Bicarbonate-responsive “soluble” adenylyl cyclase defines a nuclear cAMP microdomain

Jonathan H. Zippin,1,2 Jeanne Farrell,1 David Huron,1 Margarita Kamenetsky,1 Kenneth C. Hess,1 Donald A. Fischman,3 Lonny R. Levin,1 and Jochen Buck1

1Department of Pharmacology, 2Tri-Institutional MD/PhD Program, and 3Department of Cell and Developmental Biology, Joan and Sanford I. Weill Medical College and Graduate School of Medical Sciences of Cornell University, New York, NY 10021

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 (Bacsakai 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 CO₂. Because CO₂ 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

Abbreviations used in this paper: CREB, cAMP response element binding protein; PKA, protein kinase A; sAC, soluble adenylyl cyclase; tmAC, transmembrane adenylyl cyclase.
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 adenyl 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 antiserum 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 antiserum (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, PGE₂, 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, PGE₂ (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 PGE₂ or forskolin stimulation is thought to reflect the time...
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 (RI$\alpha$ and RI$\alpha$), 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. RI$\alpha$ was present in a diffuse pattern throughout the nucleus with small areas of enrichment (Fig. 3 A, mRI$\alpha$; and Fig. 3 B), whereas RI$\alpha$ was distributed in the nucleoplasm but more...
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 previ-
ously 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 phosphor-
ylated CREB seem to be coordinately localized; the subset
of nuclei in rat liver hepatocytes and Huh7 cells (not de-
picted) 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 hepato-
cytes positive for sAC, PKA, and phospho-CREB repre-
sent ~10% of total hepatocytes, and we have not yet iden-
tified 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 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 en-
riched for neither. Rat liver tissue immunolo-
calization was confirmed to be inside the nucleus
by confocal microscopy (not depicted).

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 Rα 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 RIIα and with monoclonal (mRIIα) and polyclonal (pRIIα) antisera against RIIα. All Westerns blots resolved only single bands of the predicted molecular mass.
Inhibition of purified sAC (circles) or purified tmAC type VII C1 representative of at least three independent experiments performed in nations with SD about the means indicated. These data are representative, per milliliter of lysate and represent averages of duplicate determinations with SD about the means indicated. (B) Cyclase assay of whole cell lysate with Mg<sup>2+</sup>-ATP alone (Basal) or Mg<sup>2+</sup>-ATP and forskolin (Fsk). cAMP values are expressed as picomoles produced per milligram of protein and represent averages of duplicate determinations with SD about the means indicated. These data are representative, per milliliter of lysate and represent averages of duplicate determinations with SD about the means indicated. (A) Adenylyl cyclase assay of whole cell lysate with Mg<sup>2+</sup>-ATP alone (Basal) or Mg<sup>2+</sup>-ATP and forskolin (Fsk). cAMP values are expressed as picomoles produced per milligram of protein and represent averages of duplicate determinations with SD about the means indicated. (B) Cyclase assay of whole cell lysate with Mg<sup>2+</sup>-ATP alone (Basal), or in the presence of 10 μM forskolin (Fsk), or 40 mM of bicarbonate (+NaHCO<sub>3</sub>) 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 milligram of protein and represent averages of duplicate determinations with SD about the means indicated. These data are representative, per milliliter of lysate 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 RIIα appeared enriched within the nucleolus (Fig. 5 C). PKA RIIα 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<sub>50</sub> ≤ 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.
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 Rp-cAMPs (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 through the cytosol to its intracellular targets. However, FRET-based (Bacskaï 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, centrocles, 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 PGE2 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 CO2 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% CO2 incubator. For PGE2 or forskolin stimulation, consisted of returning cells to normal bicarbonate-containing media and rect addition of SDS sample buffer.

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 phosphor-

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 MgCl2, and 5 mM ATP for CREB phosphorylation and 100 mM Tris, pH 7.2, 10 mM MgCl2, 5 mM ATP, and 0.5 mM IBMX for adenylyl cyclase assay with the indicated additions. For 5 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 phosphor-

CAMP produced in the cyclase assays was detected using a competition-based assay with 1HlCAMP (Amersham Biosciences) and compared with a CAMP standard curve for quantitation.

Immunocytochemistry

Nuclei were isolated by denydyl cyclase assay (Assay De-
signs, Inc.) using purified sAC protein (Litvin et al., 2003) in the presence of 10 mM NaHCO3, 0.5 mM CaCl2, 10 mM MgCl2, and 10 mM ATP in a mixture of purified catalytic domains, C1 and C2, from Type VII tmAC (Yan and Tang, 2002) in the presence of 5 mM MgCl2 and 1 mM ATP as previously described.

Quantitation of isolated nuclei (P2 fraction) immunocytochemistry

Nuclei were treated with Mg2+ATP alone or in combination with bicarbonate, forskolin, or β-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, NaHCO3) 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 Ria or RII antibodies (1:250; Becton Dickinson), or polyclonal Ria or RII antisera (1:5000; Chemicon) overnight. HRP-conjugated secondary antibodies were used and bands were visualized using ECL.

We thank Dr. Abijit Bapat for assistance quantifying nuclear phosphoryla-
tion of CREB, Kelley Rosborough for cryosectioning, Dr. Sylvia Finnemann for assistance with confocal microscopy, Dr. Yanqui Chen for helpful suggestions, and Dr. Carl Nathan for critical reading of the manuscript.

This work was supported by grants from the National Institutes of Health (AR32147 to D.A. Fischman, GM62328 and HD42060 to J. Buck, HD38722 to L.R. Levin, and MSTP-CM07739 to J.H. Zippin), the Ellison Medical Foundation (to J. Buck), the American Diabetes Association (to J.H. Zippin), and the Barbara and Stephen Friedman Fellowship Endowment (to J.H. Zippin).

Submitted: 21 November 2003
Accepted: 2 January 2004

References

Alto, N., J.J. Carlisle Michel, K.L. Dodge, L.K. Langeberg, and J.D. Scott. 2002. Intracellular targeting of protein kinases and phosphatases. Diabetes. 51(3): S385–S388.

Bacsik, B.J., B. Hochner, M. Mahaut-Smith, S.R. Adams, B.K. Kaang, E.R. Kandel, and R.Y. Tsien. 1993. Spatially resolved dynamics of cAMP and protein kinase A subunits in Aplysia sensory neurons. Science. 260:222–226.

Bailey, C.H., D. Bartsch, and E.R. Kandel. 1996. Toward a molecular definition of long-term memory storage. Proc. Natl. Acad. Sci. USA. 93:13445–13452.

Bevensee, M.O., S.L. Alper, P.S. Aronson, and W.F. Boron. 2000. Control of intracellular pH. In The Kidney, Vol. I. D.W. Seldin and G. Giebisch, editors. Lippincott Williams & Wilkins, Philadelphia, PA. 391–442.

Buck, J., M.L. Sinclair, L. Schapal, M.J. Cann, and L.R. Levin. 1999. Cytosolic adenylyl cyclase defines a unique signaling molecule in mammals. Proc. Natl. Acad. Sci. USA. 96:79–84.

Byus, C.V., and W.H. Fletcher. 1982. Direct cytochemical localization of catalytic subunits dissociated from cAMP-dependent protein kinase in Reuber H-35 hepatoma cells. II. Temporal and spatial kinetics. J. Cell Biol. 93:727–734.

Chen, Y., M.J. Cann, T.N. Litvin, V. Iourgenko, M.L. Sinclair, L.R. Levin, and J. Buck. 2000. Soluble adenylyl cyclase as an evolutionarily conserved bicarbonate sensor. Science. 289:625–628.

Constantinescu, A., I. Diamond, and A.S. Gordon. 1999. Ethanol-induced translocation of cAMP-dependent protein kinase to the nucleus. Mechanism and functional consequences. J. Biol. Chem. 274:26985–26991.

Daniel, P.B., W.H. Walker, and J.F. Habener. 1998. Cyclic AMP signaling and gene regulation. Annu. Rev. Nutr. 18:353–383.

Davare, M.A., V. Avdonin, D.D. Hall, E.M. Peden, A. Burette, R.J. Weinberg, M.C. Horne, T. Hoshi, and J.W. Hell. 2001. A beta2 adrenergic receptor signaling complex assembled with the Ca2+ channel Cav1.2. Science. 293:98–101.

De Cesare, D., and P. Sassone-Corsi. 2000. Transcriptional regulation by cAMP-responsive factors. Prog. Nucleic Acid Res. Mol. Biol. 64:343–369.

Griffioen, G., P. Anghileri, E. Imre, M.D. Baron, and H. Ruis. 2000. Nutritional properties of “soluble” adenylyl cyclase. Synergism between calcium and bicarbonate. J. Biol. Chem. 278:15922–15926.

Michel, J.J., and J.D. Scott. 2002. AKAP mediated signal transduction. Annu. Rev. Pharmacol. Toxicol. 42:255–257.

Murray, S.A., C.V. Byus, and W.H. Fletcher. 1985. Intracellular kinetics of free catalytic units dissociated from adenyl 3’,5’-monophosphate-dependent protein kinase in adenocortical hormone cells (Y-1). Endocrinology. 116:364–374.

Pastor-Soler, N., V. Beaulieu, T.N. Litvin, N. Da Silva, Y. Chen, D. Brown, J. Buck, L.R. Levin, and S. Breton. 2003. Bicarbonate-regulated adenylyl cyclase (sAC) is a sensor that regulates pH-dependent V-ATPase recycling. J. Biol. Chem. 278:49525–49529.

Qiao, J., F.C. Mei, V.L. Popov, L.A. Vergara, and X. Cheng. 2002. Cell cycle-dependent subcellular localization of exchange factor directly activated by cAMP. J. Biol. Chem. 277:26381–26386.

Rubinow, K.T., J.S. Fink, M.Z. Gilman, D.A. Walsh, R.H. Goodman, and J.R. Feramisco. 1988a. The catalytic subunit of cAMP-dependent protein kinase induces expression of genes containing cAMP-responsive enhancer elements. Nature. 336:83–86.

Rubinow, K.T., M.Z. Gilman, and J.R. Feramisco. 1988b. Microinjection of the catalytic subunit of cAMP-dependent protein kinase induces expression of the c-fos gene. Cold Spring Harbor Symp. Quant. Biol. 53:85–90.

Rich, T.C., K.A. Fagan, H. Nakata, J. Schaack, D.M. Cooper, and J.W. Karpen. 2000. Cyclic nucleotide-gated channels colocalize with adenylyl cyclase in regions of restricted cAMP diffusion. J. Gen. Physiol. 116:147–161.

Rich, T.C., K.A. Fagan, T.E. Tse, J. Schaack, D.M. Cooper, and J.W. Karpen. 2001. A uniform extracellular stimulus triggers distinct cAMP signals in different compartments of a simple cell. Proc. Natl. Acad. Sci. USA. 98: 13049–13054.

Sinclair, M.L., X.Y. Wang, M. Martia, M. Conti, J. Buck, D.J. Wolgemuth, and L.R. Levin. 2000. Specific expression of soluble adenylyl cyclase in male germ cells. Mol. Reprod. Dev. 56:6–11.

Singh, L.P., J. Andy, V. Anyamele, K. Greene, M. Alexander, and E.D. Crook. 2001. Hexosamine-induced fibroprotein synthesis in mesangial cells is associated with increases in cAMP responsive element binding (CREB) phosphorylation and nuclear CREB, the involvement of protein kinases A and C. Diabetes. 50:2355–2362.

Spector, D.L., R.D. Goldman, and L.A. Leinwand, editors. 1998. Culture and Biochemical Analysis of Cells. Vol. 1 of Cells: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Trumper, A., K. Trumper, and D. Horsch. 2002. Mechanisms of mitogenic and anti-apoptotic signaling by glucose-dependent insulinotopic polypeptide in brain(DN5)-1 cells. J. Endocrinol. 174:233–246.

Wurcke, M.S., J. Buck, and L.R. Levin. 2001. Bicarbonate-regulated soluble adenylyl cyclase. JOP. 2:154–158.

Yan, S.Z., and W.J. Tang. 2002. Construction of soluble adenylyl cyclase from human membrane-bound type 7 adenylyl cyclase. Methods Enzymol. 345: 231–241.

Yang, J., J.A. Drazba, D.G. Ferguson, and M. Bond. 1998. A-kinase anchoring protein 100 (AKAP100) is localized in multiple subcellular compartments in the adult rat heart. J. Cell Biol. 142:511–522.

Zaccomo, M., and T. Pozzan. 2002. Discrete microdomains with high concentration of cAMP in stimulated rat neonatal cardiac myocytes. Science. 295: 1711–1715.

Zhang, Q., D.W. Carr, K.M. Lerea, J.D. Scott, and S.A. Newman. 1996. Nuclear localization of type II cAMP-dependent protein kinase during limb cartilage differentiation is associated with a novel developmentally regulated A-kinase anchoring protein. Dev. Biol. 176:51–61.

Zippin, J.H., L.R. Levin, and J. Buck. 2001. CO2/HCO3- responsive soluble adenylyl cyclase as a putative metabolic sensor. Trends Endocrinol. Metab. 12:366–370.