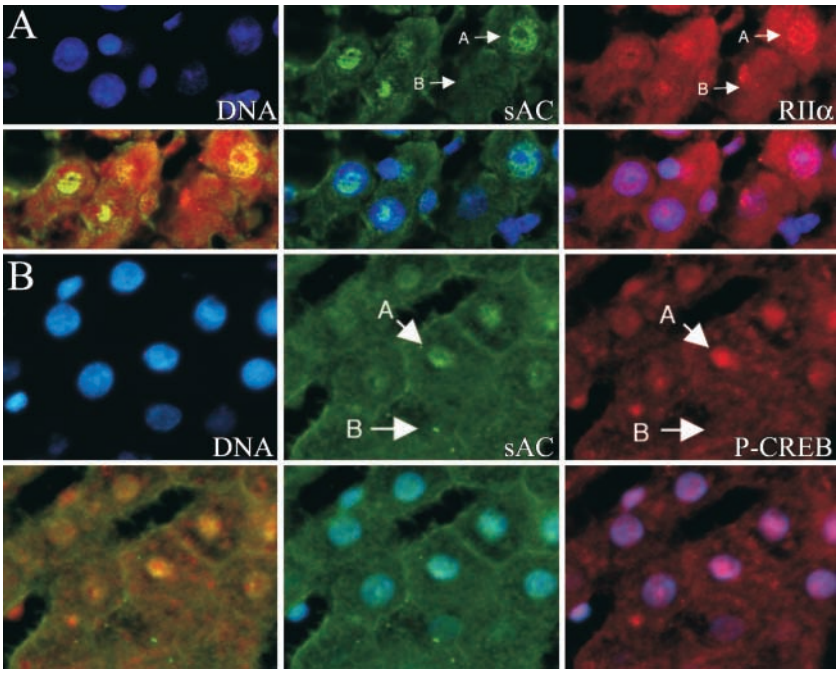

Figure 4. **Activated CREB, sAC, and PKA are present within the same rat liver nuclei.** (A) Rat liver section stained with DAPI (top left, DNA, blue), R52 biotinylated mAb (top middle, sAC, green), and polyclonal RIIα antisera (top right, RIIα, red); overlays of RIIα and sAC (bottom left), sAC and DAPI (bottom middle), and RIIα and DAPI (bottom right). A arrows indicate nuclei enriched for both sAC and PKA, whereas B arrows indicate nuclei not enriched for either. (B) Rat liver section stained with DAPI (top left, DNA, blue), R21 mAb (top middle, sAC, green), and polyclonal P-CREB antisera (top right, P-CREB, red); overlays of P-CREB and sAC (bottom left), sAC and DAPI (bottom middle), and P-CREB and DAPI (bottom right). A arrows indicate nuclei enriched for both sAC and P-CREB, whereas B arrows indicate nuclei enriched for neither. Rat liver tissue immunolocalization was confirmed to be inside the nucleus by confocal microscopy (not depicted).

enriched in nucleoli (Fig. 3 A, pRIα; and Fig. 3 C). RIIα was also detected in the nuclei of a subset of rat liver primary hepatocytes (Fig. 4 A). Consistent with our previously published data (Zippin et al., 2003), sAC was also present in the nuclei of Huh7 cells (Fig. 3 D) and a subset of rat liver hepatocytes (Fig. 4). PKA, sAC, and phosphorylated CREB seem to be coordinately localized; the subset of nuclei in rat liver hepatocytes and Huh7 cells (not depicted) positive for sAC protein (Fig. 4, A and B, A arrow) also contained R subunit (Fig. 4 A, A arrow) and CREB phosphorylation (Fig. 4 B, A arrow), whereas nuclei not enriched for sAC displayed neither R subunit nor CREB phosphorylation (Fig. 4, A and B, B arrow). Rat liver hepatocytes positive for sAC, PKA, and phospho-CREB represent ~10% of total hepatocytes, and we have not yet identified any consistency with known liver anatomy. These data demonstrate that nuclei contain all the components of a cAMP signaling cascade and suggest that sAC-generated cAMP is positioned to activate nuclear PKA holoenzymes to phosphorylate CREB proteins.

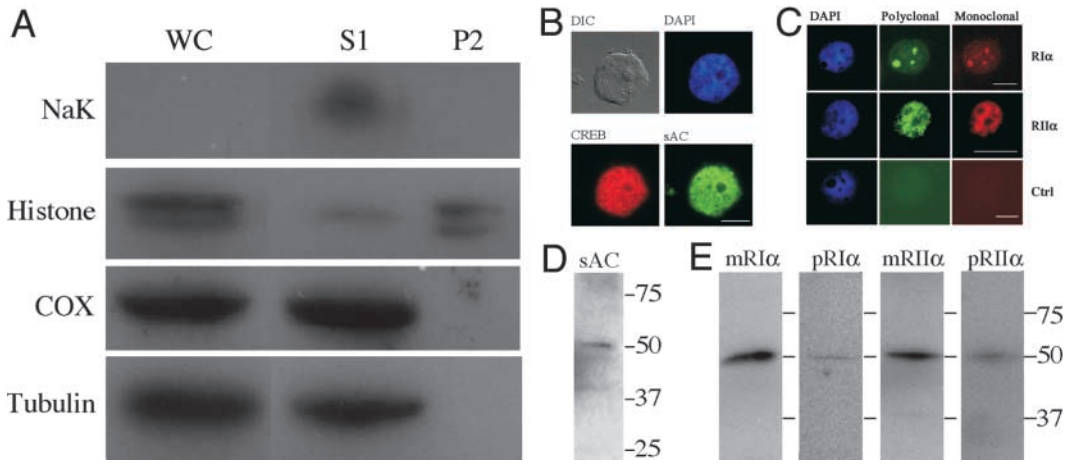

Figure 5. **sAC, PKA, and CREB coexist in mammalian cell nuclei.** (A) Western blots of cell equivalents from HeLa whole cells (WC), low speed supernatant (S1), and nuclear-enriched high speed pellet (P2) probed with antibodies against NaK ATPase (NaK), histone H1 (Histone), cytochrome oxidase subunit III (COX), and β-tubulin (Tubulin). (B) Immunocytochemistry of nuclei isolated from HeLa cells (P2 pellet) using CREB polyclonal antisera (red) and sAC R52 biotinylated mAb (green). Differential interference contrast microscopy (DIC) and DAPI (blue) images shown. Bar, 10 μm. (C) Nuclei isolated from HeLa cells (P2 pellet) immunostained with polyclonal antisera (green) and mAb (red) directed against both RIα and RIIα indicated that both proteins maintained their nucleoplasmic architecture throughout the fractionation procedure. Bottom row represents staining with goat anti-rabbit (middle) or goat anti-mouse controls (right) alone. Left column represents DAPI images in blue. Bars, 10 μm. (D) Western blot of nuclear enriched P2 for sAC with R21 mAb. (E) Western blots of nuclear enriched P2 pellet with monoclonal (mRIα) and polyclonal (pRIα) antisera against RIα and with monoclonal (mRIIα) and polyclonal (pRIIα) antisera against RIIα. All Westerns blots resolved only single bands of the predicted molecular mass.

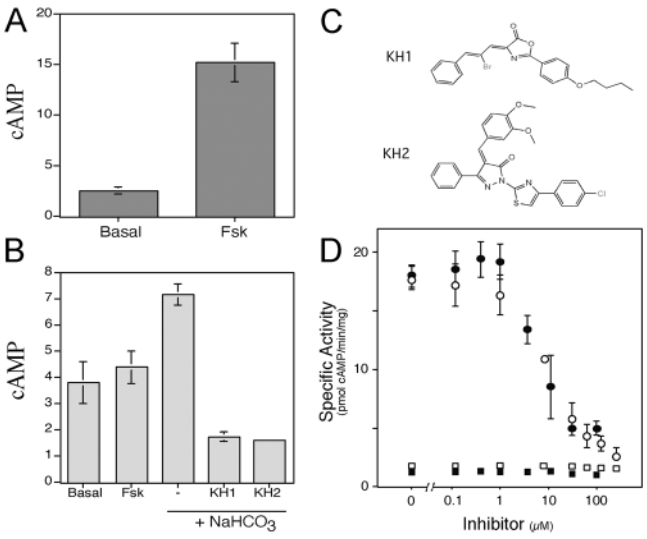

Figure 6. **sAC activity is the only detectable adenylyl cyclase activity in the mammalian cell nucleus.** (A) Adenylyl cyclase assay of whole cell lysate with Mg^{2+}-ATP alone (Basal) or Mg^{2+}-ATP and forskolin (Fsk). cAMP values are expressed as picomoles produced per milliliter of lysate and represent averages of duplicate determinations with SD about the means indicated. (B) Cyclase assay of nuclear lysate with Mg^{2+}-ATP alone (Basal), or in the presence of 10 μM forskolin (Fsk), or 40 mM of bicarbonate (+$NaHCO_3$) in the absence (−) or presence of the sAC-specific inhibitors KH1 (250 μM) or KH2 (100 μM). cAMP values are expressed as picomoles produced per milliliter of lysate and represent averages of duplicate determinations with SD about the means indicated. These data are representative of at least three independent experiments performed in duplicate. (C) Chemical structures of inhibitors KH1 and KH2. (D) Inhibition of purified sAC (circles) or purified tmAC type VII C1 + C2 (squares) by KH1 (open symbols) and KH2 (closed symbols). Values for purified tmAC type VII C1 + C2 represent basal activity, which was significantly above background. Activity values are expressed as picomoles of cAMP produced per minute per milligram of protein and represent averages of duplicate determinations with SD about the means indicated.

Isolated nuclei contain components of a cAMP signaling microdomain

Bicarbonate treatment of whole cells leads to rapid induction of CREB phosphorylation (Fig. 2). To test whether the nuclear localized sAC and PKA were responsible for this bicarbonate-induced CREB activation, we prepared isolated nuclei from suspension HeLa cells, a cell line with well-established protocols for the isolation and enrichment of nuclei. Cells were lysed using digitonin, and nuclear preparations were purified by density centrifugation through an OptiPrep gradient. Western analyses of the same cell equivalents from each fraction using cellular markers for different subcellular compartments (histone H1, NaK ATPase α1 subunit, cytochrome *c* oxidase subunit III [COX], and β-tubulin) confirmed that the nuclear fractions (P2) were positive for nuclear markers (histone) with undetectable levels of plasma membrane (NaK ATPase), mitochondrial (COX), or cytoplasmic (tubulin) contamination (Fig. 5 A). To confirm that the P2 fraction did not contain any detectable mitochondria, a possible source of both sAC and PKA contamination, we overloaded the P2 fraction, but COX antigen was still not detected (unpublished data). Visual inspection and DAPI fluorescence confirmed that the final preparation was enriched for intact nuclei (Fig. 5, B and C), and, as expected, isolated nuclei contained both CREB and sAC proteins by immunocytochemistry (Fig. 5 B) and Western blotting (Fig. 5 D).

Consistent with the aforementioned staining patterns (Fig. 3, A–C), RIIα immunostaining was present throughout the nucleus, whereas RIα appeared enriched within the nucleolus (Fig. 5 C). PKA RIα and RIIα were also detected by Western analysis as a single band of the predicted molecular mass in the P2 lysate, using monoclonal and polyclonal antibodies (Fig. 5 E), confirming the specificities of these antibodies for immunostaining. Because the staining patterns of isolated HeLa cell nuclei (Fig. 5 C) reflected the immunostaining pattern observed in intact HeLa cells (Fig. 5, B and C), we concluded that the isolation and enrichment of nuclei had little effect on nucleoplasm architecture.

sAC represents the only source of cAMP detectable in isolated nuclei

We previously demonstrated that sAC activity was present in COS7 cell nuclei (Zippin et al., 2003). We now show that bicarbonate-responsive sAC is the only source of cAMP in nuclei isolated from suspension HeLa cells. Whereas forskolin potently stimulates cAMP production in whole cell lysates (Fig. 6 A), there was no significant increase in cAMP elicited by forskolin in isolated nuclei (Fig. 6 B). There was a significant level of basal adenylyl cyclase activity in isolated nuclei, which was stimulated by bicarbonate addition (Fig. 6 B). Both the bicarbonate-stimulated and basal activities were inhibited by sAC-selective inhibitors (Fig. 6, C and D). We have identified several sAC inhibitors (Fig. 6 C), inert toward tmACs (Fig. 6 D), in a screen of a combinatorial chemical library (unpublished data). In the presence of two representative, structurally unrelated inhibitors (KH1 and KH2 each display an $IC_{50} \leq 10$ μM toward recombinant human sAC protein), the cAMP generated in the presence of bicarbonate in P2 nuclei was reduced to a level below that of basal. These results indicate that in addition to mediating the bicarbonate-induced increase in cAMP in isolated nuclei, sAC is also responsible for the observed basal adenylyl cyclase activity.

Bicarbonate induces CREB phosphorylation in isolated nuclei

CREB phosphorylation in isolated nuclei was assayed by immunocytochemistry using phospho-CREB–specific antisera (Fig. 7, A and B). Nuclei incubated in the presence of either bicarbonate or cAMP displayed at least a twofold rise in the percentage of phospho-CREB–positive nuclei relative to untreated nuclei (basal; Fig. 7 B). As expected, due to the lack of tmACs in isolated nuclei, the number of nuclei positive for CREB phosphorylation was unaffected by forskolin. These data demonstrate that a bicarbonate-responsive signaling cascade leading to CREB phosphorylation is wholly contained within the mammalian cell nucleus. In contrast, the hormone and forskolin-responsive tmAC-defined cascade is only functional in a whole cell context.

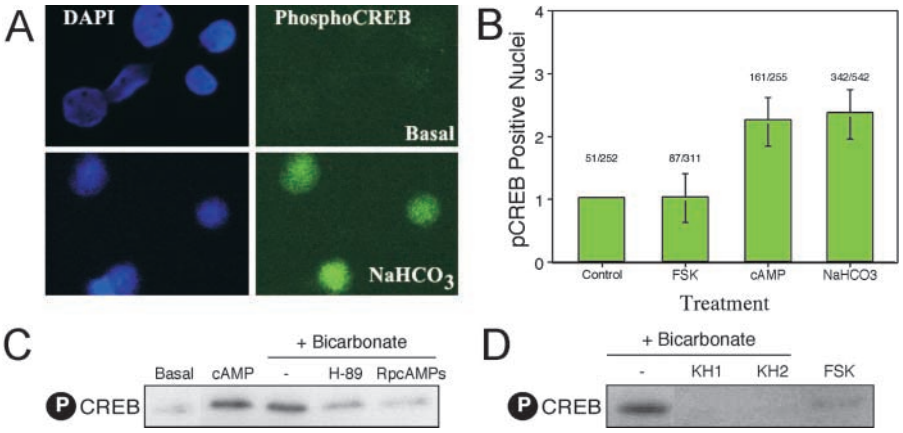

Figure 7. **Isolated nuclei contain a bicarbonate-responsive cAMP signaling microdomain dependent on both sAC and PKA.** (A and B) Equal aliquots of nuclei-enriched P2 were incubated with 40 mM NaCl (Basal), 10 μM forskolin (FSK), 1 mM 8-Br cAMP (cAMP), or 40 mM of sodium bicarbonate ($NaHCO_3$) for 10 min, smeared on a chilled glass slide, placed at −20°C, and immunostained for CREB family member phosphorylation using phospho-CREB–specific polyclonal antisera (green). (A) Intact nuclei were confirmed by DAPI staining (left). Bottom right highlights representative nuclei considered positive for CREB phosphorylation for quantitation. (B) Three microscopic fields per condition were photographed and counted by a blinded scientist. Values graphed represent the percentage of positive nuclei normalized to Basal (control) averaged from five separate experiments. Ratios above each bar represent the total number of positive nuclei divided by the total number of nuclei counted for all five experiments. (C) Western blot using phospho-CREB–specific antisera against equal aliquots of nuclei-enriched P2 treated with Mg^{2+}-ATP alone (Basal) or substrate in the presence of 1 mM 8-Br cAMP (cAMP), 40 mM bicarbonate, or 40 mM bicarbonate in the presence of either 10 μM H-89 or 1 mM 8-Br-RpcAMPs. Each band was quantitated and normalized to basal; the relative intensities are basal (1 U), cAMP (30 U), bicarbonate alone (−; 27 U), bicarbonate plus H-89 (13 U), and bicarbonate plus Rp-cAMPs (8 U). (D) Western blot using phospho-CREB–specific antisera against equal aliquots of nuclei-enriched P2 treated with 10 μM forskolin (FSK) or with 40 mM of bicarbonate alone (−) or in the presence of either sAC specific inhibitor KH1 (250 μM) or KH2 (100 μM).

Nuclear sAC activates CREB via nuclear PKA

To facilitate the use of pharmacological reagents to further evaluate bicarbonate-induced CREB phosphorylation, we monitored CREB phosphorylation by Western analysis (Fig. 7, C and D). Similar to our observations using immunocytochemistry, treatment of isolated nuclei with bicarbonate or 8-Br-cAMP elicited a 27- or 30-fold increase in CREB phosphorylation, respectively (Fig. 7 C). Once again, forskolin, which had a potent effect in a whole cell context (Fig. 1), elicited no significant stimulation of CREB phosphorylation in isolated nuclei (Fig. 7 D).

Next, we confirmed that the effects of bicarbonate on CREB phosphorylation were mediated by nuclear sAC and PKA. CREB phosphorylation induced by bicarbonate was substantially reduced by the PKA inhibitors, H89 (50%) and RpcAMPs (70%; Fig. 7 C), revealing the involvement of cAMP-responsive PKA holoenzyme. The chemical inhibitors effective at blocking sAC-generated cAMP accumulation (Fig. 6 B, KH1 and KH2) were also effective in preventing bicarbonate-induced CREB phosphorylation (Fig. 7 D), demonstrating, once again, that sAC is responsible for the bicarbonate-stimulated cAMP-dependent phosphorylation of CREB in the mammalian cell nucleus.

Discussion

Most cellular pathways in eukaryotic cells are impacted by cAMP. Effectors of cAMP mediate processes at both the plasma membrane and multiple, distinct intracellular sites. It has been widely assumed that cAMP is generated exclusively at the plasma membrane by G protein–regulated tmACs, and the second messenger then diffuses from the cell membrane through the cytosol to its intracellular targets. However, FRET-based (Bacskai et al., 1993; Zaccolo and Pozzan, 2002) and biochemical (Rich et al., 2000, 2001) methods for observing intracellular cAMP concentrations reveal that the second messenger generated by tmACs does not diffuse far from its site of synthesis. We have recently demonstrated that sAC is localized at multiple, subcellular compartments throughout the cell including mitochondria, centrioles, mitotic spindles, mid-bodies, and nuclei (Zippin et al., 2003), each of which contains targets of cAMP. These data suggest that the cell may contain multiple, independently modulated cAMP signaling microdomains; targets near the plasma membrane would depend on tmACs for second messenger generation, whereas targets inside the cell would be modulated by sAC-generated cAMP (Wuttke et al., 2001; Zippin et al., 2003). We provide data supporting this hypothesis by demonstrating the existence of a sAC-defined nuclear cAMP signaling microdomain, which can lead to CREB activation.

The nuclear cAMP signaling cascade induced by bicarbonate produced a rapid activation of CREB family members in both whole cells and nuclei, whereas PGE_2 and forskolin, tmAC-specific activators, produced a delayed response exclusively in whole cells. Therefore, cAMP-mediated activation of CREB family members by tmACs and sAC proceed via independent pathways. CREB activation by hormones or neurotransmitters via tmACs apparently requires time for movement of PKA catalytic subunit from the plasma membrane into the nucleus (Riabowol et al., 1988a; Hagiwara et al., 1993). This delayed activation is consistent with hormonal control of gene expression providing a long-term response to predominantly sustained extracellular signals (Bailey et al., 1996). In contrast, the newly described nuclear sAC activation pathway proceeds rapidly without requiring the translocation of any constituent. In this regard, the sAC nuclear microdomain is capable of responding quickly to subtle fluctuations in intrinsic signals, such as local intracellular concentrations of bicarbonate and calcium.

In tissues, sAC is not present within the nucleus of every cell. In liver, sAC appears to be predominantly extranuclear but enriched in a subset of the nuclei (Fig. 4, A and B, A arrows). PKA holoenzyme appears to be enriched within the same subset of nuclei (Fig. 4 A, A arrows), and interestingly,

these are the nuclei that are also positive for CREB phosphorylation (Fig. 4 B, A arrows). The presence of both positive and negative nuclei for sAC, PKA, and CREB phosphorylation in the same tissue suggests that there may be coordinated regulation of the presence of this newly described nuclear signaling microdomain.

The demonstration that bicarbonate treatment of whole cells leads to activation of the CREB family of transcription factors reveals that bicarbonate itself induces a signal transduction cascade. Cellular bicarbonate levels reflect intracellular pH as well as CO_2 generation (Bevensee et al., 2000); therefore, bicarbonate signaling pathways would respond to a wide variety of cellular transitions. Immunostaining revealed that sAC is present at mitochondria, centrioles, mitotic spindles, and mid-bodies (Zippin et al., 2003), suggesting the existence of multiple cAMP signaling microdomains within a single cell. A remaining challenge will be to determine whether sAC molecules in these different microdomains are subject to independent and unique modes of regulation, permitting a variety of distinct responses independently mediated by the same second messenger.

Materials and methods

Cell growth and transfections

All cell lines were grown in DME (44 mM sodium bicarbonate) supplemented with 10% FBS. Where indicated, cells were transfected using Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions. Cells were incubated with DNA for 5 h in OPTI-MEM, and then switched to normal media. Bicarbonate starvation was conducted by changing media to bicarbonate-free DME (44 mM Hepes) supplemented with 10% FBS for at least 1 h at 37°C under ambient air conditions. Bicarbonate stimulation consisted of returning cells to normal bicarbonate-containing media and placing them in a 5% CO_2 incubator. For PGE_2 or forskolin stimulation, cells were grown in normal media under 5% CO_2. Stimulation was accomplished by replacing media with normal media containing 1 μM PGE_2 or 10 μM forskolin. For Western analysis, cells were lysed immediately by direct addition of SDS sample buffer.

Immunocytochemistry

Cells or nuclei were washed in PBS and fixed for either 30 min in 4% PFA and permeabilized in 0.1% Triton X-100 or fixed for 15 min in 2% PFA and permeabilized in 0.05% Triton X-100. Liver from adult rat was rapidly excised, placed between two thinly sliced pieces of bovine liver, and snap frozen in isopentane cooled with liquid nitrogen. 6-μM-thick cryosections were collected on superfrost slides (Fisher Scientific) and stained within 1 d of sectioning. Tissue was fixed for 30 min in 4% PFA and permeabilized in 0.1% Triton X-100 for 15 min. All samples were blocked in 2% BSA for at least 1 h. Cells or tissues were stained with anti-sAC R41 or R52 biotinylated mAbs or R21 mAb (1:100) generated against human 48-kD isoform of sAC (sAC_t) antigen as described previously (Zippin et al., 2003), anti-PKA regulatory subunit (RIα and RIIα) polyclonal antisera (1:100; Chemicon and Cedarlane Laboratories Limited) or mAbs (Becton Dickinson), and anti-CREB or anti–phospho-CREB polyclonal antisera (1:500; Cell Signaling Technologies) overnight in 2% BSA, 0.01% Triton X-100; washed three times for 10 min each in 2% BSA, 0.01% Triton X-100; stained for 1 h at RT with goat anti–rabbit Alexa Fluor 488, goat anti–mouse Alexa Fluor 568, or goat anti–mouse Alexa Fluor 594 (Molecular Probes); treated with DAPI for 5 min or To-Pro 3 (1:500; Molecular Probes) for 15 min; and washed and mounted with gelvatol/DABCO (Sigma-Aldrich).

For phospho-CREB immunolocalization, cells or nuclei were fixed in 4% PFA for 30 min, permeabilized in 0.1% Triton X-100 for 15 min, blocked for at least 1 h in 3% BSA, and immunostained using phospho-CREB polyclonal antisera (1:500; Cell Signaling Technologies) overnight at 4°C. Staining was visualized by incubation with goat anti–rabbit Alexa Fluor 488 (Molecular Probes) for 1 h at RT, treated with DAPI for 5 min, and washed and mounted with gelvatol/DABCO (Sigma-Aldrich). Fluorescent images were recorded by a digital camera (Hamamatsu) connected to an inverted epifluorescent microscope (Nikon). Images were taken at the same exposure time and gain, and all photographic manipulations were performed equally. Phospho-CREB–positive nuclei were quantified in multiple fields from each stained slide by a blinded experimenter.

Confocal images were acquired with a confocal system (model LSM 510; Carl Zeiss MicroImaging, Inc.). Goat anti–rabbit Alexa Fluor 488 was excited with a 488-nM Kr/Ar laser, goat anti–mouse Alexa Fluor 568 was excited with a 568-nm Kr/Ar laser, and To-Pro 3 was excited with a 633-nm Kr/Ar laser.

Isolation of nuclei

Nuclei were isolated by cellular lysis followed by differential centrifugation (Spector et al., 1998) through OptiPrep (Axis-Shield). HeLa cells grown in suspension were lysed by detergent treatment in TM-2 buffer (0.01 M Tris-HCl, pH 7.4, 1.5 mM $MgCl_2$, 150 mM NaCl, 0.5 mM PMSF, 10 μg/ml apoprotin, and 10 μg/ml leupeptin) containing 100 μg/ml of digitonin followed by a 1,000-*g* spin. Supernatant (S1) was removed and the pellet was resuspended in 0.25 M sucrose, 25 mM KCl, 30 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.8. The resuspended pellet and 60% OptiPrep iodixanol were mixed (30% OptiPrep final) and centrifuged at 10,000 *g* for 20 min. The supernatant was removed and the nuclei-enriched pellet (P2) was resuspended in TM-2 buffer without detergent.

CREB phosphorylation and adenylyl cyclase assays

Equal aliquots of nuclei-enriched P2 preparations were incubated in 50 μl of the final volume of 100 mM Tris, pH 7.2, 10 mM $MgCl_2$, and 5 mM ATP for CREB phosphorylation and 100 mM Tris, pH 7.2, 10 mM $MgCl_2$, 5 mM ATP, and 0.5 mM IBMX for adenylyl cyclase assay with the indicated additions for 10 min (CREB phosphorylation) or 15 min (adenylyl cyclase) at 37°C. Reactions were stopped by the addition of 20 μl of SDS sample buffer (CREB phosphorylation) or by being placed into a 100°C heat block for 3 min (adenylyl cyclase).

For whole cell and isolated nuclei CREB phosphorylation assays, equal cell or nuclear equivalents were separated under reducing conditions using a 10% SDS-PAGE, transferred to a PVDF membrane, and probed for CREB (rabbit polyclonal antiserum; Upstate Biotechnology) and phosphorylated CREB (rabbit polyclonal antiserum; Upstate Biotechnology). HRP-conjugated secondary antibodies were used, and bands were visualized using ECL. Image analysis software (model Fluorchem 8800; Alpha Innotech) was used to quantitate Western results. Intensities of phospho-CREB bands were normalized to total CREB.

cAMP produced in the cyclase assays was detected using a competition-based assay with [^{3}H]cAMP (Amersham Biosciences) and compared with a cAMP standard curve for quantitation.

Inhibitor profiles were determined by adenylyl cyclase assay (Assay Designs, Inc.) using purified sAC protein (Litvin et al., 2003) in the presence of 10 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 10 mM $MgCl_2$, and 10 mM ATP or a mixture of purified catalytic domains, C1 and C2, from Type VII tmAC (Yan and Tang, 2002) in the presence of 5 mM $MgCl_2$ and 1 mM ATP as previously described.

Quantitation of isolated nuclei (P2 fraction) immunocytochemistry

Nuclei were treated with Mg^{2+}-ATP alone or in combination with bicarbonate, forskolin, or 8-Br-cAMP for 10 min, spread on a chilled slide, stored at −20°C, and immunostained using phospho-CREB–specific antisera as described. Nuclei were also treated with DAPI to differentiate intact nuclei from membrane ghosts. DAPI-positive nuclei were scored for phosho-CREB immunofluorescence. Nuclei with detectable staining (Fig. 7 A, $NaHCO_3$) were considered positive for CREB phosphorylation, whereas nuclei with no detectable staining (Fig. 7 A, Basal) were counted as negative. Multiple microscopic fields were photographed for each condition, and data was combined from three to five separate experiments.

Western analysis

Equal cell equivalents, unless otherwise noted, were separated under reducing conditions using a 10% SDS-PAGE, transferred to PVDF membrane, and blocked in 5% milk. The blots were probed with antibodies against either NaK ATPase (monoclonal, 1:50; Santa Cruz Biotechnology, Inc.), histone H1 (monoclonal, 1:100; Santa Cruz Biotechnology, Inc.), cytochrome oxidase subunit III (monoclonal, 2 μg/ml; Molecular Probes), β-tubulin (monoclonal, 1:1000; Sigma-Aldrich), sAC (R21 mAb, 1:500), monoclonal RIα or RIIα antibodies (1:250; Becton Dickinson), or polyclonal RIα or RIIα antisera (1:5000; Chemicon) overnight. HRP-conjugated secondary antibodies were used and bands were visualized using ECL.

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