β-Arrestin-recruited phosphodiesterase-4 desensitizes the AKAP79/PKA-mediated switching of β2-adrenoceptor signalling to activation of ERK

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Abstract
Using combined dominant-negative and siRNA (small interfering RNA)-mediated knockdown strategies, the functional importance of specific PDE4 (phosphodiesterase-4) isoforms in modifying signalling through the β2-AR (β2-adrenoceptor) has been uncovered. The PDE4D5 isoform preferentially interacts with the signalling scaffold protein β-arrestin and is thereby recruited to the β2-AR upon agonist challenge. Delivery of an active PDE to the site of cAMP synthesis at the plasma membrane specifically attenuates the activity of a pool of PKA (protein kinase A) that is tethered to the β2-AR via AKAP79 (A-kinase anchoring protein 79). The specific functional role of this anchored PKA is to phosphorylate the β2-AR and allow it to switch its coupling with G_i and thereby activation of ERK (extracellular-signal-regulated kinase). Our studies uncover a novel facet of the regulation of β2-AR signalling by showing that β-arrestin-recruited PDE4 provides the means of desensitizing the agonist-dependent coupling of β2-AR with G_i and its consequential activation of ERK.

Compartmentalization of cAMP signalling intermediates
Extracellular stimuli are converted into cellular responses by the action of cell-surface receptors. These relay messages into the cell using a variety of signalling pathways, which can contain many different proteins. An important mediator of such signals is the second messenger cAMP [1–4]. This is produced by membrane-associated adenylate cyclase in response to stimulation of an appropriate receptor able to couple with the heterotrimeric G-protein, G_s. Once produced, cAMP diffuses through the cell eliciting responses in the location of its effector proteins. Although cAMP is known to activate cyclic-nucleotide-gated ion channels and the GTP exchange factor EPAC (exchange protein activated by cAMP), the best-characterized downstream effects of cAMP are mediated by the selective activation of cAMP-dependent PKA (protein kinase A). PKA is targeted to specific subcellular locations and distinct PKA substrates by AKAPs (A-kinase anchoring proteins) [2,3]. At any location within the cell, the activation status of localized PKA is dependent on the concentration of cAMP in that vicinity. Cellular microdomains of cAMP are shaped by adenylate cyclases, which produce cAMP, and by PDE (phosphodiesterase) enzymes, which degrade cAMP [2–5]. The specificity and versatility of the cAMP signalling system is therefore underpinned by tightly regulated compartmentalization of membrane-localized adenylate cyclases, AKAP-tethered PKAs and anchored PDEs.

PDE4 isoforms are tailored to perform a key role in compartmentalized cAMP signalling processes
The thesis that intracellular targeting of PDEs is key to compartmentalized cAMP signalling has been developed in particular detail for the cAMP-specific PDE4 family [6]. Each isoform of this multigene PDE family contains a unique N-terminal region that serves as a ‘postcode’ to target the PDE4 protein to defined intracellular membrane compartments or cytosolic signalling complexes [6,7]. Most PDE4s are anchored via protein–protein associations as in the binding of PDE4D5 to the scaffolding protein RACK1 (receptor for activated C-kinase 1) [8], PDE4A5 to the immunophilin XAP2 [9], PDE4D3 to myomegalin [10] and both PDE4A4 and PDE4A5 [11–13] and PDE4D4 [14] to Src family tyrosine kinases. However, protein–lipid interactions, involving phosphatidic acid, mediate the membrane association and membrane targeting of PDE4A1 to the Golgi compartment [15]. Intriguingly, AKAPs can bind PDE4s and effectively tether them to PKA [16–19]. In this situation, PKA can only be activated when the concentration of cAMP is so elevated (e.g. after activation of a G_s-coupled receptor) that the activity of the associated PDE4 enzyme is ‘overcome’ and enough cAMP accesses PKA to allow for its activation. Such regulation has been observed for PDE4D3, which can bind both mAKAP and AKAP450 [20–22].

There is considerable interest in PDE4 enzymes, as selective inhibitors have the potential as therapeutic agents...
for treating asthma, chronic obstructive pulmonary disease, certain types of cancer, Alzheimer’s disease, Parkinson’s disease, Crohn’s disease and rheumatoid arthritis, for example [6,23–26]. Also the \( \text{PDE4D} \) gene has been linked to stroke, the \( \text{PDE4B} \) gene to schizophrenia and knockout of the ‘\( \text{PDE4} \)’ family in \( \text{Drosophila melanogaster} \) leads to learning and memory defects [27,28]. The exquisite nature of the distinct patterns of intracellular targeting of PDE4 isoforms, which has been shown to underpin compartmentalized cAMP signalling in various cell types [5,21], undoubtedly explains the profound physiological actions consequent upon selectively inhibiting this enzyme family.

Four PDE4 genes each encode approx. five different isoforms [6,26]. Individual PDE4 isoforms are characterized by a unique N-terminal region, which plays a key role in intracellular targeting and recruitment to signalling scaffold complexes [6,7,16]. Located between these unique N-terminal regions are the UCR (upstream conserved region) modules that can interact with each other [29] and are involved in orchestrating the functional outcome of phosphorylation by PKA [30–32] and ERK (extracellular-signal-regulated kinase) [33–35] as well as having a role in targeting [9,10,36] and dimerization [37].

**The PDE4-\( \beta \)-arrestin complex performs a crucial role in determining the ability of the \( \beta_2 \)-AR (\( \beta_2 \)-adrenoceptor) to switch its signalling to activation of ERK**

A novel aspect of PDE4 compartmentalization was uncovered when it was realized that PDE4s could bind to and translocate with the multifunctional scaffold protein \( \beta \)-arrestin [16–19,38].

The arrestin family of proteins contains four members and all have been shown to interact with seven-membrane-spanning receptors subsequent to agonist challenge [39,40]. The main function of arrestin proteins is to process the desensitization of G-protein-coupled receptors, attenuating their ability to be activated by another round of agonist challenge. \( \beta \)-Arrestins do this by translocating from the cytosol to receptors that have been phosphorylated by G-protein-coupled receptor kinases. In doing this, they physically block the signal from the receptor to its coupled G-protein. This action serves to suppress adenylate cyclase activity and ablates the production of cAMP. In this context, the observation [19] that PDE4s can form stable complexes with \( \beta \)-arrestins becomes more noteworthy as signal dampening and message removal can now be undertaken concomitantly in one translocation event.

Initially, it was discovered that \( \beta \)-arrestins could bind all known PDE4s in a region that lies in the highly conserved PDE4 catalytic unit [19]. This interaction proved essential for effective delivery of PDE activity to the receptor, as PDE4 recruitment was not detectable in MEF (mouse embryonic fibroblast) cells from knockout animals lacking \( \beta \)-arrestins, but was reconstituted when \( \beta \)-arrestin was introduced using a viral vector [19]. Translocation of PDE4 occurs concomitantly with \( \beta \)-arrestin and this process follows on after agonist-stimulated activation of adenylate cyclase. Desensitization together with the action and PKA-mediated activation of PDEs, such as PDE4, underpins the transient rise in cAMP that ensues. Indeed, it has been shown for the \( \beta_2 \)-AR that this transient rise in localized cAMP concentration is enough to activate cellular PKA [19].

We have shown that the introduction of excess levels of a dominant-negative PDE4, which lacks catalytic activity but now acts to displace active endogenous PDE4 from \( \beta \)-arrestin, prevents the delivery of endogenous (active) PDE4 to membranes [17]. This serves to greatly enhance membrane-bound PKA activation in response to \( \beta_2 \)-AR stimulation, showing the physiological importance of the agonist-stimulated \( \beta \)-arrestin-mediated recruitment of PDE4.

PKA can directly phosphorylate the \( \beta_2 \)-AR and this has two separate but complementary desensitization effects [41,42]. First, phosphorylation causes a decrease in coupling with \( G \), Secondly, it switches \( \beta_2 \)-AR coupling with \( G \). This latter effect alters the direction of the downstream signal so that it is rerouted through Src and results in the rapid activation of ERK (Scheme 1). Inhibition of PDE4s by chemical means or use of a dominant-negative species, in the presence of agonist, releases a brake on membrane PKA activity and thereby proves effective in amplifying PKA phosphorylation of the \( \beta_2 \)-AR, which results in increased efficiency of switching of receptor coupling from \( G \)/adenylate

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**Scheme 1** The central role of agonist-recruited \( \beta \)-arrestin-PDE4 is to desensitize signalling through the \( \beta_2 \)-AR

A schematic representation showing the key proteins involved in mediating and desensitizing signalling through the \( \beta_2 \)-AR both to \( G_\text{S} \)-activation of adenylate cyclase and PKA-mediated switching of signalling to \( G_\text{i} \)-dependent activation of ERK.
cyclase to the Gβ/ERK signalling pathways [17]. Hence, the complex between β-arrestin and PDE4s is integral to desensitization of the β2-AR as it not only attenuates membrane PKA signalling via Gβ but also attenuates Gα-mediated ERK stimulation by PDE4-mediated control of a privileged pool of membrane-anchored PKA that phosphorylates the β2-AR (Scheme 1).

**PDE4D5 is the PDE that selectively interacts with β-arrestin**

Early studies evaluating the association of PDE4 isoforms with β-arrestins identified a site within the conserved PDE4 catalytic region that mediated binding [18,19]. As all PDE4s contain this site, it was initially assumed [19] that there was no preference for any one PDE4 isoform as each PDE4 should bind similarly. However, further experiments on immunopurified endogenous β-arrestins from a number of cell types showed that the PDE4 species that was preferentially associated with β-arrestin was the cAMP-induced PDE4D5 isoform [43,44]. This occurs even in cells where PDE4D5 is not the predominant isoform. Using yeast two-hybrid analysis and detailed truncation experiments, it was discovered that whilst β-arrestins did indeed associate with the PDE4 catalytic unit, PDE4D5 had an additional site within its N-terminal region, underpinning its preferential interaction [18]. β-Arrestins have two spatially distinct sites [18] allowing the binding of PDE4D5, one in the N-region and one in the C-region.

**PDE4D5 controls the PKA/AKAP79-mediated switching of the β-AR**

Determining the functional roles of PDE4 enzymes as a family is commonly done using a range of PDE4-specific inhibitors that block the active site and prevent cAMP hydrolysis [6,7,26]. Although many of these selective inhibitors bind to PDE4 enzymes with high affinity, the close homology that exists between PDE4 subfamilies and the identical nature of the catalytic unit within subfamilies preclude chemical inhibition as a tool for investigating individual functional roles for specific PDE4 isoforms. Recently, RNA silencing technology has developed into a powerful technique for the analysis of functional readouts influenced by one or more gene products. Using selective siRNA (small interfering RNA) technology has developed into a powerful technique for the analysis of functional readouts influenced by one or more gene products. Using selective siRNA (small interfering RNA)-mediated knockdown of PDE4 isoforms, we showed that PDE4B and PDE4D account for approx. 90% of PDE4 cAMP in HEK-293B2 cells (human embryonic kidney 293B2 cells). Interestingly, there was no compensatory up-regulation of PDE4 expression or PDE activity when either or both of these subfamilies were knocked down. These observations support work carried out on PDE4D and PDE4B knockout mice that, similarly, did not show any PDE4 subfamily compensatory change in expression [45–47]. When we analysed immunoprecipitates of endogenous β-arrestins from HEK-293B2 cells for association of PDE4, we found that only PDE4D isoforms were pulled down. There was no compensatory binding of either PDE4B or PDE4A isoforms to β-arrestin when PDE4Ds were silenced. However, both PDE4B–β-arrestin and PDE4A–β-arrestin association could be ‘forced’ in these cells by the ectopic overexpression of recombinant PDE4A and PDE4B species. Such data support the notion [6,7] that endogenous PDE4 isoforms are tethered to complexes in cells. In this specific instance, the endogenous PDE4B and PDE4A isoforms appear to be so tightly associated with competing scaffolds that precludes their ability to interact with β-arrestin even when the PDE4D family is knocked down. However, upon overexpression of recombinant PDE4B and PDE4A isoforms, these sites become ‘swamped’ and thus a pool of free enzyme is now available for association with β-arrestin. These results indicate that the affinity of PDE4A and PDE4B isoforms for other scaffolds in HEK-293B2 cells is far higher than for β-arrestin. Thus we can expect to see cell-type-specific patterns that define whether or not PDE4 isoforms are bound to β-arrestin and, if so, which isoforms are involved. This will depend on differences in the affinity of particular PDE4 isoforms for binding to β-arrestin, their expression level and the presence of competing scaffolds capable of binding particular PDE4 isoforms, whose efficacy in doing this will depend on their concentration and affinity for specific PDE4 isoforms.

As PDE4D isoforms have preferential access to β-arrestin in HEK-293B2 cells, it was no surprise to discover that RNA silencing of PDE4D, but not PDE4B, families enhanced the isoprenaline-stimulated phosphorylation of the β-AR by PKA and the subsequent activation of ERK. Control of this process depends on the activity of the PDE4 isoform recruited by β-arrestin and membrane PKA activity, which allows for the switching of β2-AR signalling from Gα to Gβ. HEK-293B2 cells express equimolar amounts of PDE4D3 and PDE4D5 and we have exploited the differences between the N-terminal regions of these proteins to design specific siRNA reagents so as to effect their selective knockdown. Using these reagents, we showed that knockdown of PDE4D5, but not PDE4D3, enhanced the ability of isoprenaline to induce PKA phosphorylation of the β2-AR and to activate ERK. This illustrates the importance of the selective interaction between PDE4D5 and β-arrestin, conferred by the extra association site in the N-terminal region of PDE4D5, in altering the functional outcome of β2-AR stimulation.

The PKA pool that mediates phosphorylation of the β2-AR has been suggested to be anchored near the receptor by either AKAP79 [48] or AKAP250 [49,50]. However, the method of anchoring differs for these species as AKAP79 constitutively anchors PKA to the β2-AR, whereas AKAP250 translocates to the β2-AR only after it has been phosphorylated by PKA. HEK-293 cells express both AKAP79 and AKAP250 and we have been able to attenuate the ability of isoprenaline to activate ERK in these cells by disrupting the interaction between AKAPs and PKA. This was achieved using either a membrane-permeable Ht31 peptide to disrupt PKA-AKAP interaction or by overexpression of an Ht31-GFP (green fluorescent protein) chimera. These reagents also attenuated the increased isoprenaline-induced ERK...
activation seen when PDE4D was knocked down by siRNA. We have recently employed an siRNA approach to silence AKAP79 and AKAP250 in HEK-293B2 cells, showing that it is AKAP79 and not AKAP250 which anchors the pool of PKA responsible for phosphorylating the β2-AR in response to isoprenaline challenge and allowing the consequential activation of ERK.

Although we did note translocation of AKAP250 upon isoprenaline challenge, recruitment to the membrane fraction occurred more quickly than the small amount that associated with the β1-AR. This indicates that the major fraction of recruited AKAP250 is localized in a subdomain distinct from that occupied by the β2-AR, which functionally associates with AKAP79. Hence the significance of translocated AKAP250 remains to be ascertained. Notwithstanding this, the tethering of PKA by AKAP79 is clearly fundamental to the regulation of PKA phosphorylation of the β2-AR controlled by β-arrestin-recruited PDE4D5.

Conclusion

We have identified the process whereby the 'switched' coupling of the β2-AR with Gβγ-mediated activation of ERK becomes desensitized (Scheme 1). This is through the recruitment of PDE4 in complex with β-arrestin. Specifically, the cAMP-induced PDE4D5 isoform preferentially interacts with β-arrestin to achieve this. Thus β-arrestin directly uncouples the β2-AR from Gβγ whilst β-arrestin-recruited PDE4D5 concomitantly deactivates AKAP79-tethered PKA and thereby attenuates coupling with Gβγ.

We thank the Medical Research Council, U.K. (GB604010) and the European Union (QLG2-CT-2001-02278; QLK3-CT-2002-02149) for funding.

References


Received 14 June 2005