

# A key phosphorylation site in AC8 mediates regulation of Ca<sup>2+</sup>-dependent cAMP dynamics by an AC8–AKAP79–PKA signalling complex

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Accepted 3 August 2012

Journal of Cell Science 125, 5850–5859

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doi: 10.1242/jcs.111427

## Summary

Adenylyl cyclase (AC) isoforms can participate in multimolecular signalling complexes incorporating A-kinase anchoring proteins (AKAPs). We recently identified a direct interaction between Ca<sup>2+</sup>-sensitive AC8 and plasma membrane-targeted AKAP79/150 (in cultured pancreatic insulin-secreting cells and hippocampal neurons), which attenuated the stimulation of AC8 by Ca<sup>2+</sup> entry (Willoughby et al., 2010). Here, we reveal that AKAP79 recruits cAMP-dependent protein kinase (PKA) to mediate the regulatory effects of AKAP79 on AC8 activity. Modulation by PKA is a novel means of AC8 regulation, which may modulate or apply negative feedback to the stimulation of AC8 by Ca<sup>2+</sup> entry. We show that the actions of PKA are not mediated indirectly via PKA-dependent activation of protein phosphatase 2A (PP2A) B56δ subunits that associate with the N-terminus of AC8. By site-directed mutagenesis we identify Ser-112 as an essential residue for direct PKA phosphorylation of AC8 (Ser-112 lies within the N-terminus of AC8, close to the site of AKAP79 association). During a series of experimentally imposed Ca<sup>2+</sup> oscillations, AKAP79-targeted PKA reduced the on-rate of cAMP production in wild-type but not non-phosphorylatable mutants of AC8, which suggests that the protein–protein interaction may provide a feedback mechanism to dampen the downstream consequences of AC8 activation evoked by bursts of Ca<sup>2+</sup> activity. This fine-tuning of Ca<sup>2+</sup>-dependent cAMP dynamics by targeted PKA could be highly significant for cellular events that depend on the interplay of Ca<sup>2+</sup> and cAMP, such as pulsatile hormone secretion and memory formation.

**Key words:** Adenylyl cyclase, PKA, Phosphorylation, AKAP, Calcium

## Introduction

The membrane-bound adenylyl cyclase (AC) family comprises nine members each exhibiting unique structural and regulatory properties. Among them, the AC8 isoform, highly expressed in hippocampal and pancreatic tissue, is robustly stimulated by dynamic, sub-μM increases in intracellular Ca<sup>2+</sup> acting via calmodulin (CaM) (Willoughby and Cooper, 2007). This characteristic of AC8 is thought to underpin its central role in coordinating the intracellular actions of Ca<sup>2+</sup> and cAMP during major cellular events such as insulin secretion (Dyachok et al., 2006) and long term potentiation (LTP) (Wang and Storm, 2003). Recently we revealed that such interplay between Ca<sup>2+</sup> and AC8 activity can be regulated by a specific plasma membrane-localised A-kinase anchoring protein, AKAP79/150 (Willoughby et al., 2010) (AKAP150 is the rodent orthologue of human AKAP79). AKAPs are a group of over 50 scaffolding proteins that target cAMP-dependent protein kinase (PKA) to discrete cellular microdomains, thereby facilitating selective phosphorylation of PKA substrates including receptors, ion channels and enzymes (Wong and Scott, 2004). In addition to targeting PKA activity, AKAPs have been found to interact with protein kinase C (PKC), protein phosphatases, phosphodiesterases (PDEs), mitogen-activated protein kinases (MAPK) (Carr et al., 1992; Coghlan

et al., 1995; Klauck et al., 1996; Le et al., 2011; Willoughby et al., 2006; Jivan et al., 2009) and, there is now growing evidence of AKAP interactions with the ACs (Bauman et al., 2006; Kapiloff et al., 2009; Piggott et al., 2008; Willoughby et al., 2010). These latter studies have reported AKAP79 and mAKAPβ binding to Ca<sup>2+</sup>-inhibited AC5/6 (Bauman et al., 2006), Yotiao (AKAP9) binding to Ca<sup>2+</sup>-stimulable AC1 (Piggott et al., 2008) and AKAP79/150 interaction with Ca<sup>2+</sup>-stimulated AC8 (Willoughby et al., 2010). The interaction between AKAP79/150 and AC8 is thought to occur in lipid raft domains of the plasma membrane, with palmitoylation of AKAP79/150 playing an important role in targeting the AKAP to the cholesterol- and sphingolipid-rich regions of the plasma membrane where it can impact on local Ca<sup>2+</sup>-stimulated AC8 activity (Delint-Ramirez et al., 2011; Willoughby et al., 2010). The mechanism underlying the AKAP79/150-dependent attenuation of Ca<sup>2+</sup>-stimulated AC8 activity is not known, although AKAP79/150 does not significantly alter the amplitude of the Ca<sup>2+</sup> entry signal regulating AC8 (Willoughby et al., 2010) and, unlike AC5/6, there are no reports that the PKA or PKC associated with AKAP79/150 can regulate AC8 activity (Willoughby and Cooper, 2007).

Here we identify a specific action of AKAP79-targeted PKA on Ca<sup>2+</sup>-dependent AC8 activity. Although PP2A B56δ subunits

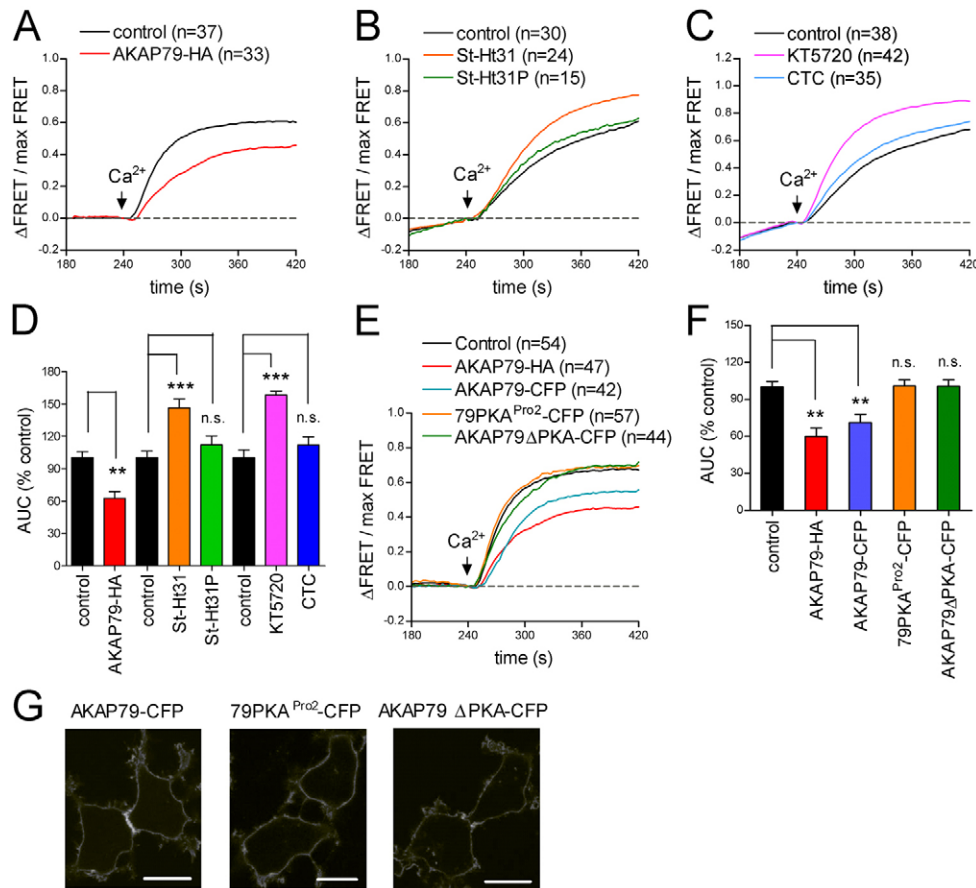
are potential substrates for PKA phosphorylation (Ahn et al., 2007), which associate with the N-terminus of AC8, they did not modulate PKA actions under our experimental conditions. Using site-directed mutagenesis and single-cell cAMP imaging we identify Ser-112 as a key residue within the N-terminus of AC8 that is directly phosphorylated by AKAP79-associated PKA. Perhaps of most significance from a physiological viewpoint, the AKAP79-targeted PKA attenuated the response of AC8 activity to transient  $\text{Ca}^{2+}$  events; this effect was precluded in AC8 mutants that were not susceptible to phosphorylation by PKA.

## Results

### AKAP79-associated PKA regulates $\text{Ca}^{2+}$ -stimulated AC8 activity

AKAP79 attenuates  $\text{Ca}^{2+}$ -stimulated AC8 activity in hippocampal and pancreatic cells, where both proteins are endogenously expressed (Willoughby et al., 2010). Here, using an HEK293 cell overexpression system, we examine whether

protein kinases that associate with AKAP79, namely PKA and PKC, mediate the AKAP79-dependent modulation of  $\text{Ca}^{2+}$ -stimulated AC8 activity. The FRET-based biosensor, Epac2-camps, was used to monitor real-time cAMP production in HEK293 cells stably expressing AC8 (HEK-AC8) during store-operated  $\text{Ca}^{2+}$  entry (SOCE).  $\text{Ca}^{2+}$ -dependent stimulation of AC8 activity is highly selective for SOCE over other modes of  $\text{Ca}^{2+}$  rise (Willoughby and Cooper, 2007). Thus, HEK-AC8 cells were pretreated with the sarco/endoplasmic reticulum (ER)  $\text{Ca}^{2+}$ -ATPase (SERCA) pump inhibitor thapsigargin (Tg, 200 nM) in the absence of external  $\text{Ca}^{2+}$ , to promote passive depletion of the ER  $\text{Ca}^{2+}$  stores. Once the ER stores had been depleted of  $\text{Ca}^{2+}$  the addition of 2 mM extracellular  $\text{Ca}^{2+}$  triggered a robust SOCE. Under control conditions, the induction of SOCE at 240 sec was accompanied by a marked increase in cAMP production in individual HEK-AC8 cells, reaching  $\sim 60\%$  of maximum FRET ratio change [Fig. 1A; saturation of the FRET sensor is determined by addition of a cocktail of AC activators and the



**Fig. 1. AKAP79-associated PKA attenuates SOCE-induced AC8 activity.** (A) Expression of AKAP79-HA decreased SOCE-mediated AC8 activity. HEK-AC8 cells expressing the cAMP sensor Epac2-camps were pretreated with 200 nM Thapsigargin +10 nM Forskolin in zero  $\text{Ca}^{2+}$  conditions to deplete intracellular  $\text{Ca}^{2+}$  stores and provide modest basal stimulation of AC8 activity. Addition of 2 mM external  $\text{Ca}^{2+}$  at 240 sec induced SOCE-stimulated AC8 activity. (B) Effects of the AKAP/PKA disruptor peptide 10  $\mu\text{M}$  St-Ht31 on AC8 activity stimulated by SOCE in response to 2 mM external  $\text{Ca}^{2+}$ . 10  $\mu\text{M}$  St-Ht31P was used as a negative control. (C) Effects of PKA inhibitor 1  $\mu\text{M}$  KT5720 and PKC inhibitor 1  $\mu\text{M}$  chelerythrine chloride (CTC) on SOCE-stimulated AC8 activity. Experiments were performed in the presence of 100  $\mu\text{M}$  IBMX to prevent any indirect effects of PKA inhibition on PDE activity, and the external  $\text{Ca}^{2+}$  concentration used for SOCE was decreased to 0.5 mM to avoid saturation of the cAMP sensor. (D) Summary of data ( $\pm$ s.e.m.) presented in A–C plotted as area under the curve (AUC) relative to control cells. \*\* $P < 0.01$ , \*\*\* $P < 0.001$  compared to controls, n.s., not significant. (E) Lack of effect of AKAP79 PKA-binding deficient mutants 79PKA<sup>Pro2</sup>-CFP and AKAP79 $\Delta$ PKA-CFP on 2 mM external  $\text{Ca}^{2+}$  SOCE-induced AC8 activity compared to wild-type AKAP79 constructs. (F) Summary of data ( $\pm$ s.e.m.) presented in E. \*\* $P < 0.01$  compared to controls, n.s., not significant. (G) Confocal images of HEK-AC8 cells expressing wild-type AKAP79-CFP and PKA-binding mutants. Scale bars represent 20  $\mu\text{m}$ .

non-selective PDE inhibitor IBMX (100  $\mu$ M)]. In wild-type HEK293 cells SOCE has no effect on cAMP production due to the absence of  $\text{Ca}^{2+}$ -stimulated AC isoforms, confirming a dependence of  $\text{Ca}^{2+}$ -stimulated cAMP production in HEK-AC8 cells on the overexpression of the  $\text{Ca}^{2+}$ -stimulated AC isoform (Willoughby et al., 2012). Under the same experimental conditions HEK-AC8 cells overexpressing AKAP79-HA displayed reduced  $\text{Ca}^{2+}$ -dependent cAMP production (Fig. 1A,D;  $P < 0.01$ ) which demonstrates the inhibitory action of the scaffold protein on AC8. In contrast, the cell-permeant PKA-AKAP disruptor peptide, St-Ht31, enhanced  $\text{Ca}^{2+}$ -stimulated AC8 activity (Fig. 1B,D;  $P < 0.001$  compared to controls), suggesting that the inhibitory effects of endogenous AKAP79 on AC8 could be mediated by an AKAP-associated PKA. Previous work from our laboratory has shown that AKAP79 is one of the major membrane-bound AKAP isoforms endogenously expressed in HEK293 cells (Willoughby et al., 2006).

Evidence of an inhibitory action of PKA on AC8 activity was provided by an augmented cAMP response during SOCE in HEK-AC8 cells pretreated with the PKA selective inhibitor 1  $\mu$ M KT5720 (Fig. 1C,D;  $P < 0.001$  compared to controls). All PKA inhibition experiments were performed in the presence of 100  $\mu$ M IBMX to remove any indirect effects of PKA-dependent PDE activity (PDE4, the major isoform of PDE in HEK293 cells, is activated by PKA). The external  $[\text{Ca}^{2+}]$  used to induce SOCE was reduced from 2 mM to 0.5 mM to limit cAMP production and prevent sensor saturation in the presence of the PDE inhibitor. As PKA has shown opposing regulatory effects on SOCE channel activity (Hahn et al., 2000; Hague et al., 2000) control experiments were performed to determine if the enhanced cAMP increase seen in the presence of KT5720 could be an indirect consequence of PKA actions on SOCE.  $\text{Ca}^{2+}$  imaging experiments using Fura-2 revealed a  $34 \pm 5\%$  decrease in the amount of SOCE seen in the presence of 1  $\mu$ M KT5720 (supplementary material Fig. S1), consistent with PKA stimulating SOCE (Hague et al., 2000; Hahn et al., 2000). Thus, the enhanced  $\text{Ca}^{2+}$ -stimulated AC8 activity seen following PKA inhibition was unlikely to be due to indirect actions of KT5720 on  $\text{Ca}^{2+}$  entry. In fact, the inhibitory effects of KT5720 on SOCE potentially muted the stimulatory effects of PKA inhibition on AC8 activity. A selective PKC inhibitor, chelerythrine chloride (CTC), was tested to determine whether PKC targeted to AKAP79 (Coghlan et al., 1995; Klauck et al., 1996) might affect AC8 activity. Pretreatment of HEK-AC8 cells with 1  $\mu$ M CTC did not affect SOCE-induced AC8 activity (Fig. 1C,D). Thus, although AKAP79 potentially recruits PKC to the site of AC8 activity, this kinase does not mediate the inhibitory effects inferred from the AC8-AKAP79 interaction.

To address the requirement for PKA-AKAP binding, two CFP-tagged mutants of AKAP79, unable to bind PKA, were useful tools. The mutant 79PKA<sup>Pro2</sup>-CFP cannot bind the RII subunit of PKA due to disruption of the helical structure of the PKA binding site (Oliveria et al., 2007) and AKAP79 $\Delta$ PKA-CFP completely lacks the PKA-binding domain (Oliveria et al., 2003). Both 79PKA<sup>Pro2</sup>-CFP and AKAP79 $\Delta$ PKA-CFP targeted appropriately to the plasma membrane, as seen by confocal imaging (Fig. 1G). However, unlike HA- or CFP-tagged wild-type AKAP79, overexpression of 79PKA<sup>Pro2</sup>-CFP and AKAP79 $\Delta$ PKA-CFP did not modulate AC8 activity, (Fig. 1E,F). Competition of the overexpressed AKAP79

mutants for AC8 binding did not, however, completely reverse the actions of AKAP79 to yield an increase in cAMP production relative to control cells. This may be due to incomplete displacement of the highly expressed wild-type AKAP79 from its site of AC8 interaction. Nevertheless, this observation is consistent with the attenuation of AC8 activity being mediated by PKA directly bound to the C-terminal region of AKAP79.

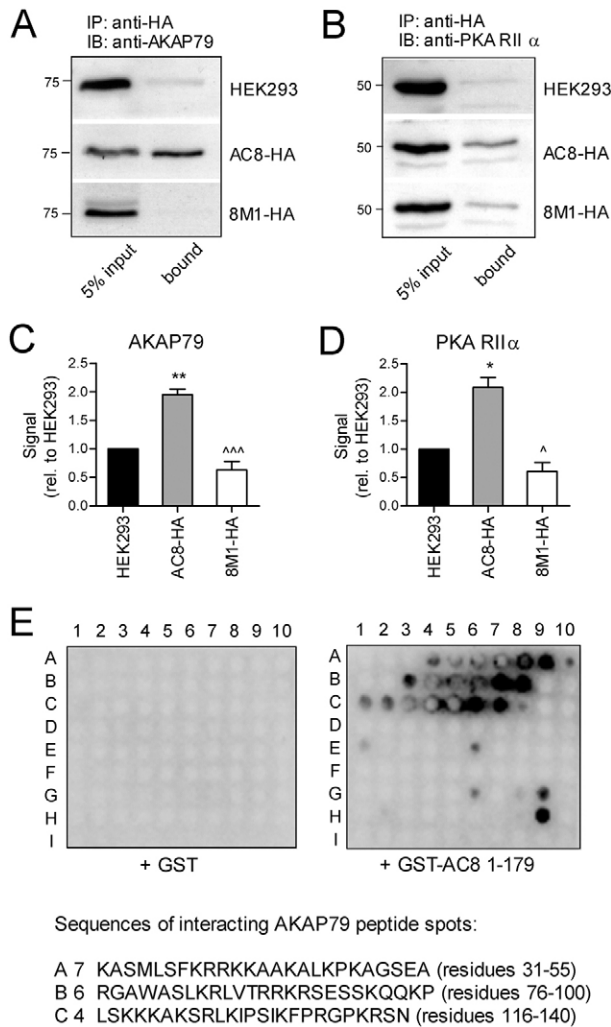
### The polybasic regions of AKAP79 interact with the N-terminus of AC8

Recent work from our group provided the first evidence of AKAP79/150 interaction with AC8 (Willoughby et al., 2010). Here, targeting of both AKAP79 (Fig. 2A) and its associated PKA regulatory subunits (Fig. 2B) to the N-terminus of AC8 was confirmed by co-immunoprecipitation; endogenous AKAP79 and PKA RII $\alpha$  were associated with wild-type AC8, but absent from an N-terminally truncated form of AC8 lacking the first 106 residues (8M1-HA, Fig. 2C,D). The 8M1 form of AC8 displays a similar  $\text{Ca}^{2+}$  sensitivity to wild type in *in vitro* assays but is insensitive to stimulation via SOCE in the intact cell (Smith et al., 2002).

Peptide array methods were utilised to identify the specific regions of AKAP79 that mediated the interaction with the N-terminus of AC8. A library of overlapping peptides (25-mers) covering the entire sequence of AKAP79 (427 residues in total) were immobilised on cellulose membranes and probed for interactions with GST alone and GST-AC8 1-179 (full-length AC8 N-terminus) (Fig. 2E). Positive interactions (represented by dark spots) were seen between GST-AC8 1-179 and peptide spots A7-9, B6-9 and C4-6 (Fig. 2E). These spots correspond to polybasic region A (residues 31-52), region B (residues 76-101) and region C (residues 116-145), which have already been shown to mediate targeting of AKAP79 to the plasma membrane (Dell'Acqua et al., 1998). Recently, palmitoylation of regions A and C have been shown to play a key role in targeting AKAP79 more specifically to the cholesterol- and sphingolipid-rich raft regions of the plasma membrane and to dendritic recycling endosomes (Delint-Ramirez et al., 2011; Keith et al., 2012).

### PKA acts independently of PP2A subunits associated with the N-terminus of AC8

Previous work from our laboratory identified an interaction between PP2A scaffolding and catalytic subunits (PP2A<sub>A</sub> and PP2A<sub>C</sub>) and the N-terminus of AC8 (Crossthwaite et al., 2006). The interaction between AC8 and PP2A<sub>C</sub> was found to be antagonised by  $\text{Ca}^{2+}$ /CaM due to over-lapping but non-identical binding domains within the N-terminus of AC8 (Crossthwaite et al., 2006). However, the physiological significance of the interaction between AC8 and PP2A, a downstream regulator of cAMP signalling, is not known. Because PKA can potentially phosphorylate and activate some forms of PP2A, in particular PP2A complexes incorporating the B56 $\delta$  regulatory subunit (Ahn et al., 2007), we considered that PP2A might provide the means for the PKA effects on AC8 regulation. GST-pulldowns confirmed a  $\text{Ca}^{2+}$ /CaM-sensitive association of PP2A<sub>C</sub> with the N-terminal cytosolic domain of AC8, GST-AC8 1-179 (supplementary material Fig. S2A). This interaction with the AC8 N-terminus was confirmed by HA-affinity co-immunoprecipitation with PP2A<sub>C</sub> subunits in lysate from cells expressing full-length AC8-HA but not in cells expressing N-terminally truncated 8M1-HA (supplementary material Fig.



**Fig. 2. Interaction between the N-terminal domain of AC8 and AKAP79.** (A,B) AC8-HA and 8M1-HA immune complexes probed for interaction with (A) AKAP79 and (B) PKA-RII $\alpha$  in HEK293 cell lysates. 8M1 is an N-terminally truncated form of AC8 lacking the first 106 residues. 100% of the bound samples was loaded in lane 2. (C,D) Densitometry analysis of western blots from co-immunoprecipitations in A and B, respectively. Data represent mean  $\pm$  s.e.m. from three or more similar experiments. \* $P$ <0.05, \*\* $P$ <0.01 compared to untransfected HEK293 cells;  $\wedge$  $P$ <0.05,  $\wedge\wedge$  $P$ <0.001 compared to AC8-HA expressing cells. (E) Peptide array data for overlapping sequences containing 25 residues from regions of AKAP79 probed for interaction with GST alone and GST-tagged AC8 N-terminus. Protein binding was detected using anti-GST and secondary horseradish-peroxidase-conjugated anti-rabbit antibodies. Positive interactions were seen for peptide spots A7, B6 and C4, corresponding to polybasic regions 'A', 'B' and 'C' of AKAP79 (Dell'Acqua et al., 1998). Data are representative of three experiments.

S2B). A selective Ca<sup>2+</sup>/CaM-sensitive interaction between AC8 N-terminus and PP2A B56 $\delta$  regulatory subunits was also observed using GST-pull-downs (Fig. 3A) suggesting that this PP2A subunit bound AC8 close to sites of Ca<sup>2+</sup>/CaM interaction.

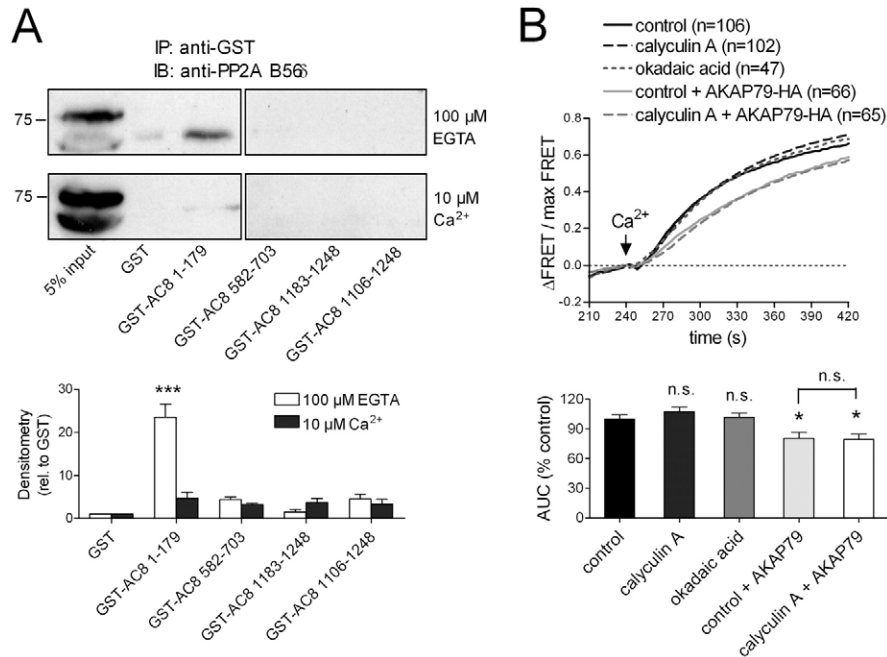
To determine whether PKA was indirectly regulating AC8, via phosphorylation of the B56 $\delta$  subunit associated with the N-terminus, we examined the effects of pharmacological inhibition of PP2A on SOCE-stimulated AC8 activity (Fig. 3B). Treatment with either calyculin A (100 nM) or okadaic acid (100 nM), both potent PP2A inhibitors, had no effect on Ca<sup>2+</sup>-stimulated AC8

activity. Furthermore, the inhibitory effects of AKAP79-HA overexpression on AC8 were insensitive to PP2A inhibition. Thus, although AC8 interacts with PP2A subunits, any such action does not mediate the inhibitory effects of PKA on AC8. The inability of calyculin A, an inhibitor of both PP2A and protein phosphatase 1 (PP1), to modulate AC8 activity indicates that the recently identified interaction between AKAP79 and PP1 (Le et al., 2011) is also not involved in the inhibitory actions of AKAP79 reported here.

#### Identification of target serines for direct PKA-mediated phosphorylation of AC8

PKA is known to directly attenuate the activity of Ca<sup>2+</sup>-inhibited ACs (AC5/6) (Chen et al., 1997; Iwami et al., 1995) but there are no reports to date of PKA regulating Ca<sup>2+</sup>-stimulated ACs such as AC8. To establish if AKAP79-targeted PKA could theoretically phosphorylate AC8, sequence analysis of AC8 was performed using NetPhos software (see Materials and Methods). This analysis identified six potential PKA phosphorylation sites within the cytosolic regions of AC8: Ser-66, Ser-112, Ser-178, Ser-611, Ser-852 and Ser-1120. Site-directed mutagenesis was used to replace each of the six serines with alanines to determine whether any of the predicted PKA phosphorylation sites were required to mediate the attenuating actions of AKAP79 on AC8 activity (Fig. 4A). PKA phosphorylation mutants containing single serine to alanine substitutions (S66A; S112A; S178A; S611A; S852A; S1120A) were tested alongside wild-type AC8 in HEK293 cells to assess any effects of the serine mutations on SOCE-stimulated AC8 activity (Fig. 4B–H). To ensure that effects on the amplitude of cAMP accumulation were not due to variable expression of the AC8 mutants (supplementary material Fig. S3A), we compared Ca<sup>2+</sup>-dependent stimulation of each AC8 construct in the presence and absence of PKA inhibition (1  $\mu$ M KT5720). All experiments were carried out in the presence of 100  $\mu$ M IBMX to discount any indirect effects of KT5720 on PKA-dependent PDE activity. For these experiments, just 0.5 mM Ca<sup>2+</sup> was used to induce SOCE to avoid possible saturation of the Epac2-camps cAMP sensor.

Since inhibition of PKA increased Ca<sup>2+</sup>-stimulated activity of wild-type AC8 (Fig. 1C; Fig. 4B), due to reversal of the attenuating actions of endogenous AKAP79-targeted PKA, a loss of sensitivity to KT5720 was interpreted as disruption of a key PKA phosphorylation site within AC8. Comparing the various single mutations, Ser-112, and to a lesser extent Ser-66, seemed likely targets for PKA phosphorylation (Fig. 4C,D,J). Ser-178, Ser-611, Ser-852 and Ser-1120 did not appear to mediate any actions of PKA on AC8 (Fig. 4E–H,J). AC8 mutants containing multiple serine to alanine substitutions (S66,112,1120A; S66,112,178,1120A; S66,112,178,852,1120A and S66,112,178,611,852,1120A – referred to as S/A<sup>[6]</sup>) were also tested in HEK293 cells and exhibited similar loss of sensitivity to PKA with respect to SOCE-induced activity as that seen with the single S112A mutant (Fig. 4I,J; supplementary material Fig. S3B–D). A maximal loss of sensitivity to PKA following the mutation of Ser-112 alone suggested that this was a key residue mediating the effects of PKA on Ca<sup>2+</sup>-stimulated AC8 activity. This conclusion was supported by a loss of the effect of AKAP79 overexpression in cells expressing either S112A or S/A<sup>[6]</sup> (AC8 mutated in all six putative phosphorylation sites) (supplementary material Fig. S4). Generation of a



**Fig. 3. Interaction of PKA-sensitive PP2A B56 $\delta$  subunit with the N-terminus of AC8.** (A) GST pull-downs using whole-cell lysate from HEK293 cells and GST-tagged cytosolic regions of AC8 were probed for interaction with PP2A B56 $\delta$  subunits. Lane 1 is 5% input and lane 2 represents GST alone. Lanes 3–6 use the following regions of AC8 fused to GST: 1–179 (N-terminus containing an amphipathic CaM-binding domain), 582–703 (C1b domain), 1183–1248 (C2b domain containing an ‘IQ-like’ CaM-binding domain) and 1106–1248 (entire C-terminus containing the C2a catalytic domain (residues 1106–1182) and terminal C2b region). 100% of the samples for each GST-pull-down were loaded. Interactions are compared in Ca<sup>2+</sup>-free conditions (100  $\mu$ M EGTA) and in the presence of 10  $\mu$ M Ca<sup>2+</sup>. \*\*\* $P$ <0.001. (B) Effects of PP2A inhibitors calyculin A (100 nM) or okadaic acid (100 nM) on SOCE-stimulated cAMP production in HEK-AC8 cells. SOCE was induced by the addition of 0.5 mM Ca<sup>2+</sup> at 240 sec; 10 nM forskolin +100  $\mu$ M IBMX were present throughout to basally stimulate AC8 and block PDE activity, respectively. \* $P$ <0.05, n.s., not significant (error bars show the s.e.m.).

phosphomimetic version of AC8 by substitution of Ser-112 with an aspartate rendered this mutant form of AC8 (S112D) insensitive to further potentiation via KT5720 treatment (supplementary material Fig. S5).

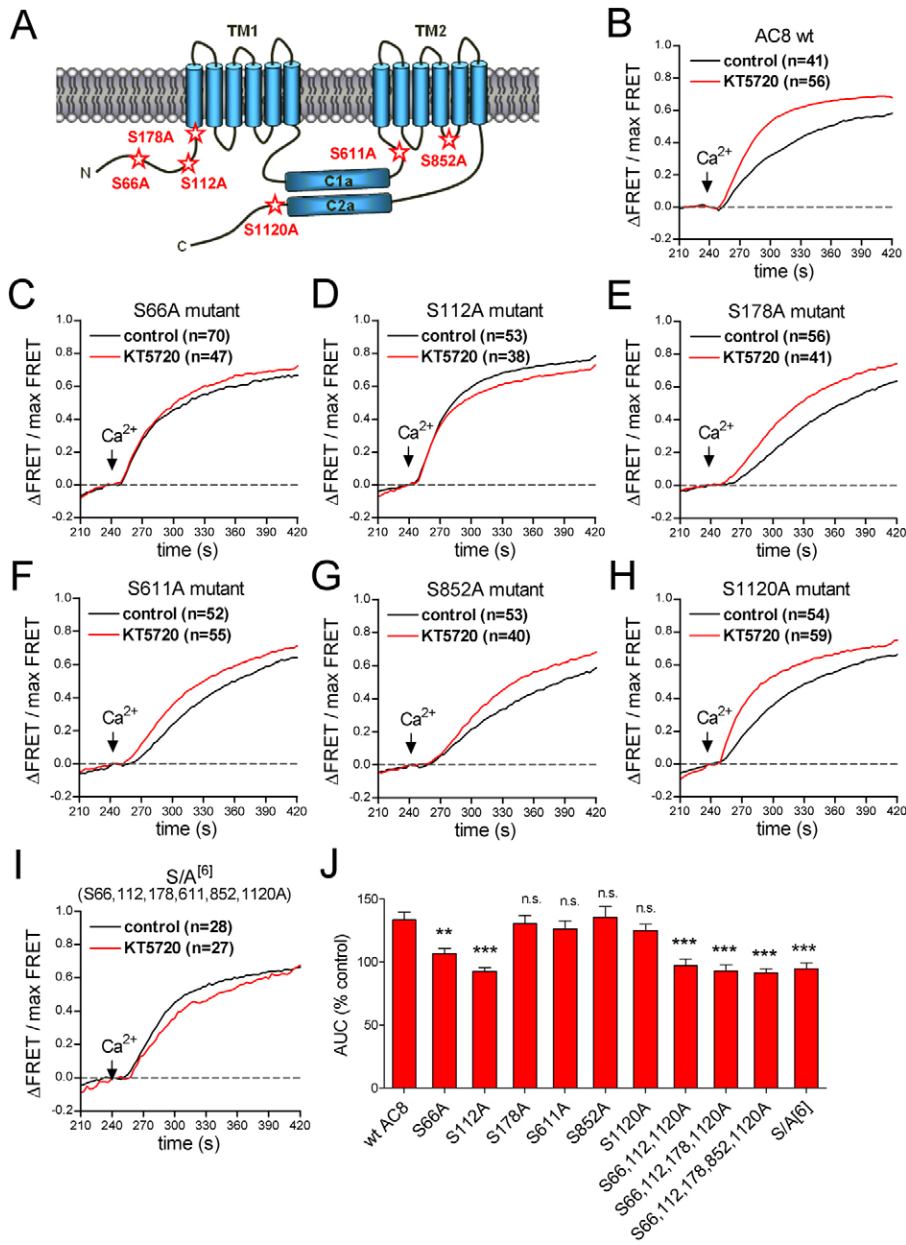
An ongoing premise of these experiments is that phosphorylation of AC8 occurs. Indeed we had shown previously (Delint-Ramirez et al., 2011) that AC8 is phosphorylated *in situ*. We also show presently that the N-terminal 1–179 region can be phosphorylated in an *in vitro* assay (supplementary material Fig. S6A). However, the S112A N-terminal fragment is phosphorylated to a similar extent, which suggests that other PKA-consensus sites, e.g. S66 and S178, obscure the contribution of the S112 residue (supplementary material Fig. S6A).

Given the importance of the S112 residue in the functional studies above, one simple hypothesis might anticipate that the interaction between AKAP79 and the AC8 N-terminus could be compromised in pulldown-type experiments. To test this hypothesis we introduced the phosphomimetic S112D mutation into GST-AC8 1–179 and compared its ability with that of wild-type GST-AC8 1–179 to pulldown overexpressed AKAP79-HA from HEK293 cell lysate. In fact no difference was detectable in this assay (supplementary material Fig. S6B). Of course this result does not imply that the interaction is not important, merely that other residues also reinforce the interaction between AC8 1–179 and AKAP79.

#### AKAP79–PKA association impacts on dynamic AC8-mediated cAMP signals

The high sensitivity of AC8 to Ca<sup>2+</sup> bestows an important ability for this AC isoform to generate cAMP oscillations during dynamic intracellular Ca<sup>2+</sup> signalling (Willoughby and Cooper, 2006). Such cAMP oscillations have a frequency of ~1–3 cAMP transients min<sup>-1</sup> and are proposed to help mediate the diverse cellular actions of cAMP (Dyachok et al., 2006; Willoughby and Cooper, 2006). As cells expressing endogenous AC8 also express

high levels of AKAP79/150 (Cali et al., 1994; Delmeire et al., 2003; Nauert et al., 2003; Ostroveanu et al., 2007) and are subject to oscillatory Ca<sup>2+</sup> events (Garaschuk et al., 1998; Thorn and Petersen, 1993) the functional interaction between AC8 and AKAP79 described here during Ca<sup>2+</sup> entry (Figs 1 and 4) is also likely to affect more dynamic cAMP signalling events. To explore this possibility, a series of three repeated Ca<sup>2+</sup> transients were imposed on HEK-AC8 cells expressing Epac2-camps to monitor real-time cAMP changes (Fig. 5). All cells were pretreated with 200 nM Tg in the absence of extracellular Ca<sup>2+</sup> to deplete the intracellular Ca<sup>2+</sup> stores, and 10 nM Forskolin was added to enhance basal AC8 activity. Periodic switching of the bath solution each minute from saline containing either 1.3 mM or zero CaCl<sub>2</sub> induced a repeated rise and fall in cAMP levels in control HEK-AC8 cells (Fig. 5A,B) as reported previously (Willoughby and Cooper, 2006). Cells overexpressing AKAP79-HA or AKAP79-CFP exhibited a marked decrease in the peak on-rate of Ca<sup>2+</sup>-evoked cAMP dynamics compared to control cells ( $P$ <0.01, Fig. 4C), and showed reduced amplitudes of response (Fig. 5A). In contrast, selective knockdown of AKAP79 levels or overexpression of the PKA-binding deficient AKAP79 mutant, AKAP79 $\Delta$ PKA-CFP, increased the peak rate of cAMP production by AC8 ( $P$ <0.01, Fig. 5C). We have previously shown that the AKAP79 selective shRNA used here reduces AKAP79 expression levels by at least 80% in HEK293 cells (Willoughby et al., 2006). These data support a downregulating role for the AKAP79–PKA–AC8 interaction with respect to cellular cAMP dynamics. Furthermore, we could confirm that reduction in the rate of cAMP production was mediated via PKA phosphorylation of AC8 at Ser-112 (Fig. 5B,C). Thus, the attenuating actions of overexpressed AKAP79 are mimicked by the phosphomimetic S112D mutant ( $P$ <0.01), and the on-rate is enhanced in phosphorylation-deficient AC8 mutants (S112A and S/A<sup>[6]</sup>;  $P$ <0.01 compared to wild-type controls; Fig. 5B,C). Mutation of S66A, which exhibited partial loss of sensitivity to PKA inhibition (Fig. 4C),



**Fig. 4. Identification of Ser-112 as a target for PKA phosphorylation.** (A) Predicted PKA phosphorylation sites in AC8. Serines were mutated to alanines to examine the contribution of each site to PKA-mediated inhibition of AC8 activity. (B–I) cAMP changes in HEK293 cells expressing wild-type AC8 or potential PKA phosphorylation deficient AC8 constructs during SOCE (induced by addition of 0.5 mM  $\text{Ca}^{2+}$  at 240 sec)  $\pm$  PKA inhibitor KT5720 (1  $\mu\text{M}$ ). 10 nM forskolin +100  $\mu\text{M}$  IBMX were present throughout to basally stimulate the AC8 constructs and to preclude any effects of PKA inhibition on PDE activity. n values are in parentheses. (J) Analyses of data ( $\pm$ s.e.m.) presented in B–I reveal loss of the stimulatory effects of KT5720 in mutants containing the S112A mutation, and to a lesser extent the S66A mutation. \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , n.s., not significant.

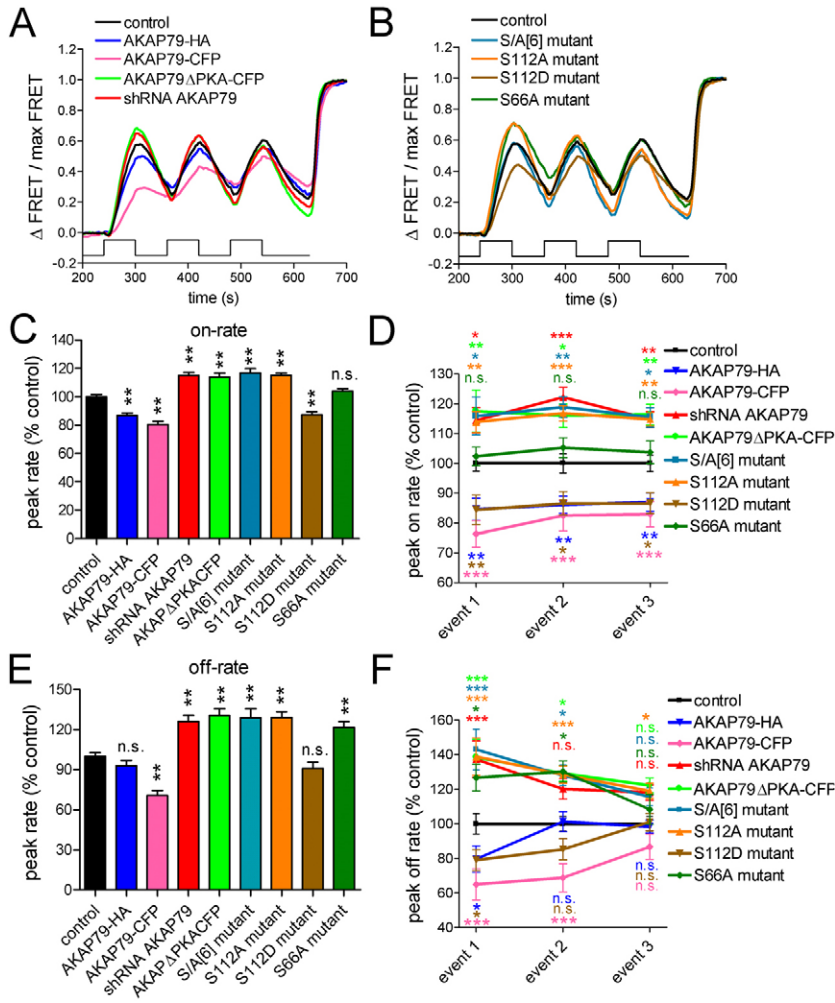
did not alter the peak on-rate of cAMP production compared to wild-type AC8 controls (Fig. 5B,C). The contribution of AKAP79-targeted PKA to the reduced on-rate of AC8 activity remained constant during all three  $\text{Ca}^{2+}$  events (Fig. 5D).

Analyses of the pooled off-rates (due to cAMP hydrolysis) of the transient cAMP signals under the various experimental conditions revealed that disruption of AKAP79–PKA actions on AC8 also enhanced the peak off-rates of cAMP signals ( $P < 0.01$  compared to wild-type controls; Fig. 5E). Although S66A did not enhance the on-rate of cAMP production, the peak off-rate was significantly increased compared to wild-type controls ( $P < 0.01$ ; Fig. 5E). However, the effect of overexpressed AKAP79, or the phosphomimetic S112D mutant, on off-rate was less clear (Fig. 5E). This is most likely due to any initial influence of AKAP79–PKA on the off-rates of AC8 (Fig. 5F) being obscured by activation of cAMP-dependent PDEs (especially PDE4

isoforms) which contribute substantially to the generation of  $\text{Ca}^{2+}$ -dependent cAMP oscillations in HEK-AC8 cells (Willoughby and Cooper, 2006).

## Discussion

Growing evidence shows that ACs can function as central components of macromolecular signalling complexes targeted to specific receptors or ion channels, where they are exposed to the highly localised activities of other scaffolded signalling molecules that function downstream of cAMP (Dessauer, 2009; Willoughby et al., 2012). AKAP79/150 is a prototypical AKAP in that it contains an amphipathic  $\alpha$ -helix that binds PKA RII subunits, it also can be targeted to discrete regions of the cell, and can interact with multiple signalling molecules. In addition to binding PKA, PKC, PP2B (CaN) and PP1 (Carr et al., 1992; Coghlan et al., 1995; Klauck et al., 1996; Le et al., 2011). AKAP79 interacts with



**Fig. 5. Contribution of AKAP79-targeted PKA activity to AC8-mediated cAMP dynamics.** (A) cAMP changes in HEK-AC8 cells during three SOCE events evoked by switching the bath solution from 0 to 1.3 mM  $\text{Ca}^{2+}$  at 1 min intervals (see bottom trace). The effects of AKAP79-HA, AKAP79-CFP and AKAP79 $\Delta$ PKA-CFP expression, or AKAP79 knockdown, are compared to control (wild-type AC8). (B) Effects of repeated SOCE events on PKA phosphorylation mutants (S/A<sup>[6]</sup>, S112A, S66A) and the phosphomimetic mutant (S112D). (S/A<sup>[6]</sup> refers to the mutant S66,112,178,611,852,1120A.) (C) Peak rate of cAMP rise relative to AC8 control, analysed from data in A,B. \*\* $P$ <0.01,  $n$  values range from 27–57 cells, n.s., not significant. (D) Comparison of peak on-rates relative to control AC8 data over three successive SOCE events. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001, n.s., not significant. (E) Peak rate of cAMP decrease relative to AC8 control, analysed from data in A,B. \*\* $P$ <0.01,  $n$  values range from 27–57 cells, n.s., not significant. (F) Comparison of peak off-rates relative to control AC8 data over three successive SOCE events. \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001; n.s., not significant.

numerous receptors and ion channels in neuronal systems (Colledge et al., 2000; Oliveria et al., 2007; Oliveria et al., 2003; Smith et al., 2006). Direct interactions between AKAP79/150 and  $\text{Ca}^{2+}$ -sensitive AC isoforms have also been reported. AKAP79/150 interaction with  $\text{Ca}^{2+}$ -inhibitable AC5/6 facilitates PKA-mediated phosphorylation of the ACs to suppress cAMP production (Bauman et al., 2006), and can also target AC5/6 to AMPA receptors to optimise their phosphorylation upon the activation of Gs-coupled receptors within the signalling complex (Efendiev et al., 2010).

We recently reported a direct interaction between AKAP79/150 and  $\text{Ca}^{2+}$ -stimulable AC8 that attenuates  $\text{Ca}^{2+}$ -dependent production of cAMP in pancreatic and hippocampal cells (Willoughby et al., 2010) where both proteins are highly expressed (Cali et al., 1994; Delmeire et al., 2003; Nauert et al., 2003; Ostroveanu et al., 2007). Here we provide evidence to suggest that the interaction occurs between the N-terminus of AC8 and key membrane-targeting polybasic sequences within the N-terminus of AKAP79, referred to as regions A, B and C (Dell'Acqua et al., 1998). Region B of AKAP79 has also been identified as a likely site of AKAP79 interaction with the N-termini of AC5 and AC6 (Efendiev et al., 2010), despite these regions sharing little homology with the N-terminus of AC8.

In hippocampal neurons both AKAP79 and AC8 are selectively targeted to dendritic regions and have been

identified as key proteins in the induction of LTP (Ostroveanu et al., 2007; Smith et al., 2006; Wang et al., 2003; Zhang et al., 2008), with loss of expression of either protein being linked to deficits in memory (Tunquist et al., 2008; Wang et al., 2003; Zhang et al., 2008). Identifying the precise consequences of the interaction between these two key signalling molecules could provide essential insights into their role in hippocampal and pancreatic function.

Here we reveal an inhibitory effect of AKAP79-targeted PKA on  $\text{Ca}^{2+}$ -stimulated AC8 activity that is mediated via selective phosphorylation of a serine residue within the cytosolic N-terminus of AC8. This provides the first evidence that AC8, primarily regulated by a well-defined interaction with  $\text{Ca}^{2+}$ -CaM (Willoughby and Cooper, 2007), is also sensitive to phosphorylation by PKA. Although phosphorylation of the relevant N-terminal domain could be demonstrated in *in vitro* assays (supplementary material Fig. S6A), attempts to measure changes in phosphorylation of specific residues in AC8-HA were considered too challenging technically due to the lability of phosphorylation of large membrane proteins during extraction. Nevertheless, the functional consequences of mutating consensus PKA phosphorylation sites make the inference of direct phosphorylation events most likely.

We assessed the contribution of six consensus PKA phosphorylation sites identified within the cytosolic domains of

AC8 and found that alanine substitution of Ser-112, and to a lesser extent Ser-66, eliminated the PKA-mediated attenuation of  $\text{Ca}^{2+}$ -dependent AC8 activity. Our study used rat AC8 cDNA, but the phosphorylation consensus sequence incorporating Ser-112 is conserved in human AC8. Ser-66 is also present in human AC8, but Arg-63 is replaced by a glycine, which reduces the likelihood of PKA phosphorylation since the arginine residue is needed to form a consensus phosphorylation sequence. Interestingly, Ser-112 is located within the N-terminus of AC8, the proposed site for AKAP79 interaction (Willoughby et al., 2010; Fig. 2), thus ensuring the adjacency of PKA to its site of action. However GST-pulldowns using a phosphomimetic mutation (supplementary material Fig. S6B) suggest that phosphorylation of S112 is unlikely to dramatically influence the interaction between AKAP79 and AC8, which is likely to be mediated by multiple residues. PKA-dependent phosphorylation has previously been documented to affect basal activity and  $G_{\alpha s}$  stimulation of the  $\text{Ca}^{2+}$ -inhibited AC5 and AC6 (Chen et al., 1997; Iwami et al., 1995). In contrast, the current study suggests that PKA significantly reduces the stimulation of AC8 by  $\text{Ca}^{2+}/\text{CaM}$ .

The effect of AKAP79-PKA on AC8-mediated cAMP signalling during imposed  $\text{Ca}^{2+}$  oscillations may be most revealing of an elegant cellular mechanism for regulating the localised and transitory actions of cAMP under more physiological conditions. Specifically, AKAP79-targeted PKA reduced the on-rate of cAMP production during  $\text{Ca}^{2+}$  entry. In contrast, AC8 phosphorylation mutants (S112A and S/A<sup>[6]</sup>) displayed increased rates of cAMP production compared to wild-type AC8 controls. Although AKAP79-targeted PKA also appeared to limit the subsequent off-rate of AC8-mediated cAMP signalling (presumably via phosphorylation at Ser-112 of AC8) any contribution was temporary and appeared to be overwhelmed by PDE4 activation (Willoughby and Cooper, 2006). The 'dampening' effects of AKAP79 on AC8 activity are likely to have significant impact on various cellular processes that depend on cAMP oscillations, such as axon guidance (Nicol et al., 2007) or gene transcription (Haisenleder et al., 1992). Thus, it would appear that feedback inhibition by targeted PKA regulation of AC8 may be a valuable fine-tuning device for the downstream consequences of AC8 activation evoked by bursts of  $\text{Ca}^{2+}$  activity. This modulation is likely to have significant impact on the activity of numerous cellular proteins, including those interacting with the scaffold protein AKAP79 such as  $\text{Ca}^{2+}$  channels (Oliveria et al., 2007) and glutamate receptors (Gomez et al., 2002), which may themselves promote  $\text{Ca}^{2+}$ -dependent activation of AC8 (Chetkovich et al., 1991).

## Materials and Methods

### Cell culture and transfection

HEK293 cells (European Collection of Cell Cultures, Porton Down, UK) were grown in minimum essential medium supplemented with 10% (v/v) foetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine and maintained at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$ , 95% air.

To produce stably expressing HEK-AC8 cells, wild-type HEK293 cells were plated on 100 mm dishes at ~60% confluency 1 day prior to transfection with 2 µg of rat AC8 using the Lipofectamine 2000 transfection method. Two days later the culture medium was replaced with fresh medium containing 800 µg/ml G-418 disulphate (Formedium Ltd, Hunstanton, UK) to select transfected cells. After selection, cells were maintained in medium containing 400 µg/ml G-418. Stable AC8 expressing HEK293 cells were established from ~2 weeks following transfection.

For single-cell cAMP measurements cells were plated onto 25 mm poly-L-lysine coated coverslips at ~60% confluency 24 hr prior to transient transfection with 1 µg of total cDNA (0.5 µg each, if transfecting two constructs e.g. Epac2-camps and AKAP). For co-immunoprecipitation assays, cells were plated onto

100 mm dishes at ~40% confluency 1 day prior to transient transfection with 2 µg of AC8-HA or 8M1-HA cDNA using the Lipofectamine 2000 method. All experiments were carried out 48–72 hr post transfection.

### Epac2-camps FRET measurements

Fluorescent imaging of Epac2-camps expressing HEK293 or HEK-AC8 cells was performed using an Andor Ixon+ EMCCD camera and an Optosplit (S05DC) to separate CFP (470 nm) and YFP (535 nm) emission images (Cairn Research, Kent, UK). For dual emission ratio imaging cells were excited at 435 nm using a monochromator (Cairn Research) and 51017 filter set (Chroma Technology Corp. VT, USA) attached to a Nikon eclipse TE2000-S microscope (×40 oil immersion objective). Emission images at 470 nm and 535 nm were collected every 3 sec (250 ms integration time) and analysed using Metamorph imaging software (Molecular Devices). Cells in which the CFP and YFP fluorescence intensity was less than twice the background signal were excluded, as were cells with excessive expression of the fluorescent probe. Single-cell FRET data were plotted as changes in background subtracted 470 nm versus 535 nm (CFP/YFP or CFP/Citrine) emission ratio relative to maximum FRET ratio change seen with saturating cAMP concentrations (seen upon addition of a cocktail containing 10 µM forskolin, 10 µM isoproterenol and 100 µM IBMX).

### Fura-2 $\text{Ca}^{2+}$ measurements

Cells were plated onto 25 mm poly-L-lysine coated coverslips 24 hr prior to loading with 4 µM Fura-2/AM and 0.02% Pluronic F-127 (Molecular Probes, Leiden) for 40 min at room temperature in extracellular buffer containing (mM): 140 NaCl, 4 KCl, 1  $\text{CaCl}_2$ , 0.2  $\text{MgCl}_2$ , 11 D-glucose, 10 HEPES, pH 7.4. After loading, cells were washed several times and then imaged using an Andor Ixon+ EMCCD camera (Andor, Belfast, UK) and monochromator system (Cairn Research, Kent, UK) attached to a Nikon Eclipse TE2000-S microscope (×40 objective). Emission images (ET510/80M) at 340 nm and 380 nm excitation were collected at 1 Hz using MetaFluor software (Molecular Devices). For zero calcium buffers, the constituents were the same as for extracellular buffer but  $\text{CaCl}_2$  was omitted and replaced by 0.1 mM EGTA.

### Confocal imaging

Live cell confocal images were obtained using an inverted Zeiss LSM 510 confocal microscope with an oil immersion 63×PlanApo objective (NA 1.4). CFP-tagged AKAP constructs were excited at 458 nm and emission collected using a 505–550 BP filter.

### GST pulldown assays

Confluent 100 mm dishes of HEK293 cells were lysed in GST-Fish buffer (10% (v/v) glycerol, 100 mM NaCl, 50 mM Tris pH 7.4; supplemented with 0.5% Tween-20 or 0.3% NP40, 100 µM EGTA, 2 mM DTT, 1 mM PMSF, 1 mM benzamide, protease inhibitors, 10 mM β-glycerophosphate and 2 mM sodium orthovanadate) by rotating for 30 min at 4°C, before centrifugation (16,000 g, 4°C, 15 min). The cell lysate was incubated with the appropriate GST-fragment for 4 hr at 4°C with rotation. GST-beads were washed twice in GST-Fish buffer, and bound proteins were eluted by adding an equivalent volume of 2×Laemmli buffer and boiled for 5 min prior to western blot analysis.

### Co-immunoprecipitation

HEK293 cells expressing AC8-HA or 8M1-HA were washed with PBS and lysed in solubilisation buffer (50 mM Tris pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.3% (v/v) NP40, and protease inhibitors) by repeatedly passing the cell suspension through a 21-gauge needle. The crude cell lysate was then centrifuged (200 g, 4°C, 5 min) to remove cellular debris. The cell lysates were rotated with 100 µl pre-washed bead slurry (50%) for 4 hr at 4°C. Beads were washed five times in NP40 solubilisation buffer, and proteins were eluted with NP40 solubilisation buffer supplemented with 1% (w/v) SDS. Laemmli buffer was added to the elution and incubated at 37°C for 30 min prior to western blot analysis.

### In vitro phosphorylation assay

GST fragments on beads were incubated with 200 µM ATP, 625 units of PKA catalytic subunit (New England Biolabs) and 1×PKA buffer containing 10 mM  $\text{MgCl}_2$  (New England Biolabs) at 25°C for 30 min with shaking. The beads were washed three times in PBS containing 0.1% Triton ×100. Bound proteins were eluted by adding an equivalent volume of 2×Laemmli buffer and boiled for 5 min prior to western blot analysis.

### Western blot analysis

Proteins were resolved using 8% or 10% SDS-polyacrylamide gels. Separated proteins were then transferred to a supported nitrocellulose membrane. Nitrocellulose membranes were then blocked in TBS (20 mM Tris, pH 7.5, 150 mM NaCl) containing 5% (w/v) skimmed milk, for 1 hr, followed by three 5 min washes in TBS supplemented with 0.05% (v/v) Tween-20 (TTBS). Membranes were incubated overnight at 4°C with anti-HA antibody (1:5000;



Sigma), anti-AKAP79 antibody (1:5000; BD Biosciences), anti-PKA RII $\alpha$  antibody (1:2000, BD Biosciences), anti-PP2Ac antibody (1:2000; BD Biosciences), anti-PP2A B56 $\delta$  (1:500; Abcam), anti-phospho-(Ser/Thr)PKA substrate (1:2000, Cell Signaling) or anti-GST antibody (1:40,000; Sigma) in TBS containing 1% (w/v) skimmed milk and 0.02% (v/v) sodium azide. Membranes were washed (3 $\times$ 5 min) in TTBS and then incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (1:10,000 for anti-AKAP79, anti-PKA RII $\alpha$ , anti-PP2Ac and anti-PP2A B56 $\delta$  antibodies; and 1:20,000 for anti-HA and anti-GST antibodies) or goat anti-rabbit IgG conjugated to horseradish peroxidase [1:10,000 for anti-phospho-(Ser/Thr)PKA substrate] in TBS containing 5% (w/v) skimmed milk for 1 hr. Finally, the membrane was washed three times in TTBS (5 min) and once in TBS (5 min), visualised with ECL Plus reagent (GE Healthcare) according to the manufacturer's protocol, and exposed to film. Immunoreactive bands were quantified by densitometry using ImageJ.

### Constructs

Potential PKA phosphorylation sites in AC8 were identified using NetPhos 2.0 (Blom et al., 1999). Mutations were introduced into pcDNA3 AC8 using site-directed mutagenesis. Primers are listed in supplementary material Table S1.

### Peptide array analysis

The entire length of AKAP79 was synthesised as overlapping peptide spots (25 mers, shift 5). Peptide SPOT arrays were generated by automated SPOT synthesis as described previously (Coin et al., 2007; Hundsruker et al., 2006; Stefan et al., 2007). To assay the binding of GST or GST-tagged AC8 N-terminal fusion protein, peptide spot membranes were blocked for 1 hr in blocking buffer (3% BSA in TTBS) before incubation with 0.02  $\mu$ M protein (GST, GST-8NT 1–179) overnight at 4°C. Detection was carried out as described previously (Hundsruker et al., 2010).

### Data analysis

Data are expressed as mean  $\pm$  s.e.m. Statistical significance was assessed with the unpaired Student *t*-test or by one-way ANOVA with post-hoc analysis using the Newman-Keuls multiple comparisons tests and GraphPad Prism software. A *P*-value of less than 0.05 was considered statistically significant.

### Acknowledgements

AKAP79-CFP, AKAP79 $\Delta$ PKA-CFP and AKAP79<sup>Pro2</sup>-CFP were gifts from Mark Dell'Acqua (University of Colorado, Aurora). AKAP79 shRNA was a gift from John Scott (University of Washington, Seattle). DMFC is a Royal Society Wolfson Research Fellow.

### Funding

This work was supported by the Wellcome Trust [grant number RG31760]. Deposited in PMC for release after 6 months.

Supplementary material available online at

<http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.111427/-/DC1>

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