Reshaping cAMP nanodomains through targeted disruption of compartmentalised phosphodiesterase signalosomes.

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Abstract

Spatio-temporal regulation of localised cAMP nanodomains is highly dependent upon the compartmentalised activity of phosphodiesterase (PDE) cyclic nucleotide degrading enzymes. Strategically positioned PDE-protein complexes are pivotal to the homeostatic control of cAMP-effector protein activity that in turn orchestrate a wide range of cellular signalling cascades in a variety of cells and tissue types. Unsurprisingly, dysregulated PDE activity is central to the pathophysiology of many diseases warranting the need for effective therapies that target PDEs selectively. This short review focuses on the importance of activating compartmentalised cAMP signalling by displacing the PDE component of signalling complexes using cell permeable peptide disrupters

Perspectives

Importance to the field: As PDEs tend to have highly conserved catalytic domains, agents that target the cellular location of this enzyme family rather than the catalytic site have the potential to be more specific than conventional PDE inhibitors. This should facilitate identification of the role of individual PDE isoforms and hence offer novel therapeutic routes to solve aberrant cAMP signalling in disease.

Current thinking: As cAMP is a ubiquitous second messenger that is used to transduce signals from many G-protein coupled receptors, specificity of receptor function is maintained by the unique spatial and temporal profile of cAMP gradients that are formed within the cell following specific receptor activation events. Three-

dimensional cAMP gradients are formed by localised PDE "pools" that are tethered in signalling complexes at define cellular locations. Dis-regulated cAMP signalling caused by lack of coordination between enzymes that produce, are activated by and degrade cAMP can lead to disease. It is possible to rescue cAMP deficits using conventional PDE inhibitors that compete with cAMP for the enzyme's catalytic site, however a new approach that seeks to displace PDEs from their cellular location is being pioneered to get round the problem of "off target" effects that arise from the lack of isoform selectivity seen with current pharmacologic approaches.

Future directions: Development of agents that can specifically "unhook" single isoforms of PDE from defined signalling complexes at one cellular location is in its "proof-of-concept" stage. Disruptor peptides have been used to show that this strategy can work in cellular models of disease, however they will have to become more "drug-like" before their true potential as therapeutics are realised.

Key Words

cAMP, PDE, compartmentalisation, signalosomes, PDE-protein complex, peptide microarray, cell permeable peptide disrupters, bs906, PPL-008C.

Abbreviations

cAMP, cyclic adenosine monophosphate; PDE, phosphodiesterase; PKA, protein kinase A; AKAP, A-kinase anchoring protein; P75 NTR, P75 neurotrophic receptor; PPL-008C, PDE8A – Cell Porter® disrupter peptide.

Introduction

Since the initial discovery of cyclic 3',5'-adenosine monophosphate (cAMP) over 60 years ago, extensive research has shown cAMP to be a master regulator of intracellular signalling pathways [1,2]. cAMP is a ubiquitously utilised second messenger that is synthesised by membrane-bound and soluble adenylate cyclase (AC) enzymes [3]. cAMP mediates it's myriad of cellular responses through cAMP-effector proteins: protein kinase A (PKA), exchange protein directly activated by cAMP (EPAC), cyclic nucleotide activated ion channels (CNGC) and Popeye domain containing proteins (POPDC) [4]. These proteins are responsible for regulating signalling cascades, which play crucial roles in cellular proliferation and differentiation, apoptosis, gene expression and metabolism. Advancements in cAMP detection methodology has reinforced the existence of discrete cAMP nanodomains that are spatially restricted within subcellular compartments [5,6].

Data from these novel cAMP reporters have bolstered the concept that specific physiological outcomes directed by individual G-protein coupled-receptors are dependent upon compartmentalised cAMP signalling that is shaped by localised "pools" of PDE. Fine-tuning of cAMP dynamics in space and time is achieved by coordination between AC and phosphodiesterase (PDE) enzyme activity [5]. PDEs are the only known enzymatic means of degrading cyclic nucleotides and hence PDEs are recognised for their ability to maintain homeostatic responsiveness of localised cAMP-effector proteins by maintaining cAMP levels below the threshold of activation [7]. In this context it is easy to conceptualise that aberrant localisation of PDE activity leads to unequilibrated cAMP signalling that results in disease. Unsurprisingly, this has led to the development of multiple pharmacological agents with the ability to target PDE activity directly via inhibition [8,9] or activation [10].
The following review will discuss the therapeutic benefit of targeting individual PDE – protein complexes, focussing on the development of novel cell-permeable peptides that disrupt PDE signalosomes in order to reshape cAMP signal-specific responses in cells.

PDE Overview: Structure, function and compartmentalisation

Phosphodiesterases are a diverse super-family of cyclic nucleotide degrading enzymes that catalyses the hydrolysis of cAMP/cGMP into inactive 5' AMP/GMP. Mammalian PDEs consist of 11 subtypes (PDE1-11), encoded by 21 genes that give rise to over 100 PDE isozymes (e.g. PDE4A-D, PDE7A-B, PDE8A-B, etc.)[11]. PDE subtypes are classified according to their structure, affinity for cAMP and/or cGMP, catalytic activity and mechanism of action[11]. Structurally, PDEs share a conserved catalytic region and form homo-dimers; with the exception of PDE6 holo-enzymes that typically form heterodimers under physiological conditions [57]. Interestingly, individual PDE isoforms contain unique amino-terminal subdomains and N-terminal regions that determine subcellular location via interaction with membranes and key protein partners within signalosomes. Of the 11 families, PDE4, 7 and 8 specifically hydrolyse cAMP, PDE5, 6 and 9 hydrolyse cGMP and PDE1-3, 10 and 11 hydrolyse both cAMP and cGMP. Further detail on individual PDEs expression, structural, functional and pharmacological properties can be found in several excellent reviews [9,12].

Compartmentalisation of PDEs is crucial to their function and enables precise spatiotemporal control of cAMP dynamics[5]. Formation of unique signalosomes, in which combinations of PDEs and cAMP-effector proteins form protein-complex interactions with each other and/or with membrane bound proteins (e.g. β-adrenergic receptors, adenylate cyclase), scaffolding proteins (e.g. AKAPs, β-arrestin, RACK1) and/or other cAMP-effector protein substrates (e.g. HSP20, C-Raf), is pivotal to the regulation of related downstream signalling pathways [13,14]. A prime example of how compartmentalised PDEs tightly regulate cAMP dynamics is the tethering of both PKA and PDE4D3 to the mAKAP scaffolding protein at the perinuclear compartment. The mAKAP "pool" of PDE4D3 exerts negative feedback control that attenuates PKA activity following an increase in cAMP. Thus, PDE4D3 acts as a cAMP 'sink' that maintains local control of PKA activity through degradation of cAMP surrounding this signalosome[15].

PDE signalosomes as therapeutic targets

Research into how recruitment of individual PDE isoforms within distinct signalosomes, sculpts cAMP gradients has led to the identification of over 100 unique PDE-specific protein-interactions[9]. Subsequently, this prompted the realisation that by targeting single PDE-protein interactions, precise regulation of

individual cAMP nanodomains could be achieved[16] . Studies looking at the cAMP-specific PDE4 family (primarily within the cardiovascular system) have helped delineate some of the underlying mechanisms involved with PDE compartmentalisation [17,18]. PDE4 is arguably the largest family of PDE enzymes encoded by four genes (PDE4A-D), giving rise to over 20 isoforms, which are ubiquitously expressed throughout many tissues. PDE4 isoforms specific functions are largely determined by their subcellular location, post-translational modifications and interaction with signalling proteins [7].

With regard to cellular localisation, PDE4 isoform-specific N-terminal sequences act as a 'post-code' for PDE4 localisation directing each unique PDE4 – protein complex association[19]. Other regions may also confer protein-protein interactions [20]. Importantly, multiple non-redundant roles of the same PDE4 isoform (e.g. PDE4D5) have been identified, where-by a single isoform exists in more than one signalosome at different locations within the same cell [19,21-23]. In this context, the advantages of agents that target single "pools" of one enzyme rather than the activity of entire global PDE4 "pool" become clear. Pharmacological inhibition by targeting the active site of PDE4 or even genetic ablation via siRNA or displacement using transfected dominant negative catalytically dead isoforms cannot achieve geographical isolation of single isoforms tethered at one cellular site. Disruption of individual PDE protein-protein interactions is the only option to achieve such specificity [16].

Some key examples of PDE4-protein interactions include: PDE4A4/5 – P75 NTR [24,25], PDE4B – DISC1 [26], PDE4D5 – Integrin α5 [27], PDE4D5 – β-arrestin [22], PDE4D5 – RACK1 [28], PDE4D5 – HSP20 [29] and PDE4D8 – β1-adrenergic receptors [30]. Many of these PDE-protein complexes are implicated in the progression of disease, and therefore represent viable therapeutic targets. We highlight 2 examples below, namely PDE4A4/5 – P75 NTR complex and the PDE4D5 – Integrin α5 complex.

P75 NTR, through sequestering of other signalling mediators, inhibits fibrin degradation; a process crucial to promoting extracellular matrix (ECM) remodelling and tissue repair following insult [24]. Direct interaction between P75 NTR and PDE4A5 (with binding sites identified in the UCR2 and catalytic domain regions) led to the realisation that local cAMP degradation attenuated PKA-mediated activation, impairing P75 NTR driven tissue repair mechanisms. This in turn suppressed tissue

plasminogen activator (tPA) and potentiated plasminogen activator inhibitor-1 (PAI-1) activity, promoting scar formation and inhibiting ECM remodelling. Further, p38 MAPK inflammatory signalling was found to regulate this interaction as MAPKAPK2 (MK2) phosphorylation of PDE4A5 (a p38 downstream signalling kinase) upregulated its association with P75 NTR [25]. Targeting of the PDE4A5 – P75 NTR complex represents a potentially promising therapeutic target in treating fibrinolysis associated with COPD and atherosclerosis.

Increased fibronectin (FN) associated inflammatory signalling has been identified as an early event in the progression of atherosclerosis. Interestingly, the cytoplasmic region of Integrin α5 (subunit of the major α5β1 FN receptor) was recognised as a direct binding partner of PDE4D5, binding within its UCR2 region (as seen with P75 NTR – PDE4A5) [27]. PDE4D5 degrades the cAMP nanodomain surrounding integrin α5, promoting FN-driven atherosclerotic pro-inflammatory signalling. Blocking the integrin α5 pathway attenuated PDE4D5 binding, suppressed endothelial pro-inflammatory signalling, reduced plaque size without impairing plaque stability and improved recovery in an animal model of atherosclerosis [27,31]. Thus, targeted disruption of PDE4D5 – Integrin α5 seems to be a promising strategy for the treatment of coronary/peripheral artery disease.

Targeted disruption of PDE protein complexes

Effective therapies aimed at regulating cyclic nucleotide dynamics through targeting the activity of PDE families has driven clinical success and regulatory approval of several family selective PDE inhibitors, with dozens in clinical trials and even more in pre-clinical/early stage development [9,32]. Of the 11 PDE families, clinically approved inhibitors are limited to PDE3 (milrinone and cilostazol), PDE4 (roflumilast and apremilast) and PDE5 (sildenafil, vardenafil, tadalafil and avanafil), as well as other non-selective PDE inhibitors (theophylline and caffeine). Further detail regarding the actions of PDE-selective inhibitors and related clinical indications/targets are extensively covered in a recent Nature review[58]. Despite the clinical achievements of many of these small molecule inhibitors, disappointing off-target side effects have restricted their use as efficient treatments. These limitations are demonstrated by roflumilast, a second-generation PDE4 selective inhibitor for the treatment of severe chronic obstructive pulmonary disease (COPD)[33]. Although this drug is a highly

potent and effective PDE4 inhibitor, roflumilast systemically targets all PDE4A-D isoforms equally. Consequently, patients taking this inhibitor commonly experience concerning side-effects including diarrhoea, nausea, headaches, emesis, pancreatitis and weight loss.

One current strategy focussed on decreasing the risk – benefit ratio of selective PDE inhibitors is aimed at targeting individual PDE – protein complexes and displacing specific PDE isoforms from signalosomes – as opposed to direct inhibition of the catalytic site [9,16,34]. This approach is believed to overcome the non-selective (for isoforms of same family) nature of current PDE inhibitors and reduce unwanted side-effects through highly selective targeting. Extensive research in this area has utilised novel techniques of disrupting specific PDE4 isoforms, including generation of catalytically inactive 'dead' PDE4 isoforms [35,36], siRNA silencing [37], murine knockout/knockdown models [38] and PDE4[29]-protein complex peptide disrupter interference. These techniques have provided invaluable insight into the individual functions of uniquely tethered PDE4 isoforms. However, as previously stated, each approach (excluding peptide disrupter interference), is disadvantaged by the fact that they target multiple subpopulations of the same isoform or subfamily. As individual subpopulations of PDE4 have multiple non-redundant roles within the same cell/tissue, investigating their niche functions become difficult using these techniques. This limitation is overcome through targeting pools of defined PDE-protein complex interactions with novel cell permeable peptide disrupters [16].

Developing novel PDE peptide disrupters

Accurate mapping of PDE-protein interfaces, and subsequent design of novel peptide disrupters, has been facilitated through the utilisation of high-throughput peptide microarray technology [39,40]. Briefly, linearised synthetic peptides (up to 25 amino acids (aa) in size), spanning the entire length of a protein of interest (e.g. PDE4D5), are covalently 'spotted' onto an immobilised cellulose support. Purified recombinant protein (i.e. a validated PDE4D5 binding partner) is then overlaid onto the microarray and a far western immunoblot is carried out to identify existing continuous and/or non-continuous binding sites. Positive 'spots' detail binding sequences that can be further analysed through rational substitution and/or peptide truncation analyses to identify key 'hot spot' regions and minimum binding

sequences. As peptides are linearised, and therefore do not display their natural secondary and tertiary structures, mutagenesis studies can be deployed to confirm binding regions and to further investigate their significance to PDE function and localisation. Incidentally, peptide microarray technology not only offers a rapid and powerful screening tool for the identification of key interaction domains between protein-protein interactions within select PDE signalosomes, but can also be used to rationally design short sequence peptides capable of disrupting specific PDE-protein complexes in vivo [41]. This approach is by far the most selective method of PDE signalosome inhibition, targeting only the localisation of a single "pool" of individual PDE isoforms and leaving all other PDEs (including PDEs of the same isoform at different locations within the same cell) untouched.

Cell permeable peptide disrupters of PDE signalosomes

Peptide therapeutics offer a highly diverse pool of potential drug candidates that possess many advantages compared with traditional small-molecule drugs [42]. Peptides tend to be highly potent and efficacious, demonstrating high specificity and selectivity toward their target and leading to fewer off-target side effects. Additionally, their natural characteristics result in fewer immunogenic responses, lower toxicity and improved tolerability. Classical disadvantages related with peptides therapeutics (i.e. cell membrane impermeability, susceptibility to degradation and poor oral availability) are slowly being addressed, with significant improvements in cell-penetrating chaperones and physicochemical modifications empowering their use within the clinic [43].

Peptide microarray mapping of the putative binding domains between PDE4D5 – RACK1, PDE4D5 – β-arrestin[39] and PDE4D5 – EPAC1 complex [44] has validated the concept of utilising cell permeable peptide disrupters as selective inhibitors of distinctly compartmentalised PDE signalosomes that can even contain the same isoform. This strategy has been further ratified, through targeted disruption of complexes such as PDE4D5 – HSP20 [29] and PDE8A – C-Raf [45]. These protein-protein interactions represent potentially efficacious therapeutic targets in the treatment of cardiovascular disease, cancer and autoimmune disease and will be discussed below.

PDE4D5 – HSP20 (Figure 1)

Small heat shock protein 20 (HSP20) is a highly ubiquitous molecular chaperone that is upregulated following cellular stress in order to trigger a broad-spectrum of protective actions in the context of cardiovascular disease, neurodegenerative disease and cancer [46]. Under basal physiological conditions, HSP20 remains inactive. However, cellular stressors promote an upregulation of active HSP20 through phosphorylation at Ser^{16} via PKA (in some circumstances, PKG and PKD) [47,48]. Tight proximity between HSP20 and PKA is maintained within the cytoplasm through complex interactions with the AKAP-Lbc scaffolding protein, facilitating HSP20's protective actions [49]. Although the phosphorylation of HSP20 has been shown to be protective against several diseases, the chaperone's qualities have predominantly been characterised within the cardiovascular system, particularly in settings of chronic β-adrenergic induced stimulation, ischaemia/reperfusion (I/R) injury and heart failure [47]. Transgenic overexpression of HSP20, and expression of constitutively active HSP20 mutants (S16D), safeguard against cardiac remodelling and attenuate apoptotic signalling in both ex vivo and in vivo models of cardiac hypertrophy and I/R injury [50,51]. Effects not seen following expression of a phospho-null HSP20 mutant (S16A).

Complimentary co-immunoprecipitation, ELISA and peptide microarray techniques identified PDE4D5 as a direct binding partner of HSP20, mapping the interaction to within aa residues 461 – 495 of PDE4D5s conserved catalytic domain [29]. The PDE4D "pool" associated with HSP20 shapes the cAMP nanodomain surrounding the chaperone, maintaining it in inactive unphosphorylated aggregates. Directly inhibiting PDE4, and/or saturating local PDE4 nanodomains through persistent cAMP production, can overcome PDE-mediated HSP20 inhibition (mentioned above). Interestingly, a rationally designed 25mer stearylated cell permeable peptide (bs906), based on the PDE4D5 binding domain (E^{468}-K^{492}), can act as a novel inhibitor targeting the disruption of PDE4D5 – HSP20 [29]. Bs906 effectively displaced PDE4D5 from HSP20 and potentiated PKA-phosphorylation of HSP20 at Ser^{16} (See Figure 1). As a result, bs906 was found to attenuate ANP gene expression (hypertrophy marker), hypertrophic remodelling and cardiac fibrosis, whilst improving left-ventricular function in an aortic-banded heart failure mouse

model [41]. Cardioprotective effects were not found in mice treated with scrambled peptide disrupter sequences.

PDE8A – C-Raf (Figure 2)

Discovery of the PDE8A – C-Raf interaction added a new level of complexity to cross-talk between cAMP-PKA and RAS-RAF-MEK-ERK signalling axes [52]. In short, PDE8A could be detected in complex with C-Raf (Raf-1) and influence the activity of the kinase [45]. PKA phosphorylation of C-Raf at Ser^{259} inhibits it, attenuating downstream MEK-ERK signalling. PDE8A binds C-Raf with high picomolar affinity (<61pM), protecting C-Raf from PKA-mediated inhibition. This protection is increased following overexpression of PDE8A in HEK293 cells. Conversely, expression of a catalytically inactive PDE8A mutant displaced the endogenous active PDE pool, suppressing ERK activation through a marked increase in C-Raf phosphorylation [45]. This concept was further ratified in mouse and drosophila PDE8 knock-out models. Peptide microarray mapping identified a key C-Raf binding region within the conserved PDE8A catalytic domain (N^{442} – I^{476}), narrowing the minimum required binding site down to 9 amino acids (R^{454} – Y^{461}). Subsequent rational design of a stearylated PDE8A peptide (R^{454} – T^{465}) convincingly disrupted the endogenous PDE8A – C-Raf interaction, potentiating inhibitory C-Raf phosphorylation and suppressing ERK activation. This effect was not observed with a scrambled control peptide[45]. As C-Raf activity plays a central role in the regulation of mechanisms involved in multiple cancers[53], and as PDE8A is thought to make up only ~5% of total PDE8 expression within cells, targeted disruption of PDE8A – C-Raf has potential as a highly selective anti-cancer treatment.

Optimisation of the PDE8A peptide disrupter, through C-terminal conjugation with a novel HOXD12 homeodomain protein-based cell penetrating peptide (Cell Porter®; Portage Pharmaceuticals Limited), validated the therapeutic potential of disrupting the PDE8A – C-Raf complex in B-Raf inhibitor resistant melanoma [54] (See Figure 2). Co-treatment with PDE8A – Cell Porter® peptide (PPL-008C) robustly suppressed B-Raf inhibitor (PLX4032/Vemurafenib) induced paradoxical ERK activation and attenuated cell proliferation in MM415 human malignant melanoma cells (NRAS Q61L, KRAS WT, BRAF WT). Moreover, PPL-008C monotherapy demonstrated a rapid and long-lasting abrogation of ERK activation in an MM415

xenograft mouse model [54]. As gain-of-function RAS mutations exist in over 25% of human cancers [55], and with C-Raf activity inhibition being recognised as a novel target in overcoming B-Raf inhibitor resistant cancer, therapeutic use of PPL-008C is currently being investigated in mutant RAS pancreatic, lung and colorectal cancers.

Interestingly, a recent report using original PDE8A-stearylated peptide, was found to inhibit the adhesion and migration of T-effector cells to brain endothelial cells in an auto-immune mouse model of multiple sclerosis[56]. Disruption of this PDE8A signalosome produced a more potent effect than PF-04957325, a highly selective PDE8 enzyme inhibitor. These findings add to the therapeutic value of attenuating ERK activity through PDE8A – C-Raf disruption in the setting of inflammatory disease. Follow-up studies using the PDE8A-Cell Porter® peptide are underway.

Conclusion

Family selective small molecule PDE inhibitors can be limited by their indiscriminate targeting of multiple PDE-signalosomes within non-target tissues, resulting in dose-limiting side-effects. Development of novel and highly selective cell permeable peptide disrupters that target individual PDE-protein complexes within defined cellular compartments have the unique ability to reshape cAMP gradients on a nanoscale. Targeted disruption of PDE4D5 – HSP20 and PDE8A – C-Raf exemplify the therapeutic potential of such an approach in the setting of cardiovascular disease, cancer and inflammation. Rational design of peptide disrupters targeting other key-disease linked to PDE-protein complexes (e.g. PDE4D5 – Integrin α5 [27] and PDE4A5 – P75 NTR[24]) will help characterise novel functions of defined PDE signalosomes and offer a proof-of-concept of therapeutic approaches that seek to displace localised pools of specific PDEs.

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Figure 1: Potentiating HSP20-mediated cardioprotection through targeted disruption of PDE4D5 – HSP20.
A [LEFT]: PDE4D5 – HSP20 interact at the site of AKAP-Lbc. β-adrenergic receptor (AR) stimulation upregulates cAMP production, activating PKA. PDE4D5 degrades cAMP surrounding HSP20, attenuating PKA-mediated serine 16 phosphorylation/HSP20 activation. **A [RIGHT]:** Targeted disruption with stearylated PDE4 peptide (bs906) binds HSP20, displacing endogenous PDE4D5. PDE4D5 – HSP20 disruption exposes HSP20 Ser16 to PKA phosphorylation, potentiating HSP20 activity (represented as a dimer) and its subsequent cardioprotective actions. **B:** Simple schematic of PDE4D5 (long PDE4D isoform) protein structure. *UCR1/2, upstream conserved regions 1/2; LR1/2, linker regions 1/2; Catalytic domain with cAMP binding pocket.* **C:** Simple schematic of HSP20 protein structure. *Ser16, serine 16 residue; conserved α-crystallin domain, flexible C-terminal region*

Figure 2: Combined inhibition of B-Raf/C-Raf heterodimers in B-Raf inhibitor resistant cancer.

A [LEFT]: B-Raf inhibition leads to the paradoxical activation of C-Raf through a negative feedback RAS-driven switch. PDE8A degrades cAMP, protecting C-Raf from PKA-mediated serine 259 inhibitory phosphorylation. Both allow the continuation of tumour growth. A [RIGHT]: PPL-008C (PDE8A-Cell Porter®) peptide disrupter displaces endogenous PDE8A – C-Raf protein complex, exposing C-Raf – serine 259 to inhibitory PKA phosphorylation. Combined B-Raf and C-Raf inhibition suppresses MEK-ERK driven oncogenesis. B: Simple schematic of PDE8A monomeric protein structure. REC, signal regulatory domain; PAS, PAS domain. Catalytic domain with cAMP binding pocket C: Simple schematic of C-Raf monomeric protein structure. RBD, Ras binding domain; s259, inhibitory serine residue 259; NKD and CKD, N-terminal and C-terminal lobes of catalytic tyrosine kinase domain.