Mechanisms for restraining cAMP-dependent protein kinase revealed by subunit quantitation and cross-linking approaches

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Protein phosphorylation by cyclic AMP-dependent protein kinase (PKA) underlies key cellular processes, including sympathetic stimulation of heart cells, and potentiation of synaptic strength in neurons. Unrestrained PKA activity is pathological, and an enduring challenge is to understand how the activity of PKA catalytic subunits is directed in cells. We developed a light-activated cross-linking approach to monitor PKA subunit interactions with temporal precision in living cells. This enabled us to refute the recently proposed theory that PKA catalytic subunits remain tethered to regulatory subunits during cAMP elevation. Instead, we have identified other features of PKA signaling for reducing catalytic subunit diffusion and increasing recapture rate. Comprehensive quantitative immunoblotting of protein extracts from human embryonic kidney cells and rat organs reveals that regulatory subunits are always in large molar excess of catalytic subunits (average ~17-fold). In the majority of organs tested, type II regulatory (RII) subunits were found to be the predominant PKA subunit. We also examined the architecture of PKA complexes containing RII subunits using cross-linking coupled to mass spectrometry. Quantitative comparison of cross-linking within a complex of RII β and C β , with or without the prototypical anchoring protein AKAP18 α , revealed that the dimerization and docking domain of RII β is between its second cAMP binding domains. This architecture is compatible with anchored RII subunits directing the myristylated N terminus of catalytic subunits toward the membrane for release and recapture within the plane of the membrane.

cAMP | protein kinase | cross-linking | XL-MS | protein structure

Protein kinase A (PKA), also known as cAMP-dependent protein kinase, is the major intracellular receptor for the second messenger cAMP (1). Activation of PKA by cAMP underlies responses throughout the body, including sympathetic regulation of the heart downstream of β -adrenergic receptor (β -AR) activation (2) and changes in the strength of synaptic connections between neurons (3). PKA consists of regulatory (R) subunit constitutive dimers that sequester catalytic (C) subunits before cAMP activation. There are two types of R subunit. Type II (RII) subunits associate with the low-speed particulate fraction after tissue homogenization, whereas RI subunits do not (4). This results from anchoring of RII but generally, not RI at subcellular sites that include the cell membrane by A-kinase anchoring proteins (AKAPs) (2). Imbalances in the expression or activity of R (5) and C (6) subunits or disruptions in PKA anchoring (7) lead to disease. An enduring challenge in cAMP/PKA research is to understand how PKA is directed to its cellular substrates. Experiments using fluorescent reporters have confirmed that elevations in cAMP concentration and PKA activity are localized within the cell (8, 9), with PKA activity clustering close to anchoring sites (9). However, the mechanism for restraining C subunits after their release is still unclear.

After activation of an R–C complex by cAMP, the range over which the C subunit can phosphorylate substrates will depend on its rate of diffusion and the rate of recapture by R subunits. New potential mechanisms for reducing diffusion and increasing C-subunit recapture have been investigated in recent years.

N-terminal myristylation is thought to restrict some free C subunits to the intracellular face of the membrane bilayer (10), slowing their diffusion velocity (11) and restricting their activity to the plane of the membrane. Consistent with this model, RII but not RI subunits increase binding of myristylated C subunits to liposomes (12) probably by stabilizing the “myr-out” conformation of the myristylated C-subunit A helix (13, 14). Many AKAPs also localize to the cell and organellar membranes (15). AKAPs present amphipathic helices that bind to the dimerization and docking (D/D) domain formed by the first 45 amino acids of RII subunits (16–18). However, the first ~100 amino acids of RII are not visible in the electron density of the most complete crystal structures of RII–C (13, 19), and therefore, it is not clear whether association with membrane-tethered AKAPs is compatible with inserting C subunits into the membrane.

An alternative proposed mechanism for limiting C-subunit diffusion is that C subunits are never released from RII subunits but instead, access nearby substrates while tethered to RII on cAMP activation (20, 21). This theory was supported by experiments showing no effect of β -AR stimulation on C-subunit coprecipitation with anchored RII subunits (20, 21). However, this experiment does not exclude the possibility that R and C subunits reassociate during coimmunoprecipitation after cell lysis. This possibility could not be excluded, as before our study, there has been no method to monitor association of endogenous PKA subunits in cells with temporal precision. Final aspects of PKA that could support rapid R- and C-subunit association are the stoichiometry and concentrations of its subunits. PKA is

Significance

Protein phosphorylation by cAMP-dependent protein kinase (PKA) triggers cellular changes, including fight-or-flight responses in heart cells, and synaptic potentiation in neurons. Uncontrolled activity of PKA catalytic subunits is pathological; however, the mechanism for directing PKA in cells is unclear. Using an approach for monitoring cellular PKA subunit interactions, we show that—contrary to recent proposals—catalytic subunits are released from regulatory subunits by cAMP. Instead, we identify mechanisms for rapid recapture of liberated catalytic subunits. Regulatory subunits are expressed much more highly than catalytic subunits to support rapid catalytic subunit reassociation. Furthermore, analysis of global PKA architecture reveals that type II regulatory subunit anchoring is compatible with catalytic subunit release and recapture within the cell membrane.

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unusual among the protein kinase A, G, C (AGC) group in that its regulatory and catalytic elements are formed by separate polypeptides (22). Binding studies show that increasing the concentration of RI subunits, with fixed C-subunit concentration, increases the fraction of C subunits bound to R subunits, even in high concentrations of cAMP (23). This means that higher R-subunit concentration increases the rate of R–C complex formation irrespective of cAMP concentration. Surprisingly, little information is available regarding the concentrations of endogenous PKA subunits.

We used a three-pronged approach to investigate mechanisms for restraining C subunits. This combined approach has enabled us to more clearly define which mechanisms do—and do not—contribute to restraining C subunits.

Results

Light-Activated Cross-Linking Indicates That C Subunits Dissociate from R Subunits in Cells Following β -AR Stimulation. To investigate the notion that C subunits remain tethered to R subunits in cells during cAMP elevation (20, 21), we developed an approach that uses the UV light-activated cross-linker succinimidyl 4,4'-azipentanoate (SDA). We focused on human embryonic kidney (HEK) cells, which are a model cell line for studying cAMP/PKA signaling (24). An overview of this process is shown in Fig. 1 A–C. Cells are first incubated with membrane-permeable SDA, leading to covalent attachment at primary amines including lysine side chains on RI (red in Fig. 1A), RII (blue in Fig. 1A), and C (green in Fig. 1A) subunits. During 365-nm illumination, the diazirine moiety of SDA may react with nearby molecules, leading to covalent cross-links (Fig. 1B). Since the SDA spacer arm is 3.9 Å, R–C cross-linking is strongly favored when the two

proteins are associated (Fig. 1B). After cell lysis, either RI subunits or RII subunits are precipitated using immobilized GST fusions (Fig. 1C) to the RI-anchoring disruptor (RIAD) sequence (25) or the C-terminal 93 amino acids of RII-selective AKAP79 (AKAP79_{c93}). R-subunit precipitation is performed with cAMP, and therefore, C-subunit coprecipitation only occurs through R–C cross-linking. The efficacy of this approach is shown for HEK293T cells in Fig. 1D. GST alone precipitated neither RI nor RII (Fig. 1D, lanes 2 and 3), whereas GST-RIAD (Fig. 1D, lanes 4 and 5), and GST-AKAP79_{c93} (Fig. 1D, lanes 6 and 7) pulled down either RI or RII, respectively. Control experiments confirmed that GST-RIAD pulls down RI α and RI β with similar efficiency (Fig. S1 A and B). UV exposure led to coprecipitation of covalently linked C subunits, visible as a band at 90 kDa [Fig. 1D, immunoblot (IB):C] corresponding to either 1RI-1C (Fig. 1D, lane 5) or 1RII-1C (Fig. 1D, lane 7) cross-linked heterodimers.

We applied this approach to determine if RII and C subunits remain associated when β -ARs are stimulated with isoproterenol. We incubated SDA-treated HEK293T cells for 5 min with either 1 μ M isoproterenol or vehicle. UV light illumination was either performed immediately or delayed until after cell lysis. Anti-C IB signal intensity at ~90 kDa after RII subunit pull down shows how much C subunit was bound to RII during UV illumination (indicated by the arrow in Fig. 1E). C–RII association was reduced after isoproterenol stimulation (Fig. 1E, lanes 2 and 3) by $78 \pm 7\%$ ($P = 0.0015$, $n = 4$) according to densitometry (Fig. 1F, prelysis). However, C subunits were found to have returned to RII after lysis: when UV light illumination was delayed until postlysis (Fig. 1E, lanes 4 and 5), isoproterenol led to a small increase in RII–C cross-linking ($27 \pm 3\%$, $P = 0.0016$, $n = 4$) (Fig. 1F). Reassociation was abolished when we supplemented lysis buffer with 10 μ M exogenous cAMP 10 min before UV-induced cross-linking postlysis (Fig. S1 C and D). We also performed experiments in which we coupled SDA cross-linking to GST-RIAD pull down. These experiments showed that C subunits also dissociate from RI on isoproterenol stimulation (Fig. S1 E and F). Together, our experiments using SDA cross-linking show that strong β -AR stimulation triggers C-subunit dissociation from both RI and RII subunits in cells and that coprecipitation of C in anti-RII immune complexes after isoproterenol stimulation (20, 21) likely occurs because of dilution of cAMP after cell lysis.

Fig. 1. Light-activated cross-linking of PKA subunits in HEK293T cells. (A) SDA covalently binds to free amine groups through its *N*-hydroxysuccinimide (NHS) moiety, including to lysines presented by RI (red), RII (blue), and C (green) subunits. (B) UV light triggers cross-linking between associated subunits. (C) After lysis, isoform-selective R-subunit pull down is performed with immobilized GST fusions. (D) R-subunit pull down was performed with control GST, GST-RIAD, or GST-AKAP79_{c93}. R–C heterodimer precipitation (marked by arrow) required both R-subunit pull down and UV light exposure. (E) SDA-treated HEK293Ts were incubated with vehicle or 1 μ M isoproterenol. Cells were then immediately exposed to UV light (prelysis), or exposure was delayed until postlysis. RII subunits were selectively pulled down in all cases. (F) Densitometry showing that isoproterenol reduced RII–C cross-linking in live cells (prelysis). RII–C cross-linking was slightly increased by isoproterenol ($n = 4$) when UV exposure was delayed until after lysis. *** $P < 0.001$.

RI and RII Subunits Are in Large Excess of C Subunits. The results of our SDA cross-linking experiments with live cells led us to consider alternative mechanisms for restricting C-subunit activity. We next used quantitative immunoblotting to explore the possibility that R subunits outnumber C subunits. We took advantage of highly specific antibodies, which we independently validated (Fig. S2), to detect RI, C, and either the α or β isoforms of RII subunits. Our approach is exemplified for analysis of homogenates extracted from HEK293T cells (Fig. 2). HEK293T cell extract was run alongside reference concentrations of highly purified C (Fig. 2A, row 1), RI (Fig. 2A, row 2), RII α (Fig. 2A, row 3), or RII β (Fig. 2A, row 4). Antibody binding was determined using a chemiluminescent substrate in tandem with a charge-coupled device imager, and intensity at reference subunit concentrations was used to fit Hill function calibration curves for C (Fig. 2B, Upper Left), RI (Fig. 2B, Upper Right), RII α (Fig. 2B, Lower Left), and RII β (Fig. 2B, Lower Right). All protein extracts were quantified using this approach (Fig. 2B and Fig. S3). In the case of HEK293T cells, it was possible to approximate subunit copy numbers per cell (Fig. 2C and Table S1). Numbers from four independent sets of experiments revealed that there are $2.42 \pm 0.21 \times 10^5$ C, $7.34 \pm 0.13 \times 10^5$ RI, $15.4 \pm 0.23 \times 10^5$ RII α , and $3.00 \pm 0.69 \times 10^5$ RII β subunits per cell (Fig. 2C and Table S1). These copy numbers are the same order of magnitude as the GTPase Ras (26), another wide-acting signaling protein.

Fig. 2. PKA subunit stoichiometry in HEK293T cells. (A) Subunit-selective IBs for PKA subunit quantitation in HEK293T cell extract. Reference concentrations of purified Cβ, RIβ, RIIα, or RIIβ were run alongside HEK293T cell extract. In the example shown, the amount of extract loaded per lane varied depending on the IB as follows (micrograms total protein): 32.3 (Pan C), 33.3 (Pan RI), 26.0 (RIIα), and 35.4 (RIIβ). (B) Calibration curves derived from reference protein intensities in A. According to the reference curves, the respective HEK293T extract lanes contained (nanograms subunit) 1.54 (Pan C), 4.61 (Pan RI), 12.6 (RIIα), and 2.29 (RIIβ). (C) Subunit copy numbers per HEK293T cell ($n = 4$). * $P < 0.05$.

HEK293 cell volume has been determined by different methods (27, 28) to be ~ 2 pL per cell. This equates to cellular PKA subunit concentrations of ~ 1.5 μ M for RII, ~ 0.6 μ M for RI, and ~ 0.2 μ M for C subunits.

To investigate whether this uneven subunit ratio is a general feature of PKA signaling, we analyzed protein extracts collected from Sprague–Dawley rats. Brain tissue was extracted and separated into forebrain and cerebellum, and a fraction enriched in nerve endings was also collected from forebrain. Additional extracts were prepared from heart, liver, lungs, and skeletal muscle. An identical protein extraction method was used in all cases. In sum, subunit concentrations were determined in protein extracts from eight different sample types (Fig. S3). Average concentrations ($n = 4$) are shown in nanograms per milligram protein extract in Fig. 3A. PKA subunit concentrations are relatively high in forebrain, with RIIβ accounting for 0.29% total forebrain protein, and combined forebrain PKA subunits constitute 0.41% forebrain protein (Fig. 3A). These concentrations are approaching those of calmodulin-dependent protein kinase II, which constitutes 0.86% cerebral cortex (29), and calcineurin, which is $\sim 1\%$ of cerebral cortical and hippocampal protein (30).

We next calculated copy numbers of RI (Fig. 3B, red), RIIα (Fig. 3B, light blue), and RIIβ (Fig. 3B, dark blue) relative to C subunits (Fig. 3B, green) in each extract type. In all cases, R subunits greatly outnumber C subunits, with a combined average of 17.2 ± 1.7 -fold more R than C subunits ($P = 4.8 \times 10^{-6}$) (Fig. 3B). When the average copy numbers are compared by Student's t test in each extract, both RI and RII separately outnumber C subunits, with $P < 0.05$ in every case except RI in liver, in which the comparison is underpowered ($P = 0.051$). R subunits are in greater than 20-fold excess of C subunits in forebrain, cerebellum, and lungs. A combined analysis of all ratios reveals that RII subunits typically outnumber RI subunits by ~ 2 :1. Cerebellum and heart are notable exceptions to this rule. The approximately twofold higher expression of RI than RII in cerebellum may be related to the different (presynaptic) role that PKA plays in synaptic plasticity in cerebellar granule cells (31, 32). The elevated expression of the β isoform of RII in brain extracts is consistent with previous reports (33). Overall, our stoichiometric analysis reveals that C subunits are greatly outnumbered by R subunits across tissue types.

Insights into Anchored Type II PKA Holoenzyme Structure from Cross-Linking Coupled to MS. We next aimed to resolve uncertainties in the structure of anchored RII–C complexes in an effort to

determine whether anchoring of RII subunits—the predominant R-subunit isoform in most organs (Fig. 3B)—is compatible with release and capture of C subunits in cellular membranes. There are two conceivable positions for the D/D domain within the RIIβ–C complex: between the N lobes of the C subunits or between the second cyclic nucleotide binding (CNBB) domains of the RII subunits (13, 19). To resolve this uncertainty, we turned to cross-linking coupled to MS (XL-MS), which is a rapidly developing technique suited to structural investigation of large dynamic multiprotein complexes (34). In XL-MS, protein samples are cross-linked and digested into peptides, and the sequence of cross-linked peptides is determined by MS. Cross-links reveal which regions of the protein complex are close in space. We used the homobifunctional cross-linker disuccinimidyl suberate (DSS), which links amines, including those at the termini of lysine side chains, with a maximum span of ~ 30 Å (34). We first cross-linked PKA holoenzymes comprising RIIβ–Cβ either alone or in complex with AKAP18α. AKAP18α possesses typical AKAP properties that make it a suitable prototype for studying PKA structure (18, 35), including lipid modification sites in its first six amino acids that enable membrane insertion (36). Samples were imaged using Coomassie staining, immunoblotting, and RII overlay after electrophoresis either before (odd-numbered lanes in Fig. 4A) or after (even lanes in Fig. 4A) DSS incubation. Cross-linking of RIIβ–Cβ led to prominent bands at ~ 90 and 140 kDa, with a weaker band at ~ 180 kDa. Anti-C (Fig. 4A, lane 6) and anti-RIIβ (Fig. 4A, lane 10) IBs indicate that these three species represent 2RII, 2RII–1C, and 2RII–2C. Inclusion of the AKAP shifts all three bands higher (lanes 4, 8, and 12 in Fig. 4A) by approximately the mass of AKAP18α. RII overlay confirmed that AKAP18α was effectively cross-linked within the complex (Fig. 4A, lane 16). Sequences of cross-linked peptides identified in the RIIβ–Cβ and AKAP18α–RIIβ–Cβ samples are listed in Tables S2 and S3, respectively. A total of 126 cross-linked peptides were identified after cross-linking of RIIβ–Cβ, including 42 R–R intralinks, 74 C–C intralinks, and 10 R–C interlinks. Fig. 4B shows the overall pattern of

Fig. 3. PKA subunit quantitation in panel of protein extracts. (A) Average subunit concentrations ($n = 4$) are shown at nanograms per milligram total extracted protein, including the nerve ending (N.E.) rich fraction. Protein concentration is indicated according to the heat bar on the right. (B) Relative copy numbers of RI (red), RIIα (light blue), and RIIβ (dark blue) subunits are shown relative to C subunits (green). Numbers above the bars were determined from quantitation of extracts from four rats. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Fig. 4. Structural insights into type II β PKA anchoring from XL-MS. (A) The first two lanes of each subpanel correspond to mixtures of PKA C β and RII β ; the latter two lanes correspond to AKAP18 α -C β -RII β . Samples in even-numbered lanes were subjected to DSS cross-linking before electrophoresis. The samples were visualized by Coomassie staining (5 μ g sample per lane), anti-C IB (1.67 ng in lanes 5 and 7; 83 ng in lanes 6 and 8), anti-RII β IB (50 ng per lane), or far-Western blotting with PKA RII α subunits (40 ng per lane). DSS cross-linking interfered with anti-C-subunit antibody recognition, and therefore, it was necessary to load 50-fold more material in lanes 6 and 8 than lanes 5 and 7. (B) Distribution of intralinks (purple) and interlinks (black) within C β -RII β . C-E were assembled using Protein Data Bank ID codes 3TNP (37) and 4ZP3 (18), with proteins colored black (AKAP18 α), green (C), and blue (RII β) and lysine carbon- α atoms represented as spheres. (C) Location of interlinks detected between AKAP18 α and C β -RII β . (D) Pattern of C β -RII β cross-links (red) reduced by inclusion of AKAP18 α . (E) Location of three intralinks (purple) within C β that were increased by AKAP18 α addition.

intralinks (Fig. 4B, purple) and interlinks (Fig. 4B, black) after cross-linking RII β (Fig. 4B, blue) and C β (Fig. 4B, green) in the absence of AKAP18 α .

Nine different types of cross-link were identified linking RII β K46 at the C terminus of the D/D domain to lysines visible in crystal structures of RII β -C (14, 37). In six cases, RII β K46 is coupled to sites in CNBB (positions 263, 266, 276, 326, 328, and 357), with another partner lysine (C β K285) that projects over CNBB (Fig. 4C). This pattern of cross-linking is consistent with the D/D domain lying between the CNBB domains of the RII β dimer. XL-MS with purified RII β -C β -AKAP18 α led to identification of two interlinks involving AKAP18 α (Table S3): AKAP18 α K19 linked to C β K285 and AKAP18 α K65 linked to RII β K263. AKAP18 α K19 is between the N-terminal lipid attachment sites and the R-subunit anchoring helix of the AKAP (positions 27–42), whereas K65 is near to the C terminus of the anchoring protein. These interlinks are consistent with AKAP18 α docking to a D/D located between the CNBB domains of the RII dimer (Fig. 4C). We also analyzed a complex of RII α and C β using XL-MS (Fig. S4A and Table S4). This analysis was less powerful, as the only reference lysine within the first 100 amino acids of RII α (K71) is midway between the autoinhibitory sequence and D/D domain (Fig. S4B). Furthermore, there are no crystal structures of tetrameric RII α -C for reference. Nevertheless, the pattern of interlinks between RII α K71 and C β (Fig. S4C) is consistent with the D/D adopting a similar position relative to the C subunit in both RII isoforms.

Quantitative XL-MS Confirms the Position of the RII β D/D. We next quantified how addition of AKAP18 α altered the abundance of cross-links within RII β -C β . We anticipated that dynamic links would help to establish where the AKAP binds and if it alters the

conformation of RII β -C β . We used the xTract algorithm (38) to identify changes in the abundance of 45 unique cross-linked sites that we were able to reliably quantify over the different replicate samples (Table S5). Five cross-linking sites decreased to less than one-half their original abundance on addition of AKAP18 α (Fig. 4D, red links and Table 1). The greatest decrease was observed for a link between RII β lysines 285 and 333 (5.4-fold decrease, $P = 1.32 \times 10^{-9}$) (Fig. 4D). The four other decreasing links involved conjugation of RII β K46 to lysines either within (RII β lysines 263, 285, and 357) or adjacent to (C β K266) RII β CNBB (red in Fig. 4D). This pattern of down-regulated links confirms that the D/D domain of RII β is between its CNBB domains: binding of AKAP18 α to the D/D domain sterically impedes DSS from bridging between lysines in this region of PKA (Fig. 4D). Surprisingly, three cross-links were increased by more than 50% ($P < 0.01$) on addition of AKAP18 α (Fig. 4E, purple and Table 1). All three links fall within the C-subunit N lobe, suggesting that binding of AKAP18 α propagates a structural rearrangement in this region that enables DSS to cross-link some N-lobe lysines more efficiently. Two of the up-regulated links involve lysines within the C-subunit A helix (lysines 16 and 21 in Fig. 4E), which is a locus for posttranslational modification, including myristylation. The N terminus of AKAP18 α , which is separated by ~ 20 amino acids from the anchoring helix, is also lipidated at three sites for insertion into the cell membrane. RII subunits, anchored to this AKAP at least, are, therefore, likely to orient C subunits with the myristylated A helices pointing toward the cell membrane (Fig. 5).

Discussion

This study presents three sets of experiments that clarify how PKA C subunits are controlled in cells. First, experiments using SDA show that C subunits are released from both RI and RII during cAMP elevation, suggesting that tethering to R subunits during cAMP activation does not constitute a cellular mechanism for restricting C-subunit activity. This is consistent with many in vitro measurements showing cAMP-induced R-C subunit dissociation using methods including scintillation proximity assay and surface plasmon resonance (23, 39) and with FRET changes between microinjected and genetically encoded R and C subunits bearing fluorescent labels (40, 41). Second, subunit quantitation in a range of protein extracts reveals that R subunits typically exist in an ~ 17 -fold excess of C subunits, with very high concentrations in tissues, including forebrain. High subunit concentrations and ratios heavily skewed toward R subunits will support high rates of R-C association in cells, thereby limiting the distance from point of release over which C subunits can phosphorylate substrates. Typically, membrane-associated RII

Table 1. Cross-links in RII β -C β affected by addition of AKAP18 α

Dynamic cross-link	Ratio ($\pm$ AKAP18 α)	P value
Decreased abundance + AKAP18 α		
RII β K285-RII β K324	0.186	1.32×10^{-9}
C β K266-RII β K46	0.345	2.58×10^{-3}
RII β K263-RII β K46	0.387	1.58×10^{-5}
RII β K285-RII β K46	0.424	1.3×10^{-6}
RII β K357-RII β K46	0.462	1.17×10^{-4}
Increased abundance + AKAP18 α		
C β 16-C β 292	2.12	1.26×10^{-6}
C β 21-C β 83	1.92	2.23×10^{-4}
C β 76-C β 83	1.64	4.07×10^{-6}

Unique cross-linking sites are listed if abundance either increased by more than 50% or decreased to less than 50% on addition of AKAP18 α . P was < 0.01 for all sites. Data for all 45 unique cross-linking sites that could be reliably quantified are listed in Table S5.

Fig. 5. Updated model of AKAP18 α -Rli β -C β complex. Proposed orientation of Rli β (blue) dimer with associated C β (green) in relation to its AKAP18 α (black) anchoring site and the cell membrane (gray). The location of the D/D domain, as determined from XL-MS data, suggests that myristate (yellow) and palmitate (pink) groups attached to AKAP18 α and C β point in the same direction, supporting membrane insertion of C β .

subunits outnumber RI subunits by ~2:1. XL-MS analysis of PKA tetramers containing RII subunits (Fig. 5 and Fig. S4) suggests that, in general, anchored type II isozymes orient with the N terminus of the C subunit pointing toward the anchoring site. In the case of AKAP18 α -RII β -C β , this architecture suggests that myristate (yellow in Fig. 5) and palmitate (pink in Fig. 5) groups attached at the N termini of AKAP18 α (black in Fig. 5), and the C subunit (green in Fig. 5) could simultaneously insert into the cell membrane in type II β holoenzymes anchored to this AKAP.

We found that PKA subunit concentrations and ratios vary with tissue type (Fig. 3*B*). The four PKA R-subunit isoforms are structurally and functionally different. Studies with genetically modified mice (42) suggest that RII β is more functionally critical than RII α . Type II β tetramers are also more compact (1) and less sensitive to cAMP (37) compared with RII α . We found that RII β subunits are the predominant PKA subunit in forebrain, whereas RII α subunits predominate in lungs and skeletal muscle (Fig. 3*B*). It should be noted that, within the forebrain, RII α and RII β exhibit marked neuron-specific patterns of expression (43). Genetic studies suggest that RI is more important than RII for regulating nuclear C-subunit entry and concomitant gene expression (1, 23, 43). We found that RI subunits outnumber RII subunits in only heart and cerebellar extracts (Fig. 3*B*). Cardiac myxoma is a common symptom of Carney complex, which is caused in most cases by inactivation of the gene coding for RI α (5, 44). This is consistent with a prominent role for RI subunits in inhibiting cardiac C subunits. Anchored type II PKA isozymes are thought to be responsible for rapid PKA signaling processes, including ion channel regulation (15). Consistent with this model, we found very high expression levels of RII subunits in forebrain. Our XL-MS measurements show that anchoring of RII β and potentially, RII α subunits is compatible with membrane insertion of C subunits within anchored type II tetramers. Substrate concentration affects the cAMP sensitivity of type I—but not type II—PKA holoenzymes (45). The insensitivity of RII subunits to substrate concentration may support rapid C-subunit binding and release within the plane of neuronal membranes where local substrate concentrations are high.

Membrane tethering of C subunits released from RII subunits could potentially explain why nuclear C-subunit activity is more dependent on RI subunits (23, 43). Consistent with this model, binding of C subunits to RII but not RI favors the myr-out conformation that enables efficient C-subunit membrane insertion (12). A-kinase interacting protein 1 (AKIP1) and protein kinase inhibitor peptide (PKI) will also influence the cellular localization of C subunits. AKIP1 is nuclear (46) and binds to the N terminus of the PKA C subunit, whereas the nuclear localization of PKI is cell cycle-dependent (47). PKI could potentially inhibit $\geq 20\%$ C in some neurons, but there is uncertainty regarding its exact concentration in brain extracts (47); therefore, it is difficult to relate our calculated concentrations for R and C subunits to PKI.

In vitro binding studies have previously shown that the fraction of 30 nM C α bound to RI α rises with increasing [RI α] in the presence of 50 μ M cAMP, with K_d = 0.24 μ M (23). Our estimates for PKA subunit concentrations in HEK293T cells (2 μ M RII, 0.7 μ M RI, and 0.2 μ M C) show that PKA subunits are present at substantially higher concentrations than this K_d in cells. Consistent with a prediction by Kopperud et al. (23) that a minority of C subunits remain associated with R subunits during maximal cAMP elevation in cells, we detected residual RI–C/RII–C cross-linking on strong β -AR stimulation at ~16/22% of the basal cross-linking intensity according to semiquantitative densitometry (Fig. 1E and Fig. S1E). In comparison, addition of exogenous 10 μ M cAMP to postlysis material led to a more pronounced reduction in RII–C cross-linking (Fig. S1C and D). Therefore, the residual R–C cross-linking observed in cells probably represents partial R–C association during strong β -AR stimulation of HEK293T cells. Together, our subunit quantitation and SDA measurements suggest that abundant R subunits support high rates of R–C association, such that even maximal β -AR stimulation does not fully dissociate R and C subunits.

The phosphorylation state of RII subunits is also emerging as an important determinant of the rate at which C subunits bind to RII subunits (14). A recent study quantified k_{on} constants for C-subunit binding to RII α using surface plasmon resonance (14). Remarkably, dephosphorylation of RII α Ser112 increased the k_{on} coefficient for C-subunit binding by 60-fold (14). We detected molar excesses of RII subunits relative to C subunits in every rat extract tested (Fig. 3B). Therefore, typically, the autoinhibitory sequences of most RII subunits will be unencumbered by C subunits and accessible to cellular phosphatases. Phosphatase access is not relevant to RI, since the autoinhibition sequence of these regulatory subunits contains an alanine at the equivalent position to Ser112 (1). A recent study showed that U2OS cells expressing a fusion of RII α and C α subunits ("R2C2"), in place of endogenous C α and RII subunits, exhibit PKA activity according to a cytoplasmic AKAR4 reporter after isoproterenol stimulation (21). Nuclear AKAR4 responses are blunted in cells expressing the R2C2 fusion (21). A possible explanation for these findings is that residues corresponding to the C subunit are still able to sufficiently dissociate from the regulatory elements of RII within the context of the fused R2C2 polypeptide to phosphorylate AKAR4 in the cytosol. An analogy would be activation of other AGC protein kinases by dissociation of regulatory and catalytic elements within a single polypeptide, such as in activation of protein kinase G (22). The artificially high (equimolar) ratio of C to RII and potentially raised RII phosphorylation in R2C2 may counterbalance reductions in cAMP-induced dissociation caused by fusing the two subunits within a single polypeptide. Since the C subunit cannot diffuse away from RII after dissociation in the context of R2C2, it will be unable to pass into the nucleus, consistent with blunted nuclear AKAR4 responses in cells expressing R2C2 (21). Nevertheless, these experiments suggest that, at least in the cytosol, PKA phosphorylation can be achieved with diffusion of the C subunit over a very short distance from RII.

Rapid fluctuations in local cAMP concentration, supported by colocalization of PKA with adenylyl cyclases and phosphodiesterases,

will also support faster release and binding of C subunits to R subunits. For example, type IV phosphodiesterases (PDEs) can anchor to AKAPs (48), and direct PKA–PDE coupling has been detected (49). XL-MS is developing rapidly, and it is plausible that the technique could be applied to study cAMP signaling complexes in cells. In the future, it will be exciting to determine how anchored PKA complexes are oriented in relation to interaction partners, including cyclases, phosphodiesterases, and receptors (50).

Materials and Methods

For quantitative immunoblotting, samples were collected from HEK293Ts and male 4-wk-old Sprague–Dawley rats and homogenized using a D1 25 Basic rotor/stator homogenizer (Yellowline) and 20-kHz sonication. Experiments involving rats were done in accordance with the United Kingdom Animals Act, 1986 and with University College London Animal

Research guidelines. For XL-MS measurements, digested PKA complexes were analyzed on Orbitrap Elite and Fusion Tribrid mass spectrometers (Thermo). For quantitative XL-MS analysis, we compared the intensities of peaks eluting for cross-links between the RII β –C β and RII β –C β –AKAP18 α samples using xTract (38). Results are presented throughout as mean $\pm$ SEM. Data were analyzed by two-sided Student's *t* test. *P* values are **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. Detailed methods can be found in *SI Materials and Methods*.

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