Different cAMP sources are critically involved in G protein–coupled receptor CRHR1 signaling

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Corticotropin-releasing hormone receptor 1 (CRHR1) activates G protein–dependent and internalization-dependent signaling mechanisms. Here, we report that the cyclic AMP (cAMP) response of CRHR1 in physiologically relevant scenarios engages separate cAMP sources, involving the atypical soluble adenylyl cyclase (sAC) in addition to transmembrane adenylyl cyclases (tmACs). cAMP produced by tmACs and sAC is required for the acute phase of extracellular signal regulated kinase 1/2 activation triggered by CRH-stimulated CRHR1, but only sAC activity is essential for the sustained internalization–dependent phase. Thus, different cAMP sources are involved in different signaling mechanisms. Examination of the cAMP response revealed that CRH-activated CRHR1 generates cAMP after endocytosis. Characterizing CRHR1 signaling uncovered a specific link between CRH-activated CRHR1, sAC, and endosome-based signaling. We provide evidence of sAC being involved in an endocytosis-dependent cAMP response, strengthening the emerging model of GPCR signaling in which the cAMP response does not occur exclusively at the plasma membrane and introducing the notion of sAC as an alternative source of cAMP.

Introduction

G protein–coupled receptors (GPCRs) are the largest family of membrane proteins and mediate most cellular responses to neurotransmitters, peptide hormones, lipids, and sensory stimuli, as well as therapeutic drugs. The classical role of GPCRs is to couple the binding of ligands to the activation of specific heterotrimeric G proteins, leading to the regulation of downstream effector proteins. Signaling responses are attenuated by desensitization via a series of steps that uncouple GPCR from G proteins and lead to receptor internalization/down-regulation. However, this traditional view has been replaced by a much more complex signaling model. G protein–independent mechanisms and, more recently, signaling from endosomal compartments have been described for several GPCRs (Rajagopal et al., 2010; Lohse and Calebiro, 2013; Vilardaga et al., 2014).

Corticotropin-releasing hormone (CRH) is a 41-aa peptide that plays a critical role in the integration of neuroendocrine, autonomic, and behavioral responses to stress. Hypothalamic CRH-secreting neurons drive both basal and stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis. In addition, CRH is widely distributed in the brain, where it functions as a neuromodulator, integrating a complex system that regulates several aspects of the behavioral stress response. Dysregulation of CRH action through its high-affinity type 1 receptor (CRHR1) is crucial in the pathogenesis of affective disorders (Holsboer and Ising, 2010).

CRHR1 is a class B/secretin-like GPCR that, upon ligand activation, signals mainly by Gs coupling, leading to cyclic AMP (cAMP) increase and activation of multiple signaling cascades (Bonfiglio et al., 2011). In particular, CRH-stimulated CRHR1 signals through extracellular signal regulated kinase 1/2 (ERK1/2) to induce proopiomelanocortin (Pomc) transcription and activate the HPA axis in pituitary corticotrophs and in specific brain structures related to behavioral aspects of stress in response to intracerebroventricular CRH administration (Refojo et al., 2005). We have previously described that in a hippocampal neuronal context, CRH1-mediated ERK1/2 activation triggered by CRH is biphasic, with an early phase peaking at 3–6 min that is dependent on B-Raf and protein kinase A (PKA) and a late phase of sustained ERK1/2 phosphorylation (20–60 min) dependent on CRHR1 internalization and β-arrestin2 (Bonfiglio et al., 2013).
The ubiquitous second messenger cAMP regulates numerous, often concurrent, cellular processes. Its activity is tightly regulated and localized to discrete microdomains within the cell to ensure precise spatiotemporal signaling. GPCR-mediated cAMP production was initially thought to depend exclusively on activation of transmembrane adenylyl cyclases (tmACs) at the plasma membrane. More recently, a wealth of research has shown that some activatedGPCRs continue to signal by cAMP generation after internalization, thereby defining endocytic cAMP microdomains (Vilardaga et al., 2014). There are nine G protein–regulated tmACs that have different regulatory properties (Willoughby and Cooper, 2007). Furthermore, a second source of cAMP exists in mammalian cells. Soluble adenylyl cyclase (sAC) is an evolutionarily conserved enzyme that is insensitive to G protein regulation but is directly activated by bicarbonate (Chen et al., 2000) and calcium ions (Jaiswal and Conti, 2003; Litvin et al., 2003). sAC is localized throughout the cell, enabling the production of cAMP in different intracellular locations close to specific cAMP targets (Zippin et al., 2004; Tresguerres et al., 2011; Lefkimmiaitis et al., 2013).

Combining molecular and pharmacologic tools with Förster resonance energy transfer (FRET)–based biosensor technology, we show that the CRHR1-triggered cAMP response does not exclusively depend on tmACs but also involves sAC as a second source of cAMP. Both cAMP sources play a role in the early ERK1/2 response, but only sAC activity is required for the late ERK1/2 activation phase. Our results indicate that CRH-activated CRHR1 generates cAMP from endocytic compartments. By characterizing the CRHR1 signaling response, we show a functional association between sAC-generated cAMP, ERK1/2 activation, and endosome-based GPCR signaling.

**Results**

sAC contributes to CRHR1-triggered cAMP response in HT22-CRHR1 cells

It is now widely accepted that sAC, which was first identified in testis, is ubiquitously expressed (Tresguerres et al., 2011). RT-PCR results confirmed that sAC mRNA was present in total mouse brain and several mouse cell lines (Fig. 1 A), including HT22 cells stably expressing CRHR1 (HT22-CRHR1 cells). Using an anti-sAC monoclonal antibody (R21), a 48-kD protein was detected by Western blot in HT22-CRHR1 cells (Fig. S1 A), and the immunoreactive band was reduced in cells transfected with a SAC-specific siRNA (Fig. 1 B).

We used the FRET-based biosensor Epac-SH187, which localizes diffusely throughout the cytoplasm (Klarenbeek et al., 2015), to assess CRH-triggered cAMP production at the single-cell level in real time, without phosphodiesterase inhibitors. CRH stimulation of HT22-CRHR1 cells resulted in a rapid increase of intracellular cAMP levels that stayed elevated for at least 40 min after ligand bath application (Fig. S1 B). CRH addition produced a rapid decrease of acceptor emission (cp173Venus) and a corresponding increase in donor emission (mTurquoise2), confirming that the observed changes were caused by a FRET reduction, indicating a rise in cytoplasmic cAMP concentration (Fig. S1 B).

When the tmAC-selective inhibitor 2′,5′-dideoxyadenosine (ddA) was added during the time course, the cAMP response was inhibited (P < 0.001 with respect to control after 5 min) but not completely blocked (Fig. 1 C). Interestingly, the sAC-specific inhibitor (Bitterman et al., 2013) 2-hydroxyestradiol (2-HE) also significantly reduced cAMP levels (P < 0.01 with respect to control after 5 min; Fig. 1 C). We also determined cAMP content by means of competition with [3H] cAMP for PKA in HT22-CRHR1 cells preincubated with ddA or sAC-specific inhibitor KH7 (Hess et al., 2005). Both inhibitors significantly reduced the cAMP response triggered by CRH (Fig. S1 C). sAC contribution to the cAMP response triggered by CRH was also indicated by depleting cellular levels of endogenous sAC (Fig. 1 E).

We tested whether isoproterenol, an agonist of β-adrenergic receptors (which are also Gs coupled), triggered a sAC-dependent cAMP response. We observed that only the tmAC inhibitor significantly affected the cAMP increase elicited by isoproterenol (Fig. 1 D; P < 0.001 with respect to control and 2-HE after 5 min), showing that sAC is not a general mediator of the cAMP response generated by GPCRs in the HT22 cellular context. Collectively, our data reveal that sAC is specifically involved in the CRHR1-mediated cAMP response triggered by CRH.

We examined the blocking effect and the specificity of ddA and 2-HE on the cAMP response generated by tmACs and sAC. cAMP responses in cells stimulated with CRH or treated with the tmAC-specific activator forskolin were nearly identical in the presence of ddA (Fig. 1 F, top), reaching about a 50% inhibition at the highest inhibitor concentration assayed (200 µM), 2-HE concentrations up to 40 µM had no effect on the cAMP response generated by forskolin but caused about a 30% inhibition of the cAMP response to CRH (Fig. 1 F, bottom). These results indicate that concentrations of 100 µM ddA and 20 µM 2-HE are sufficient to assess and discriminate the contribution of sAC- and tmACs-mediated processes with our experimental approach.

Do tmACs and sAC engage different biochemical cascades?

Multiple cAMP sources may contribute to a common cAMP pool, or they may act distinctly to elicit specific cellular responses. Having established that, in addition to tmACs, sAC is necessary for the CRH-activated CRHR1 cAMP response, we decided to evaluate whether specific cAMP sources have different biological meanings in CRHR1 signaling. We investigated the role of both cAMP sources in ERK1/2 phosphorylation in HT22-CRHR1 cells. We have previously reported that ERK1/2 can be activated via CRHR1 by both G protein–dependent and –independent mechanisms (Bonfiglio et al., 2013).

The dependence of ERK1/2 phosphorylation on different cAMP sources was first assessed using a pharmacologic approach (Fig. 2 A). As reported previously, specific inhibition of tmACs with ddA attenuated only the CRH-induced early (5 min) ERK1/2 activation phase (Bonfiglio et al., 2013). Two pharmacologic inhibitors of sAC, KH7 and 2-HE, reduced pERK1/2 at the early and late time points (Figs. 2 A and S1 D). When both inhibitors were used in combination, a stronger reduction of pERK1/2 was observed at the early time point (Fig. 2 A).

Furthermore, when the CRH-triggered cAMP response was analyzed with simultaneous addition of both AC inhibitors, a stronger inhibition was observed, further supporting the participation of both cAMP sources in the response (Fig. 2 B).

Next, we analyzed the time course of CRH-induced ERK1/2 phosphorylation after depleting endogenous sAC (Fig. 1 B). Knockdown of sAC significantly affected the early
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but also the late phase of CRH-mediated pERK1/2 (Fig. 2 C). In HT22-CRHR1 cells, isoproterenol triggered a biphasic ERK1/2 activation response similar to the one described in HEK293 cells (Shenoy et al., 2006). However, sAC depletion did not affect ERK1/2 phosphorylation triggered by isoproterenol in these cells (Fig. 2 C), consistent with sAC not being involved in the cAMP response to isoproterenol in HT22-CRHR1 cells (Fig. 1 D). Interestingly, in these cells, CRH-induced neurite outgrowth was blocked by KH7, but ddA had no effect, showing that the morphologic change requires sAC but not tmACs (Fig. 2 D). Together, these results show that tmACs and sAC can mediate different functions in CRH signaling.

sAC was identified in a search for B-Raf partners with a potential role in CRH signaling (Bonfiglio et al., 2013). B-Raf is the MAPK kinase kinase of the cascade leading to ERK1/2 activation and also acts as a scaffold for assembling signaling complexes. Analysis of CRH-elicited signaling mechanisms reveals that whereas sAC contributes to the cAMP response to a small extent, it has a significant role in the phosphorylation of ERK1/2. Thus, the sAC-generated cAMP pool might be biased toward activation of the ERK1/2 pathway. Consistent with the fact that CRHR1 is a Gs-coupled GPCR, the classic signal transduction mechanism involves the generation of cAMP at the plasma membrane by G protein–regulated tmACs, which is apparent in the early phase of ERK1/2 activation (Bonfiglio et al., 2013). The effect of sAC inhibition or depletion on the late phase of pERK1/2 reveals the requirement of CRH-activated sAC in the sustained signaling phase of ERK1/2 activation, suggesting that cAMP is required for ERK1/2 response in the receptor endocytosis-dependent phase.

sAC is involved in CRH signaling in AtT20 corticotrophs but not in 3T3L1-CRHR1 cells

Is sAC a specific component of CRHR1 signaling in a hippocampal neuronal context, or does it have a general role in CRHR1 function? To answer this question, we selected the mouse pituitary-derived corticotroph cell line AtT20 as a second physiologically relevant context of CRH action. In AtT20 cells, CRHR1 (Peeters et al., 2004) and sAC (Fig. 1 A) are endogenously expressed, and CRH elicits a transient increase in cAMP levels and activates ERK1/2 (Fig. S2, A and B).
To determine the involvement of different cAMP sources, we analyzed ERK1/2 activation in response to CRH, which is critical for 
Pomc expression (Kovalovsky et al., 2002) and is mediated by cAMP (Van Kolen et al., 2010). Using inhibitors, we found that tmAC-specific ddA, as well as two sAC-selective inhibitors, 2-HE and KH7, impaired CRH-mediated ERK1/2 phosphorylation (Fig. 3 A). sAC knockdown also significantly reduced CRH-triggered pERK1/2 levels, confirming that sAC participates in CRHR1 signal transduction in AtT20 cells (Fig. 3 B). In contrast, depletion of sAC had no effect on...
PACAP38-mediated ERK1/2 activation (Figs. 3 B and S2 C). Given that PACAP38 also signals through a class B GPCR that generates a cAMP response (Fig. S2 A), these results further highlight that sAC is a specific component of CRHR1 signaling.

We next tested whether sAC was involved in the CRH-mediated activation of Pomc, a key CRH target gene in corticotroph cells for HPA axis activation. The transcriptional activity of a Pomc–Luc reporter construct (Liu et al., 1992) was significantly decreased in cells pretreated with sAC inhibitors before CRH stimulation, whereas no effects on basal activities were observed (Fig. 3 C). These findings are consistent with the participation of sAC in a second signaling scenario and suggest that sAC has a key role for CRHR1 action.

We used the mouse fibroblast 3T3L1 cell line stably expressing CRHR1 (3T3L1-CRHR1), which endogenously expresses sAC (Fig. 1 A), as a third cellular context to explore a role of sAC in CRHR1 signaling. Treatment with the cell membrane–permeable cAMP analog 8-CPT-cAMP enhances ERK1/2 phosphorylation in cells in which ERK1/2 activation is dependent on increased cAMP levels. ERK1/2 response to 8-CPT-cAMP in the three cell lines was determined (Fig. 3 D). Although CRH activated ERK1/2 and CREB in 3T3L1-CRHR1 cells (Fig. S2 D), pERK1/2 was not linked to increased cAMP levels in this system, unlike in AT20 and HT22-CRHR1 cells (Fig. 3 D).

We tested the participation of sAC in 3T3L1-CRHR1 cells by measuring the CRH-mediated cAMP response using the FRET-based biosensor. CRH stimulation triggered a rapid and sustained increase in cAMP levels. Interestingly, whereas the tmAC-selective inhibitor dDA attenuated the cAMP response, the addition of sAC-specific inhibitor 2-HE did not have a significant effect on the cAMP time course (Fig. 3 E). To rule out the possibility that this result was caused by low sAC expression levels, the experiment was also performed with overexpression of the highly active sACT isoform, with similar results (Fig. 3 F).

CRH-activated CRHR1 has been shown to trigger an increase in calcium in several cellular contexts (Kovalovsky et al., 2002; Gutknecht et al., 2009; Bonfiglio et al., 2013). Because calcium activates sAC (Jaiswal and Conti, 2003; Carlson et al., 2002; Gutknecht et al., 2009; Bonfiglio et al., 2013), we asked whether a CRHR1-dependent calcium response was present in 3T3L1-CRHR1 cells. Using Fluo-4 calcium indicator, we verified that, upon CRH stimulation, there was a calcium increase that could be completely blocked with the extracellular calcium chelator EGTA (Fig. S2 E). In conclusion, despite the fact that CRH-driven responses in 3T3L1-CRHR1 and HT22-CRHR1 cells are comparable in terms of cAMP (Fig. 1 C and Fig. 3, E and F) and calcium responses (Bonfiglio et al., 2013), sAC is not involved in cAMP generation in 3T3L1-CRHR1 cells. These findings suggest that the involvement of sAC is dependent on the cellular context and that there is a functional association between the sAC-generated cAMP pool and the ERK1/2 pathway.

**CRH-stimulated sAC activates PKA and EPACs**

Both PKA and EPACs (cAMP-stimulated guanine nucleotide exchange factors) can trigger the ERK1/2 pathway (Kawasaki et al., 1998). It has been shown that sAC-generated cAMP signals through PKA during capacitation in mammalian sperm (Wertheimer et al., 2013), during leucocyte transendothelial migration (Watson et al., 2015), and in H2O2-stimulated airway epithelia (Ivonnet et al., 2015), whereas the sAC-EPAC-
Rap1 axis has been associated with cell proliferation in prostate and breast cancer cells (Flacke et al., 2013; Appukuttan et al., 2014; Onodera et al., 2014).

In HT22-CRHR1 cells, pharmacologic inhibition of PKA reduced CRH-mediated early ERK1/2 activation, without affecting late time points (Fig. 4, A and B). To study PKA activation directly, we expressed the FRET-based PKA activity sensor AKAR4 (Allen and Zhang, 2006; Depry et al., 2011). Preincubation with ddA as well as 2-HE significantly prevented CRH-triggered PKA activation (Fig. 4, C). Thus, cAMP generated through tmAC and sAC contributes to PKA activity in CRH-stimulated HT22-CRHR1 cells.

To determine the role of EPACs on CRH action, we used the EPAC1 and EPAC2 inhibitor ESI09 (Zhu et al., 2015) at its lowest effective concentration to avoid reported nonspecific effects (Rehmann, 2013). Preincubation with ddA as well as 2-HE significantly prevented CRH-triggered PKA activation (Fig. 4, C). Thus, cAMP generated through tmAC and sAC contributes to PKA activity in CRH-stimulated HT22-CRHR1 cells.

To test this hypothesis, we used two experimental approaches to inhibit endocytosis and measured the time course of cAMP accumulation mediated by CRH with the FRET-based biosensor.

In AT20 cells, CRH-induced ERK1/2 activation was slightly decreased in the presence of the PKA inhibitor RpcAMPS (Fig. 4 H). Treatment with the EPAC inhibitor impaired pERK1/2 in response to CRH (Fig. 4 I). Thus, in line with previous studies (Van Kolen et al., 2010), the main cAMP effector underlying CRH-mediated ERK1/2 activation in AT20 cells is EPAC. Collectively, these results indicate that sAC-generated cAMP does not signal preferentially through a specific cAMP effector, being able to activate both PKA and EPAC in response to CRH.

**Endocytosis-dependent cAMP is generated in response to CRH**

CRHR1–β-arrestins interactions are involved in CRHR1 desensitization (Perry et al., 2005; Holmes et al., 2006; Oakley et al., 2007). We have shown previously that, upon ligand stimulation, CRHR1 is internalized and stays in endocytic compartments 30 min after CRH addition. We examined agonist-induced CRHR1 internalization in HT22-CRHR1 cells. CRH activation promoted a fast internalization of the receptor, with a time course that overlapped that of cAMP production: a significant fraction of CRHR1 was internalized after 5 min and remained constant for at least 30 min of CRH stimulation (Fig. 5 A). The fact that both the early and late phases of ERK1/2 activation were affected when sAC activity was inhibited or silenced led us to postulate that CRHR1 endocytosis and sAC activity may be linked. To test this hypothesis, we used two experimental approaches to inhibit endocytosis and measured the time course of cAMP accumulation mediated by CRH with the FRET-based biosensor.
First, we used a genetic approach, expressing a dominant-negative mutant of dynamin (DynK44A) that is required for later stages of clathrin-dependent endocytosis. DynK44A blocked CRHR1 internalization upon ligand stimulation (Fig. S3, A and B) and the sustained ERK1/2 activation phase in HT22-CRHR1 cells (Bonfiglio et al., 2013). Cells transfected with DynK44A showed a diminished cAMP response compared with control cells (Fig. 5 B). Next, we followed a pharmacologic approach using Dyngo-4a, which chemically inhibits dynamin’s GTPase activity, blocking endocytosis (Fig. S3, A and C). As in cells expressing DynK44A, the cAMP response to CRH was impaired by Dyngo-4a treatment, showing a profile similar to the one observed with DynK44A (Fig. 5 D).

The early increase of CRH-mediated cAMP accumulation did not seem to be affected by dynamin-deficient mutant or Dyngo-4a. The differences in the time course of the cAMP response when endocytosis was inhibited were significant 3.5 min after CRH stimulation (Fig. 5, B and D), in line with fast CRHR1 endocytosis (Fig. 5 A; Kotowski et al., 2011; Bonfiglio et al., 2013). Importantly, DynK44A expression or Dyngo-4a treatment did not significantly affect the cAMP response elicited by receptor-independent activation of adenyl cyclases with forskolin (Fig. 5, C and E), confirming that the effect of endocytosis inhibition required pathway stimulation by the activated receptor.

Changes in the CRH-dependent calcium response cannot be ascribed to a decreased cAMP response in these cells because a similar calcium response was observed in both control and DynK44A-expressing cells (Fig. S3 D). Thus, inhibition of endocytosis produces a significant decrease of cellular cAMP accumulation mediated by CRHR1.

For the class B GPCR PTHR, PTH-triggered cAMP production continues after internalization by a mechanism dependent on receptor interaction with β-arrestins and prolonged ERK1/2 signaling (Feinstein et al., 2011). We investigated whether activated ERK1/2 was involved in the CRH-cAMP response in HT22-CRHR1 cells. When cells were pretreated with the MEK1/2 inhibitor U0126, a complete blockage of CRH-activated ERK1/2 was observed (Fig. S3 E). However, no differences were found between control and treated cells in the CRH-mediated cAMP response (Fig. S3 F), indicating that cAMP generated by CRH-activated CRHR1 does not depend on ERK1/2 activation.

**sAC activity is essential for endocytosis-dependent CRHR1 signaling**

We investigated the cAMP source involved in CRHR1 signaling after internalization, combining ACs inhibitors with endocytosis blockage. HT22-CRHR1-Epac-SH187 cells transfected with an empty vector or DynK44A mutant (coexpressed with mCherry) were mixed so that both types of cells could be treated, CRH-stimulated, and analyzed in the same microscope field (Fig. 6 A). When cells were preincubated with vehicle or the tmAC inhibitor ddA, cAMP levels were reduced faster in cells transfected with DynK44A compared with neighboring control cells. However, preincubation with the sAC inhibitor 2-HE led to a loss of the endocytosis effect on the cAMP response to CRH (Fig. 6 B). This result strongly suggests that the endocytosis-dependent component of the cAMP response is largely dependent on sAC activity. Importantly, 2-HE did not affect CRH-activated CRHR1 endocytosis, as shown by flow cytometry analysis and subcellular local-
The differences between CRH-elicited cAMP responses in the presence or absence of a functional endocytosis machinery can be measured for at least 20 min (Fig. S3 G). As a control, when ddA was added after 10 min, cAMP responses were reduced, but the difference in cAMP levels between control and DynK44A cells was maintained (Fig. S6 H). This further indicates that tmACs are still active but are not critical for endocytosis-dependent cAMP generation.

The effect of sAC inhibition and endocytosis blockage on ERK1/2 activation was assessed (Fig. 6 E). As previously shown (Fig. 2 A), preincubation with the sAC-specific inhibitor KH7 diminished ERK1/2 activation at early and late time points, whereas the endocytosis inhibitor Dyngo-4a caused a significant reduction of late pERK1/2. Simultaneous inhibition of sAC activity and endocytosis resulted in lower ERK1/2 activation at the early time point compared with Dyngo-4a treatment alone, showing that sAC plays a role in the early endocytosis-independent phase. Remarkably, both treatments, separately or combined, reduced late pERK1/2 to a similar extent, suggesting that sAC activity and endocytosis are involved in the same mechanism that operates at the second signaling phase.

CRH-triggered response depends on sAC modulators: calcium and bicarbonate

sAC is stimulated by calcium (Jaiswal and Conti, 2003; Carlson et al., 2007; Kleinboelting et al., 2014), which may regulate sAC activity in neurons (Wu et al., 2006; Corredor et al., 2012). In HT22-CRHR1 cells, CRH elevates intracellular calcium (Bonfiglio et al., 2013). To monitor cAMP and calcium responses simultaneously, we used the FRET-based cAMP sensor in combination with a genetically encoded red calcium indicator, REX-GECO1 (Wu et al., 2014). CRH stimulation resulted in rapid and synchronized responses of both second messengers, albeit with different profiles: intracellular calcium increased sharply upon stimulation and returned to basal levels after 60 s, whereas cAMP levels were sustained (Fig. 7, A and B). Preincubation with the cell-permeable calcium chelator BAPTA-AM reduced pERK1/2 at both early and late time points (Fig. S4 A), showing that calcium is critical for the ERK1/2 response. Remarkably, the ERK1/2 activation profile in the presence of calcium chelators was reminiscent of the one silencing sAC (Fig. 2 C).

In addition to sAC, two tmACs (AC1 and AC8) are also positively regulated by calcium (Willoughby and Cooper, 2007). In accordance with our previous study that the tmAC-specific
inhibitor ddA affected only the early phase of ERK1/2 activation (Bonfiglio et al., 2013), knockdown of AC1 significantly diminished pERK1/2 at early time points but did not alter the sustained phase (Fig. 7C). We could not detect AC8 expression in this system or an effect on the ERK1/2 response using an siRNA against AC8 (Fig. S4, B and C).

If CRH-mediated calcium influx were critical for sAC activation, a reduced cAMP response would be expected when CRH-elicited calcium increase is blocked. CRH-triggered cAMP response was diminished to a similar extent by extracellular EGTA and cell-permeable BAPTA-AM calcium chelators in pretreated HT22-CRHR1 cells (Fig. 7D), indicating that the cAMP response depends, at least partially, on a calcium-sensitive mechanism. Notably, the calcium response elicited by CRH was not altered in HT22-CRHR1 cells preincubated with ddA or 2-HE (Fig. 7E), suggesting that calcium, required for sAC activity, would not depend on cAMP stimulation by CRH-activated CRHR1.

Next, we examined the effect of the sAC-specific modulator bicarbonate (Steegborn et al., 2005; Kleinboelting et al., 2014) on the ERK1/2 response to CRH. Bicarbonate concentration in the culture medium used to grow cells and perform experiments was 25 mM, which reproduces the bicarbonate concentration in vivo. When we stimulated HT22-CRHR1 cells with CRH in bicarbonate-free medium, ERK1/2 activation was strongly attenuated. Strikingly, the late phase of ERK1/2 activation almost disappeared (Fig. 8A).

Pharmacologic blockage of anion exchangers with stilbene derivative DIDS modulated bicarbonate levels and sAC activity (Choi et al., 2012). Recently, DIDS has been shown to inhibit sAC by competition with bicarbonate (Kleinboelting et al., 2014). CRH-mediated pERK1/2 in the presence of DIDS resulted in a modest decrease of the ERK1/2 response at early time points and almost null ERK1/2 activation at late time points (Fig. 8B). By either silencing sAC (Fig. 2C) or blocking bicarbonate transport (Fig. 8B), we found reduced CRH-dependent ERK1/2 activation, which provides further evidence for a role of bicarbonate-sensitive sAC activity in CRHR1 signaling in HT22-CRHR1 cells.

Collectively, these data are consistent with a mechanism in which CRH-activated sAC is regulated by calcium and bicarbonate and support the notion of sAC activity being required in the late phase of ERK1/2 activation.
In this study, we demonstrate that sAC is a second source of cAMP in CRHR1 signaling mechanisms elicited by CRH in two physiologically relevant contexts of CRH action. We show that cAMP generated by sAC contributes to the overall cAMP response and that it is critical for ERK1/2 activation upon CRH treatment.

Our work in the hippocampal neuronal context, HT22 cells, also indicates that CRH-activated CRHR1 continues to generate cAMP from endocytic compartments, as recently demonstrated for several GPCRs (Irannejad and von Zastrow, 2014; Vilardaga et al., 2014). The study of CRH-activated CRHR1 uncovers a previously unanticipated link between sAC and endosome-based GPCR signaling. These findings revise the canonical model in which GPCR-elicited cAMP production relies solely on tmACs.

A role of sAC in GPCR signaling is just beginning to emerge; only scarce and indirect evidence has been provided so far (Halm et al., 2010; Ivonnet et al., 2015). sAC is unique among the ACs in being activated by bicarbonate and calcium (Jaiswal and Conti, 2003; Han et al., 2005). Moreover, unlike tmACs, sAC has no membrane-spanning domains and is distributed throughout the cell. Despite growing evidence that sAC might be responsible for cAMP-mediated functions previously attributed to tmACs, little is known about the mechanisms involved. Here, we identified a key role of CRHR1-induced calcium for sAC activation with both PKA and EPAC effector proteins downstream of sAC (Fig. 8 C).

In HT22-CRHR1 cells, PKA is involved only in the early ERK1/2 activation phase, whereas EPAC participates in both phases. In the neuroendocrine cell model AtT20, ERK1/2 activation was dependent on both PKA and EPAC. Notably, cAMP and ERK1/2 responses to CRH are short-lived in these cells, resembling the acute phase of pERK1/2 in HT22-CRHR1. Although our data do not rule out an endosome-based CRHR1 signaling mechanism in AtT20 cells, which remains to be explored, they show that ERK1/2 activation requires sAC.

How is sAC physically integrated in signaling complexes at specific cellular locations? Recent work describes a complex assembled by ezrin, an A-kinase anchoring protein that holds sAC at the plasma membrane (Watson et al., 2015). A systematic analysis of sAC interactions will provide insight into specific functions of spatially restricted cAMP pools in cellular responses.

We show that sAC is not a general mediator of GPCRs that activate ERK1/2 downstream of cAMP. Alternative sources of cAMP involved in signaling generated by GPCRs from the endocytic pathway have not yet been explored. Even though sAC is likely to be engaged by a variety of GPCRs, its involvement may depend on specific features of the integral cellular response rather than just the mode of signaling or the cellular context.

β-Arenergics and CRHR1 internalization contribute to ERK1/2 activation in response to CRH, suggesting sustained CRHR1 signaling in endosomes (Bonfiglio et al., 2013). Our results show that CRHR1 endocytosis contributes to the overall cAMP response, in line with the emerging paradigm of endosomal generation of cAMP by GPCRs, the mechanisms of which are just beginning to be understood. For the prototypical class A GPCR β-adrenergic receptor 2, the existence of a second phase of Gs coupling from endosomes has been elegantly shown (Irannejad et al., 2013), whereas for the class B PTHR,
a prolonged Gs coupling favored by ERK1/2 and β-арrestin was demonstrated (Feinstein et al., 2011).

Our present findings reveal a Gs-independent mechanism of cAMP production with an essential role in ligand-activated GPCR endocytosis-mediated signaling. Although there is no doubt that CRHR1 activates G protein–responsive tmACs at the plasma membrane (Punn et al., 2012), our data support a critical contribution of G protein–independent sAC to the cAMP response to CRH that is linked to ERK1/2 activation in cellular contexts of CRHR1 signaling.

Notably, most sustained cAMP responses have been reported for class B GPCRs that mediate the functions of therapeutically relevant peptide hormones (Ferrandon et al., 2009; Feinstein et al., 2013; Kuna et al., 2013; Merriam et al., 2013). Future work needs to focus on defining factors that modulate kinetics and spatial organization of the CRHR1–mediated cAMP response, which is thought to have significant physiological implications (Feinstein et al., 2011; Tsvetanova and von Zastrow, 2014). Furthermore, understanding the role of G protein– and endosome-dependent signaling, together with the identification of ligands with functional selectivity, may result in clinically valuable tools for pathologies involving the CRH/CRHR1 system.

Materials and methods

Cell culture and transfection

HT22-CRHR1, AtT20, and 3T3-L1-CRHR1 cell lines were cultured in DMEM supplemented with FBS (5% for HT22-CRHR1 and 10% for AtT20 and 3T3-L1-CRHR1), 2 mM l-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (Invitrogen) at 37°C in a humidified atmosphere containing 5% CO2.

Plasmids were transfected using Lipofectamine and Plus Reagent (Invitrogen) according to the manufacturer’s instructions and as previously described (Bonfiglio et al., 2013). Experiments were performed 48 h after plasmid transfection. When single-cell measurements were performed in cells transiently transfected with plasmids, cells were cotransfected with a fluorescent protein–expressing plasmid, and only fluorescent cells were used for the studies. siRNA transfection with Lipofectamine 2000 (Invitrogen) was performed as previously described (Bonfiglio et al., 2013) and according to the manufacturer’s protocol. Experiments were performed 72 h after siRNA transfection. The efficiency of knockdown was assessed by quantitative RT-PCR normalized to HPRT, and experiments with at least 65% reduction of mRNA levels were considered.

Plasmids and siRNA

Plasmids were provided as follows: the mouse CRHR1 expression vectors c-Myc-mCRHR1 and myc-GFP-CRHR1 by J. Deussing (Max Planck Institute of Psychiatry, Munich, Germany); mTurquoise2-Epac-cp173Venus-Venus (Epac-S8145) by K. Jalink (Netherlands Cancer Institute, Amsterdam, Netherlands); AKAR4 by J. Zhang (Johns Hopkins University, Baltimore, MD); REX-GECO1 by R. Campbell (University of Alberta, Edmonton, AB, Canada); and dynamin-K44A expression vector by J. Benovic (Thomas Jefferson University, Philadelphia, PA); mCherry by M. Rossi (Instituto de Investigación en Biomedicina de Buenos Aires, Buenos Aires, Argentina) and EPAC1 and N-EPAC by D.L. Altschuler (University of Pittsburgh School of Medicine, Pittsburgh, PA).

Rat sACII plasmid was obtained from Addgene (pSAC5.02, plasmid #16077). A truncated, catalytically active form of sAC (sAC; Tresguerres et al., 2011) tagged with the epitope HA at the C terminus was generated by PCR (sAC-HA). The first 468 aa were amplified via PCR from sACII using Apal and NotI restriction sites for N and C termini, respectively, and with the C terminus of sAC and the sequence of the epitope (YPYDVPDYA) encoded in the reverse primer. The insert was ligated into pcDNA3.1(+)/Zeot (Invitrogen).

sAC knockdown was achieved using a 21-nucleotide siRNA (5′-AAUGUAUUGCCUCAUGUAtt-3′), siRNAs against AC1 and AC8 were purchased from GE Healthcare as pools of four siRNAs (SMART pools; Thermo Fisher Scientific). For EPAC knockdown, the following siRNAs were used: EPAC1 (5′-GGUCAAUUCGCAGGUUAgAtt-3′ and 5′-CGCAACACAGAGUUGGAAtt-3′) and EPAC2 (5′-GCUGGU AGACUGGAUAGAtt-3′ and 5′-GCUAUCCACACAUUUt-3′). An siRNA against GL3 (5′-CUUACCGUGAGUACUUGATT-3′) was used as control.

Generation of stable cell lines

HT22 stable clones expressing cMyc-CRHR1 were previously described (Bonfiglio et al., 2013). Stable clones expressing both cMyc-CRHR1 and FRET biosensors (Epac-S8145 or AKAR4) were obtained by transfection of HT22-CRHR1 cells with pcDNA3.1/Zeo(+)-Epac-S8145 or pcDNA3.1/Zeo(+)-AKAR4 and selection with Zeocin (100 µg/ml, Invitrogen). 3T3-L1 stable clones expressing cMyc-CRHR1 were obtained by transfection of pcDNA-3-c-Myc-mCRHR1 and selection with Geneticin (800 µg/ml). In both cases, after 14 d of antibiotic treatment, clonal colonies were isolated and subcultured.

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Blue 2011 software (ZEISS) for live-imaging incubation. Data acquisition was controlled by Zen digital CCD camera, a Stage Controller XY STEP SMC 2009 scanning fluorescence microscope (ZEISS). Images were acquired with a Axio Observer Z1 inverted Epi-Fluo-4-AM and 0.14% Pluronic F-127 (Molecular Probes) in Ringer glass-bottom dishes were loaded for 30–60 min in darkness with 6 µM for experiments testing bicarbonate dependence, bicarbonate-free DMEM supplemented with 25 mM Hepes was used to prepare media at 0- and 25-mM sodium bicarbonate concentrations. Each condition was adjusted to physiological pH 7.2 with NaOH and HCl and allowed to equilibrate in the incubator for additional 30 min, and then the pH was checked again and adjusted to 7.4. HT22-CRHR1 cells were serum-starved for 6 h in bicarbonate-free DMEM or 25 mM bicarbonate DMEM before stimulation with 10 nM CRH.

Preparation of cellular extracts and immunoblotting
After treatments, cells were washed with ice-cold PBS and lysed in Laemmli sample buffer. Whole-cell lysates were sonicated and heated to 95°C for 5 min. Samples were resolved by SDS-PAGE and transferred to 0.45-mm nitrocellulose membranes (EMD Millipore) for immunoblotting. Membranes were blocked in TBS–TWEEN 20 (0.05%) containing 5% milk at RT for 1 h under shaking and probed overnight at 4°C with the primary antibodies. The following antibodies were used: anti-sAC (R21; CEP Biotech), anti-c-Myc (9E10; sc-40) and anti-pERK1/2 (E-4; sc-7383) from Santa Cruz Biotechnology, Inc.; anti-total-ERK1/2 (9102; Cell Signaling Technology), anti-phospho CREB (06-519; EMD Millipore), and anti–total-CREB (9104; Cell Signaling Technology).

Chemiluminescent signals were detected by HRP-conjugated secondary antibodies and enhanced chemiluminescence (SuperSignal West Dura; Thermo Fisher Scientific) using a GBOX Chemi XT4 (Syngene). Phosphorylation of MAPK and CREB was detected with the Odyssey Fc Imaging System (LI-COR Biosciences), using anti-mouse–IRDye700DX and anti-rabbit–IRDye800CW secondary antibodies (Rockland). Phosphorylated proteins were relativized to the total protein level, and results were expressed as the percentage of maximum pERK1/2 after stimulation. Immunoreactive signals were analyzed digitally using ImageJ software (National Institutes of Health).

Neurite outgrowth assay
HT22-CRHR1 cells seeded at 40% density in 12-well plates were stimulated with 100 nM CRH in the presence of vehicle or tmACs- or sAC-specific inhibitors. After 20 h of treatment, cells were imaged under bright-field illumination using an Olympus IX81 inverted epi-fluorescence microscope with a 20x air objective and Metamorph software for image acquisition. For each treatment, at least 15 random fields were imaged. Quantification of morphological changes was performed using Simple Neurite Tracer plugin for Fiji software. Neurite outgrowth was determined as the ratio between the longest neurite and the soma diameter per cell after 20 h, measuring at least 100 cells per treatment. For statistical analysis, repeated-measures one-way analysis of variance followed by Tukey’s post hoc test (n = 3) was performed.

Single-cell intracellular calcium imaging
For experiments using ester calcium indicators, cells plated in glass-bottom dishes were loaded for 30–60 min in darkness with 6 µM Fluo-4-AM and 0.14% Pluronic F-127 (Molecular Probes) in Ringer buffer. Images were acquired with an Axio Observer Z1 inverted Epi-fluorescence microscope (ZEISS), equipped with an AxioCam HRm3 digital CCD camera, a Stage Controller XY STEP SMC 2009 scanning stage, and an Incubator XLmulti S1 (D) and Heating Unit XL S1 (D) for live-imaging incubation. Data acquisition was controlled by Zen Blue 2011 software (ZEISS), configured at a bit depth of 14 bits. Cells were imaged with a 20x air objective (Plan-Apochromat NA 0.8 M27) and illuminated using Colibri.2 470-nm LED excitation (5% power), with a 50-ms exposure acquired every 5 s and a 38HE Filter set for a duration of 10 min. When indicated, cells were preincubated with different reagents. Approximately 30 s after the start of the experiment, CRH was added to a final concentration of 100 nM. Image analysis was performed with Fiji by measuring calcium-dependent changes in fluorescence intensity from resting levels (ΔF/F0) in 30–40 cells randomly selected in each experiment.

Spectral FRET and coimaging of cAMP and calcium
HT22-CRHR1, AtT20, or 3T3-CRHR1 cells expressing fluorescent proteins and FRET biosensors were seeded in glass-bottom dishes. Cell imaging was performed on an inverted LSM 710 (ZEISS) equipped with an automated stage, an incubation chamber, and Definite focus system. Data acquisition was performed with ZEN Black 2011 software. Images were acquired with a C-Apochromat 40x/1.2 water immersion and temperature-corrected objective lens at 1,024 × 1,024 pixels, 16 bits, pixel dwell time 3.15 µs, with open pinhole (600 µm). The emission spectra of the fluorescent proteins Cerulean, mTurquoise2, Venus, and REX-GECOI bound to calcium were determined using 405- or 458-nm lasers for the excitation and lambda mode for the detection using a 32-channel QUasar detector arranged with bandwidth channels of 9.7 nm.

For FRET experiments, cells were illuminated with a 30-mW, 405-nm diode laser at 2% laser power (550–650 gain) and a 405-nm dichroic mirror; emission was collected at 413- to 723-nm wavelengths every 15 s for a duration of 15 min. For coimaging cAMP and calcium dynamics, a 25-mW argon laser for 458-nm excitation (2.5% power, 650 gain) and a 458-nm dichroic mirror were used; emission was collected at 463- to 723-nm wavelengths every 5 s for a duration of 10 min. The saturation level was verified for each image.

For the reference spectra and the experiments, phenol red-free DMEM/F12 medium supplemented with 20 mM Hepes was used, and imaging was performed at 37°C and 5% CO2. Approximately 2.5 min after the start of the experiment, CRH was added to the final concentration indicated. When indicated, cells were preincubated for 15 min with different reagents. AC inhibitors were also added after CRH stimulation to evaluate the change in the FRET time course.

For lambda FRET experiments, the linear unmixing tool of ZEN Black 2011 software was used to obtain an image for each fluorophore using the reference spectra. Image quantification was performed with Fiji software. After background subtraction, REX-GECOI, FRET, and donor intensities were measured for single cells for each time point. The FRET/donor ratio or REX-GECOI intensity was calculated and normalized to resting levels (before stimulation).

Flow cytometry assays
For CRHR1 surface staining, HT22-CRHR1 cells on 60-mm plates were starved for 1 h in OptiMEM before drug pretreatments or stimulation. When pharmacological inhibitors were used, cells were pre-treated with drugs or vehicle 15–30 min before stimulation with CRH for the indicated concentration and time. After incubation, cells were washed with ice-cold PBS and harvested in 1 mM PBS-EDTA. 2.0 × 106 cells were incubated at 4°C with 10% FBS in PBS for 1 h to reduce nonspecific binding. Subsequently, cells were incubated with 0.2 µg anti-c-Myc monoclonal antibody (Santa Cruz Biotechnology, Inc.) for 2 h and, after washing with 1% FBS in PBS, were stained with 1.4 µg anti-mouse Alexa Fluor 647–conjugated secondary antibody (Thermo Fisher Scientific). All solutions contained 0.01% sodium azide. Controls for autofluorescence, primary antibody staining on parental HT22 cells, and secondary antibody staining in absence of primary
antibody were performed for each experiment. Flow cytometry data were acquired on a FACsCANTO II (BD). Data were analyzed using FlowJo software (Tree Star).

Confocal microscopy
HT22-CRHR1 cells transiently transfected with cMyc-GFP-CRHR1 expression vector were seeded on glass cover slips. After treatments, cells were washed with ice-cold PBS, fixed with 4% paraformaldehyde in PBS, permeabilized with 0.01% Triton X-100/PBS, and stained with DAPI. Cover slips were washed with PBS, and cells were mounted with Mowiol and analyzed by confocal microscopy. Images were acquired on an inverted LSM 710. Data acquisition was performed with ZEN Black 2011 software. Images were acquired with C-Apochromat 63×/1.2 water immersion and temperature-corrected objective lens at 1,024 × 1,024 pixels, 12 bits, pixel dwell time 0.52 μs, with 1 Airy unit. For each treatment, 15–20 individual cells were randomly selected and examined. No fluorescence was observed for control cells transfected with an empty vector.

Competition cAMP binding assay
cAMP determination by means of competition with [3H]cAMP for PKA was performed as previously described (Copsel et al., 2011; Bonfiglio et al., 2013). In brief, HT22-CRHR1 cells were pretreated for 30 min with the indicated inhibitors, stimulated with CRH, and subjected to ethanol extraction followed by 15-min centrifugation at 3,000 g. Supernatants were dried and resuspended in 50 mM Tris-HCl buffer, pH 7.4. cAMP content was determined by means of competition with [3H]cAMP for PKA and expressed as the percentage of stimulation relative to maximum response. The standard curve was performed using eight cAMP amounts ranging from 0.1 to 90 pmol. Duplicate samples in at least three independent experiments were analyzed.

Pomc-Luc reporter assay
Luciferase reporter assays were performed as previously described (Riebold et al., 2015). In brief, AtT-20 cells were seeded in 48-well plates and transfected with 0.3 µg Pomc-Luc and 0.1 µg RSV-Gal (Addgene) for normalization for 3 h using Superfect (QIA GEN) according to the manufacturer’s instructions. Transfection mix was replaced with serum-free culture medium overnight. 24 h after transfection, cells were pretreated with vehicle or sAC-specific inhibitors and stimulated with 100 nM CRH for 6 h. Cells were lysed in Passive Lysis Buffer (Promega), followed by measurement of reporter activity according to the manufacturer’s instructions (PKJ GmbH). Relative Luc values (Luc/β-Gal) were used to normalize for transfection efficiency in all experiments.

Statistics
Each experiment was performed at least three independent times. The results are presented as the mean ± SEM of each measurement. Comparisons between treatments were performed using Student’s t test and one- or two-way analysis of variance (GraphPad Prism) followed by post hoc Tukey’s test. Statistically significant differences are indicated.

Online supplemental material
Fig. S1 shows the optimization of cAMP measurements by FRET, CRH-triggered cAMP response by competition with [3H]cAMP for PKA, sAC detection by Western blot, and effect of sAC-specific inhibitors in CRH-mediated ERK1/2 activation. Fig. S2 shows cAMP and ERK1/2 responses triggered by CRH and PACAP38 in AtT20 cells, ERK1/2 and CREB activation, and calcium response to CRH in 3T3L1-CRHR1 cells. Fig. S3 shows CRH-dependent CRHR1 endocytosis blocked by DynK44A and Dyngo-4a, calcium response to CRH when endocytosis is blocked, cAMP and ERK1/2 response in the presence of MEK1/2 inhibitors, and sustained cAMP response in the presence and absence of ddA. Fig. S4 demonstrates the role of calcium in ERK1/2 activation in response to CRH and the expression of AC8 in HT22-CRHR1 cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201512075/DC1.

Acknowledgments
We thank E. Arzt for providing intellectual input and critical advice for experimental development and data interpretation, M. Pérez-Pereda for advice on Pomc-Luc experiments, C. Davio for contributing expertise and reagents for experiments of binding assay for cAMP determination, A. Bruzzone and I.A. Lithy for providing isoproterenol, and A. Attorreis and C. Pollack for excellent technical assistance with microscopy and flow cytometry experiments.

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (Max-Planck 10:2791 and 13:0392) to S. Silberstein and C.W. Turk, the Max Planck Society, the University of Buenos Aires, the Consejo Nacional de Investigaciones Científicas y Técnicas, and the Fondo para la Convergencia Estructural del Mercosur (COF 03/11).

The authors declare no competing financial interests.

Submitted: 23 December 2015
Accepted: 10 June 2016

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