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Dynamics of phosphodiesterase-induced cAMP dissociation from protein kinase A: Capturing transient ternary complexes by HDXMS☆,☆☆

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ABSTRACT

cAMP signaling is a fundamental cellular process necessary for mediating responses to hormonal stimuli. In contrast to cAMP-dependent activation of protein kinase A (PKA), an important cellular target, far less is known on termination in cAMP signaling, specifically how phosphodiesterases (PDEs) facilitate dissociation and hydrolysis of bound cAMP. In this study, we have probed the dynamics of a ternary complex of PKA and a PDE–RegA with an excess of a PDE-nonhydrolyzable cAMP analog, Sp-cAMPS by amide hydrogen/deuterium exchange mass spectrometry (HDXMS). Our results highlight how HDXMS can be used to monitor reactions together with mapping conformational dynamics of transient signaling complexes. Our results confirm a two-state model for active RegA-mediated dissociation of bound cAMP. Further, our results reveal that Sp-cAMPS and RegA mediate mutually exclusive interactions with the same region of PKA and at specific concentrations of Sp-cAMPS, RegA is capable of blocking Sp-cAMPS reassociation to PKA. This provides a molecular basis for how PDEs modulate levels of intracellular cAMP so that PKA is better suited to responding to fluxes rather than constant levels of cAMP. This study underscores how HDXMS can be a powerful tool for monitoring reactions together with mapping conformational dynamics in signaling proteins. This article is part of a Special Issue entitled: Mass spectrometry in structural biology.

The second messenger cyclic 3′,5′ adenosine monophosphate cAMP plays an important role in mediating cellular responses to hormonal stimuli in mammalian cells [1,2]. Protein kinase A (PKA) is a major intracellular target for cAMP and binding of cAMP results in its activation [1] (Fig. 1A). In the absence of cAMP, PKA is maintained as an inactive complex of regulatory (R) and catalytic (C) subunits. cAMP binding to the R-subunit results in dissociation of the inactive complex whereby the active kinase is unleashed [3] to catalyze phosphorylation of numerous intracellular substrates. Upon removal of the hormonal stimulus, rates of intracellular cAMP synthesis level off and the action of phosphodiesterases catalyze the conversion of cAMP to 5′AMP leading to lowering of intracellular cAMP. Under these conditions, signal termination results from hydrolysis of cAMP bound to the R-subunit and reassociation of the R and C-subunits to regenerate the PKA holoenzyme (Fig. 1A). While the structural biology [4–8], biochemistry and dynamics of cAMP binding to the PKA R-subunit leading to PKA activation have been studied extensively [9–14], very little is known on how "active" PKA is reset to its "inactive" state to complete the signaling cycle. The critical step in cAMP signal termination requires cAMP that is tightly bound to the R-subunit to be released [15] and hydrolyzed by the large family of cAMP phosphodiesterases (PDEs) [16]. This is a critical step as cAMP binds the PKA R-subunit singularly with high affinity (K_D = 2–10 nM) [17–19] and cAMP once bound to the R-subunit does not readily dissociate ($t_{1/2}$ > 5 days) [17–19].

In a recent study, our group reported that PDEs catalyze dissociation of cAMP from the R-subunit and this constitutes the signal termination phase of cAMP signaling [19]. This occurs through a two-step process involving PDE-induced dissociation of bound cAMP followed by its hydrolysis. The cAMP-free R-subunit then remains bound to the PDE (Fig. 1B). The final step in the process is reassociation of the C-subunit with the cAMP-free R-subunit and dissociation of the PDE to complete the cAMP signaling cycle. For this study, the catalytic domain of RegA, a PDE from *Dictyostelium discoideum* [19] was used as a model PDE as it shows high homology to the entire PDE superfamily. Furthermore, it has been known to interact with both *D. discoideum* as well as the mammalian PKA R-subunit isoform RIα [20].

Abbreviations: BME, β-mercaptoethanol; CNB, cyclic nucleotide binding; cAMP, cyclic adenosine 3′,5′-monophosphate; D_2O, deuterium oxide; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; HDXMS, amide hydrogen/deuterium exchange mass spectrometry; IPTG, isopropyl β-D-1-thiogalactopyranoside; LC–ESI–Q-TOF, liquid chromatography–electrospray ionization–quadrupole-time of flight; NMR, nuclear magnetic resonance; PBC, phosphate binding cassette; PKA, protein kinase A; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; UPLC, ultra-performance liquid chromatography

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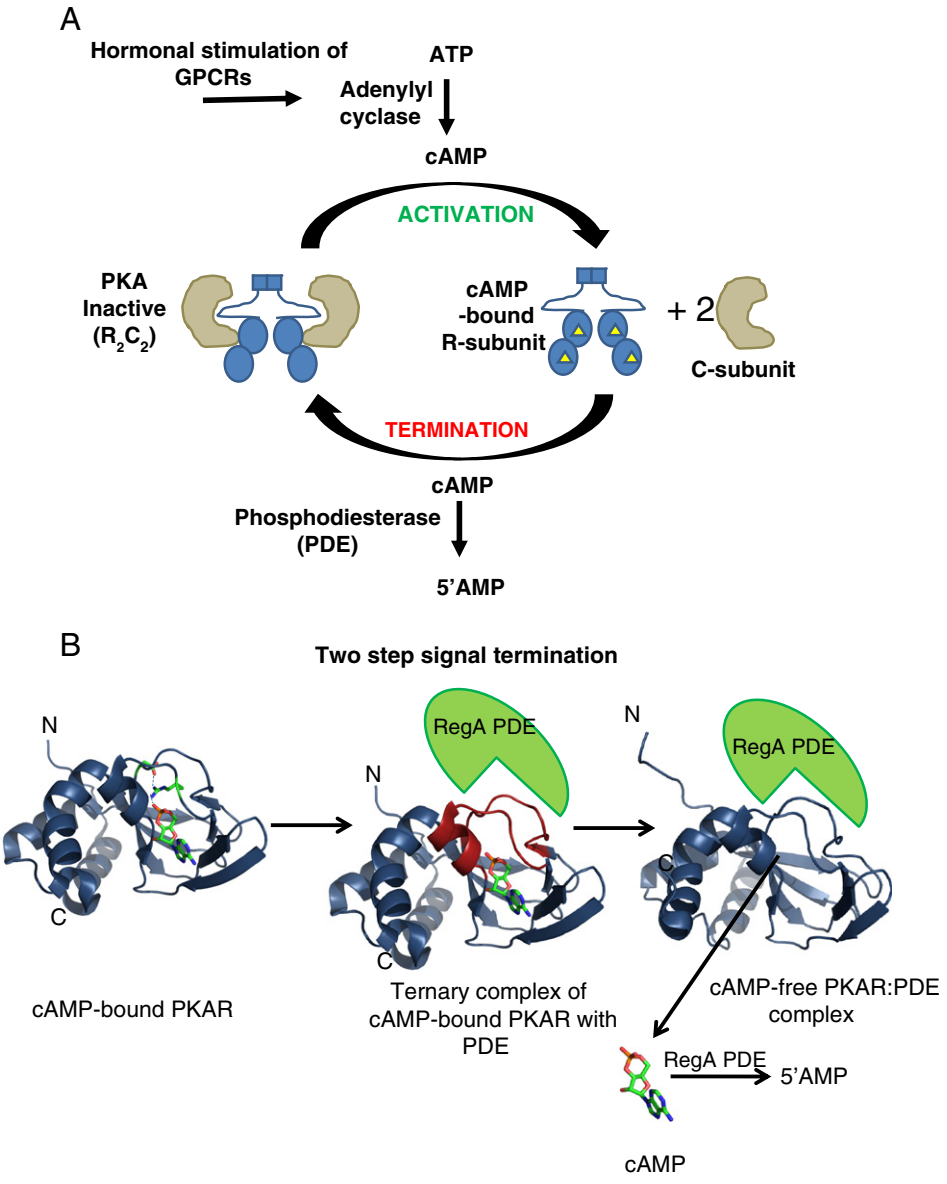

Fig. 1. A: Overview of activation and termination phases in cAMP signaling. Second messenger cAMP signaling by hormonal stimulation of G-protein-coupled receptors (GPCRs) which activate adenylyl cyclases which in turn catalyze the synthesis of cAMP from ATP. The principal target of cAMP in eukaryotes is protein kinase A (PKA) which exists in the absence of cAMP as an inactive complex of regulatory (R) (blue) and catalytic (C) subunits (tan). Binding of cAMP to PKA R-subunit results in dissociation of the R-subunit dimer and two C-subunits (activation phase). Phosphodiesterases mediate dissociation and hydrolysis of cAMP bound to the R-subunit to 5′AMP and reassociation with the C-subunit to complete the cAMP signaling cycle (termination phase). 1B: Two-state model for PDE-induced dissociation of cAMP from PKA R-subunit. Mechanism of PDE-induced dissociation of bound cAMP from RIα (91–244). Structure of cAMP-bound PKA RIα (91–244) (PDB ID: 3PNA) in blue showing cAMP interacting with two critical anchoring residues, Arg 209 and Asp 170. Interaction of the catalytic domain of RegA PDE (green) with RIα (91–244) by HDXMS (red), leads to formation of a transient ternary complex of RegAc, RIα (91–244) and cAMP. Binding of RegA facilitates dissociation of cAMP. The cAMP is released from the cAMP binding pocket and is subsequently hydrolyzed by RegA to 5′AMP.

The RIα isoform is widely prevalent in mammalian cells and exists as a dimer in solution and each monomer consists of two tandem cAMP binding domains (CNB domains). In order to monitor the kinetics of cAMP dissociation, the double deletion mutant, RIα (91–244) lacking the N-terminal 90 residues and C-terminal residues 245 to 379, has served as an excellent prototype for cAMP-binding (CNB) domains. This construct contains only one cAMP binding domain with high affinity binding site for cAMP. RIα (91–244) contains two subdomains, a β-sheet containing subdomain with an eight stranded β-sheet encompassing a high affinity cAMP binding motif referred to as the phosphate binding cassette (PBC) and a noncontiguous α-helix subdomain [1,21]. This deletion mutant still retains full functionality with respect to its ability to inhibit the PKA C-subunit in a cAMP-dependent manner [22]. The C-subunit binding surface is contributed nearly entirely by the α-helix subdomain [5] and an N-terminal C-subunit binding PKA pseudo-substrate site (RIα residues 94–98, RRGAI) [23]. This deletion mutant was also shown to interact with the catalytic domain of RegA (RegAc) via a distinct interface contributed primarily by the β-sheet containing subdomain [19]. This together with deletion mutagenesis and fluorescence polarization assays confirmed that RegAc interacted with cAMP-bound RIα, facilitated dissociation of the bound cAMP (k_{off} = 0.5 min^{-1}) followed by hydrolysis, in a two-step reaction (Fig. 1B). Given that we now had structures and dynamics of the apo and cAMP-bound R-subunit [21] as well as an HDX-MS-based map of RegAc-bound to apo R-subunit [19], we were interested in mapping the ternary complex of RegAc-bound to cAMP-bound R-subunit by