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Protein-protein interactions of PDE4 family members - Functions, interactions and therapeutic value

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– functions, interactions and therapeutic value

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Abstract

The second messenger cyclic adenosine monophosphate (cAMP) is ubiquitous and directs a plethora of functions in all cells. Although theoretically freely diffusible through the cell from the site of its synthesis it is not evenly distributed. It rather is shaped into gradients and these gradients are established by phospodiesterases (PDEs), the only enzymes that hydrolyse cAMP and thereby terminate cAMP signalling upstream of cAMP's effector systems. Miles D. Houslay has devoted most of his scientific life highly successfully to a particular family of PDEs, the PDE4 family. The family is encoded by four genes and gives rise to around 20 enzymes, all with different functions. M. Houslay has discovered many of these functions and realised early on that PDE4 family enzymes are attractive drug targets in a variety of human diseases, but not their catalytic activity as that is encoded in conserved domains in all family members. He postulated that targeting the intracellular location would provide the specificity that modern innovative drugs require to improve disease conditions with fewer side effects than conventional drugs.

Due to the wealth of M. Houslay's work, this article can only summarize some of his discoveries and, therefore, focuses on protein-protein interactions of PDE4. The aim is to discuss functions of selected protein-protein interactions and peptide spot technology, which M. Houslay introduced into the PDE4 field for identifying interacting domains. The therapeutic potential of PDE4 interactions will also be discussed.

Keywords: phosphodiesterase, A-kinase anchoring protein; drug targets; cAMP; compartmentalisation; protein kinase A; protein-protein interaction;

1. Introduction to compartmentalized cAMP signalling

Cells perceive changes of their environment through sensing chemical cues such as hormones or neurotransmitters *via* receptors. Stimulation of receptors then causes a rise in the level of second messengers, which modulate intracellular signalling to enable cellular responses that are specific for each of those cues.

Many of the cues stimulate G protein-coupled receptors (GPCRs), causing activation of the stimulatory G protein G_S. The activated G protein, in turn, stimulates adenylyl cyclases to synthesise cyclic adenosine monophosphate (cAMP). This ubiquitous second messenger activates its effectors: protein kinase A (PKA), Exchange factors directly activated by cAMP (Epac1 and Epac2) and cyclic nucleotide-gated channels (CNGC), which will then initiate signalling cascades that orchestrate a cellular response that is specific to a particular cue.

Considering that most of the cues selectively activate their cognate receptors, it is not surprising that each elicits a specific cellular response. However, the question arises of how a ubiquitous second messenger such as cAMP can elicit specific responses if its level rises in response to numerous stimuli and upon activation of a whole variety of receptors, often at the same time in the same cell. A classical example is in cardiac myocytes. While prostaglandin E_1 (PGE_1) through E-prostanoid (EP) receptors and adrenergic agonists through β-adrenoceptors both increase cAMP to similar levels, only the stimulation of β-adrenoceptors increases contractility. It is now clear that the level of cAMP does not increase uniformly but rather in microdomains where then cAMP effectors are activated. In the case of cardiac myocytes, stimulation with PGE_1 and β-agonists leads to activation of different pools of PKA [1-4].

Microdomains of cAMP are established by adenylyl cyclases synthesizing it and by phosphodiesterases (PDEs). PDEs are the sole means for cAMP degradation and prevent its uniform cellular distribution as they are strategically positioned and thereby establish gradients of cAMP that are sensed by its effectors. PDEs are tethered to subcellular compartments through direct association with membranes [5, 6], or through interactions with proteins that direct them to defined compartments. Protein binding partners include scaffolding proteins such as the A-kinase anchoring proteins (AKAPs), a heterogeneous family of around 50 proteins characterised by their common ability to directly interact with PKA [7, 8]. In addition to a conserved domain for the interaction with PKA, AKAPs posses docking domains for direct binding of additional proteins such as PDEs and they possess anchoring domains tethering the AKAP-based protein complex to defined cellular compartments [9, 10]. It is this tethering of PDEs to defined cellular compartments that is a crucial component of the safeguard sys-

tem conferring specificity to cAMP signalling. PDEs are critical for maintaining cAMP micro-domains.

2.   PDEs

2.1   The PDE4 family of phosphodiesterases

There are 21 genes encoding 11 families of PDEs, PDE1-11. The genes contain multiple promoters giving rise to >100 mRNAs and thus to >100 isozymes. PDEs hydrolyse cAMP and/or cGMP. The PDE4, PDE7, and PDE8 family enzymes specifically hydrolyse cAMP, the PDE5, PDE6, and PDE9 enzymes are cGMP-specific, while the PDE1, PDE2, PDE3, PDE10, and PDE11 family members hydrolyse both cAMP and cGMP. The individual enzymes differ in structure, regulation, location and pharmacological properties [11-14].

The PDE4 family is the largest of the eleven PDE families. Four genes encode the PDE4A, B, C and D family isoforms; due to alternative start sites and alternative splicing around 25 isozymes are generated. The isoforms are long, short, super-short or dead-short, depending on which domains are present (Fig. 1A). All isoforms contain a highly conserved catalytic domain, N-terminally from it upstream conserved regions (UCR) 1 and 2 of variable length and a unique N-terminal region (NTR). They possess unique C termini [15]. The unique N-terminal regions are involved in targeting the enzymes to defined cellular compartments. UCR1 largely mediates dimerization of the long isoforms. The absence of UCR1 renders the short forms of PDE4 monomeric. Additional regions within the catalytic domain were mapped which mediate dimerization of short forms (see below) [16]. PDE4 isoforms
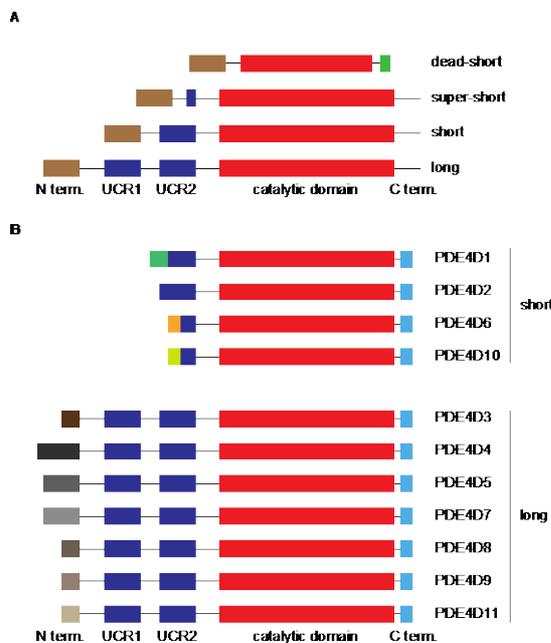

Fig. 1. Schematic representation of long, short, super-short and dead-short isoforms encoded by A) the 21 PDE genes and B) by the PDE4D encoding gene. N and C term., N and C terminus, respectively; UCR, upstream conserved region.

are constitutively active enzymes. The K_M cAMP values for the PDE4 enzymes range from 1-20 μM [17]. The K_M cAMP hydrolysis for human PDE4D3 is 1.2 μM and the V_{max} approximately 30 nmol/min/mg [17, 18]. PKA phosphorylation of the long isoforms enhances the hydrolytic activity. For example, PKA phosphorylation of S54 in UCR1 of PDE4D3 increases the V_{max} for cAMP hydrolysis of rat PDE4D3 by about 50 % without affecting the K_M. [19, 20].

In contrast, ERK2 phosphorylation of a conserved site in the C terminus of long isoforms of PDE4B, C and D inhibits their activity. ERK2 phosphorylation of S579 of human PDE4D3 inhibits it by around 75 % [21, 22]

2.2 Protein-protein interactions tether PDE4 isozymes to subcellular compartments

Light was shed on the mechanisms underlying the compartmentalisation of PDE4 isozymes when interacting partners were identified. Classical scaffolding proteins tethering their interacting partners to defined cellular locations are AKAPs. An early joint discovery by the groups of Houslay and Scott was that mAKAP tethers PDE4D3 together with PKA to the perinuclear compartment [23]. This protein assembly establishes a negative feedback loop for the local control of cAMP hydrolysis. An increase of cAMP leads to activation of mAKAP-bound PKA, which in turn phosphorylates PDE4D3, enhancing its activity and thereby terminating cAMP-mediated activation of PKA. The mAKAP complex controls several cardiac myocyte functions. For example, in response to β-adrenoceptor stimulation mAKAP-bound PKA can phosphorylate Ryanodin type 2 receptors (RyR_2) and enhance Ca^{2+}-induced Ca^{2+} release through from the sarcoplasmic reticulum. This increases contractility of the myocyte [24].

Around the same time the groups of Conti and Tasken discovered an interaction of AKAP450 with PDE4D3 on centrosomes [25]. Similar as the mAKAP interactions with PKA and PDE4D3 the AKAP450 complex introduces a negative feedback loop for cAMP signalling in close proximity of centrosomes.

Antidiuretic hormone (arginine-vasopressin, AVP) induces the redistribution of the water channel aquaporin-2 (AQP2) from intracellular vesicles into the plasma membrane of renal collecting duct principal cells. The membrane insertion of AQP2 facilitates water reabsorption from primary urine and fine-tunes body water homeostasis. AVP induces a rise of cAMP followed by PKA activation and phosphorylation of AQP2 by PKA, which is the trigger for the redistribution [10, 26, 27]. Together with M. Houslay we discovered an AKAP18δ-based signalling module on AQP2-bearing vesicles. [28]. AKAP18δ directly interacts with PDE4D3. It tethers PDE4D3 together with PKA to AQP2-bearing vesicles where PDE4D3 maintains low levels of cAMP under resting conditions and thereby prevents inappropriate activation of PKA and thus inappropriate water reabsorption. AVP stimulation of the principal cells increases cAMP and leads to PKA phosphorylation of PDE4D3 to enhance its activity. If cAMP reaches threshold levels PKA phosphorylates AQP2 and AQP2 redistributes into the plasma membrane. The PKA-phosphorylated form of PDE4D3 co-translocates to the plasma membrane and hydrolyses cAMP to basal levels and resets the system, i.e. PKA becomes inactivated and AQP2 is endocytosed [28-30].

PDE4 species can directly interact with plasma membrane receptors such as PDE4A4/5 with

the p75 neurotrophin receptor (p75NTR) [31]. Tissue repair may be limited by scar formation. A critical component in the process of scarring is an initial fibrin deposition. During tissue repair fibrin is cleared by the serine protease plasmin. p75NTR is up-regulated upon tissue damage and regulates the proteolytic activity of plasmin by simultaneously down-regulating tissue plasminogen activator (tPA) and up-regulating plasminogen activator inhibitor-1 (PAI-1) *via* a cAMP/PKA pathway. p75NTR directly interacts with PDE4A4/5, which locally decreases cAMP and reduces extracellular proteolytic activity. Thus the p75NTR-PDE4D5 interaction contributes to pathology [31].

While mAKAP and AKAP450 are located constitutively at a particular cellular compartment and thus maintain their PDE4D interacting partner at that location PDE4D species may also alter their location depending on the state of the cell. Stimulation of β-adrenoceptors activates the G_s/adenylyl cyclase system and leads to a rise in cAMP. For signal termination the receptor is desensitized by binding of β-arrestin. In HEK293 cells, PDE4D directly binds β-arrestin; under resting conditions the complex resides in the cytosol. This redistribution of β-arrestin to the receptor is associated with a co-redistribution of PDE4D5 to the receptor and local cAMP hydrolysis. Thus PDE4D contributes to terminating β-adrenoceptor signalling [32]. The β-adrenoceptor-induced rise in cAMP leads to PKA activation and PKA-catalysed phosphorylation of the receptor, which, in turn, switches receptor coupling from G_s to the inhibitory G protein, G_i. G_i activates the extracellular signal-regulated kinases ERK1/2 and Akt. In cardiac myocytes activation of these pathways contributes to stress-induced hypertrophy. G_i inhibits adenylyl cyclase and thus cAMP synthesis. The β-arrestin-dependent recruitment of PDE4D to the receptor and the local cAMP hydrolysis contributes to inhibition of PKA and reduction of receptor phosphorylation. Thus the β-arrestin-associated pool of PDE4D is associated with a switch from G_S to activation of G_i [33].

β-arrestin is not the only scaffolding protein that directs PDE4 isoforms into the cytosol. Receptor of activated C kinase 1 (RACK1) is a cytosolic scaffolding protein, although also associated with ribosomes and plasma membrane receptors. It does not posses own catalytic activity but represents a platform for protein complex assembly, and supports docking, scaffolding, stabilizing, inhibiting and linking components of the complexes together. RACK1 plays a role in diverse processes such as virus infection, neuronal development, cell migration, and angiogenesis [34, 35]. The 88 amino acids long unique N terminus of PDE4D5 contains a RACK-binding site (RIAD1) [36, 37], and also the binding site for β-arrestin. The binding of PDE4D5 to RACK1 and β-arrestin is mutually exclusive [38]. In HEK293 cells, the interaction of PDE4D5 with these two binding partners seems in equilibrium. Disturbing this equilibrium, e.g. by knockdown of RACK1 causes increased association of PDE4D5 with β-arrestin, which upon stimulation of β-adrenoceptors leads to a decrease in PKA phosphorylation of the β-adrenoceptor since β-arrestin causes an enhanced accumulation of PDE4D5 at

the receptors [38].

The given examples of protein-protein interactions compartmentalising PDE4 isozymes are by no means exhaustive. For example, the N termini of PDE4A4/5 and PDE4D4 interact with the SH3 domains of Src family tyrosine kinases such as Lyn, Fyn, and Src [39, 40]; further interactions such as with myomegalin, Shank2 and Disc1 have been identified [11, 13-15]. In addition, direct membrane association can also compartmentalize a few PDE4 isozymes. One example is the tryptophan anchoring phosphatidic acid selective-binding domain 1 (TAPAS-1 domain) of PDE4A1. The N terminus of PDE4A1 contains two helices separated by a hinge region. The second helix contains the TAPAS-1 domain, which mediates an interaction with phosphatidic acid and insertion into lipid bilayers. Selectivity for phosphatidic acid is achieved by a network of charges on the surface of the helical TAPAS-1 domain [5, 6]. The PDE4 orthologue of Aplysia, ApPDE4 exists in short and long forms, which are distinctly targeted to different membrane compartments. The long form is targeted to intracellular membranes through an N-terminally 16 amino acids long hydrophobic region, whereas the short form seems to exclusively interact with the plasma membrane through electrostatic interactions of the N terminus with negatively charged membrane lipids such as PI4P and PI(4,5)P2. The membrane association of both the long and the short forms is enhanced by oligomersation through UCR1 and UCR2 [41]

2.3 Peptide Spot arrays for mapping protein-protein interactions down to the level of individual amino acids

Crucial to the understanding of protein-protein interactions of PDEs and thus to understanding the molecular mechanisms underlying compartmentalization is the characterisation and mapping of the interacting domains. The mapping can be achieved by incubation of truncated recombinant versions of two interacting partners and co-precipitation. Truncating both proteins to a size that precludes co-precipitation will define the interacting regions. An alternative to this approach is to initially attempt co-precipitation of two proteins either from cell lysates or of recombinant proteins to show complex formation, and then to test whether the interaction is direct. Direct interactions can be elucidated by a combination peptide spot array technology and overlay with recombinant proteins. This approach simultaneously maps the interacting domains down to the single amino acid level.

Ronald Frank originally invented the peptide spot technology [42, 43]. Libraries of peptides each up to 30 amino acids in length can be generated by automatic SPOT synthesis on cellulose membranes or glass supports using Fmoc (9-fluorenylmethyloxycarbonyl) chemistry and automatic devices (e.g. Autospot-Robot ASS 222, Intavis Bioanalytical Instruments, 50933 Koeln, Germany) [44]. An advantage of the technology is the possibility of parallel synthesis of hundreds or thousands of peptides with different sequences. Each amino acid in

any peptide can be substituted with any other amino acid. Single, double and multiple substitutions may be introduced. Particular informative and widely used are alanine substitutions. An interaction of the spot-synthesised peptides with a putative binding partner is detected by a modified Western blotting procedure (Far Western blot). The peptides are often overlaid with fusions of the recombinant putative binding partner and glutathione S-transferase (GST). Then a GST overlay would be carried out as a negative control. A specific antibody directed against the putative binding partner and a suitable secondary antibody are applied. The secondary antibody is either linked to an enzyme such as horseradish peroxidase or alkaline phosphatase, catalysing a reaction that permits detection of a colour or the secondary antibody can be linked to a fluorescent moiety. Either detection of a developing colour or of fluorescence allows for visualisation of the interaction. This method can detect continuous and discontinuous binding sites. Identified interactions are validated by co-precipitation studies. The binding sites are usually located on the surface of proteins and if 3D structures are available can be mapped on the protein structure.

Moreover, analysis of posttranslational modifications such as ubiquitination, sumoylation and phosphorylation of peptides may be carried out by incubating spot-synthesised peptides with the enzymes catalysing such reactions. For example, 25mer peptides encompassing amino acid residues 22-28 of PDE4D5 were sumoylated at a sumoylation consensus motif, VKTEQED motif [45]; PKA phosphorylated sites (S428 and S438) in 30 mer peptides derived from PDE3A [46].

A drawback of the method is the limit of peptide length, 30 amino acids are currently considered the maximum. The synthesis of longer and difficult peptides can lead to impurities, synthesis termination and thus low yields. Inter- and intramolecular aggregation and sterical hindrance is a further potential problem. To minimize this cysteines are usually replaced by serines for example [44].

Identification of interacting domains and defining the contribution of individual amino acids to the interactions by a combination of the peptide spot technology with overlay techniques has been of utmost importance for understanding PDE4 isozyme interactions. For example, the interaction of p75NTR and PDE4A5 [31], of AKAP18δ with PDE4D3 [28] and that of MEK1 and β-arrestin-1 [47] have been mapped using this technology; and the technology also allowed for defining overlapping binding sites for β-arrestin and RACK1 in PDE4D5 [38].

3. PDE4 isozymes' protein-protein interactions as drug targets in human diseases

PDE4 enzymes are expressed almost ubiquitously. In particular they are found in airway and vascular smooth muscle cells, leukocytes and in the brain and are involved in major diseases such as inflammatory and neurological disorders. The ubiquitous expression and the high conservation of the catalytic domains of PDE4 family members represent the major obstacles

for clinical applications of PDE4 inhibitors. The use of rolipram, a selective PDE4 inhibitor that targets the catalytic domain, has documented, catalytic domains of PDE4 enzymes are difficult targets for the treatment of human disease. Rolipram causes nausea and emesis, side effects not tolerated by patients. However, over the last few years inhibitors that overcome the dose-limiting side effects caused by rolipram have emerged and a few have reached the market. The first approved PDE4 inhibitor was roflumilast in 2010 as an add-on therapy for chronic obstructive pulmonary disease (COPD). With apremilast a second PDE4 inhibitor reached the market in 2014. It is approved for the treatment of psoriatic arthritis. Both roflumilast and apremilast are non-selective inhibitors of PDE4A-D. Although they are effective drugs they still can cause emetic side effects. Current developments of a new generation of anti-PDE4 drugs follow different strategies. One concept is to develop conformational state-directed ligands. Roflumilast is an example for an inhibitor that binds preferentially to a state of PDE4 enzymes that is associated with low affinity binding of rolipram. In addition, antagonists are being developed that isoform-selectively target catalytic sites of PDE4A, B, C or D, and allosteric inhibitors. In any case, the aim of all of these developments is to separate the unwanted side effects, in particular nausea and emesis, from the therapeutically beneficial ones.

Maybe the most promising strategy to reach this goal is displacement of PDE4 enzymes from their cognate cellular location to which they are tethered through a protein-protein interaction. Protein-protein interactions are highly specific and diverse and, therefore, provide superb opportunities for a highly selective pharmacological interference. A drawback is that proteins often interact through extended shallow surfaces of around 1.000-2.000 $Å^2$, which cannot be blocked by binding of a small molecule. Small molecule drugs preferably possess a molecular weight of around 500. However, specific amino acid residues within the interacting domains often provide the critical energy for the interaction. These "hot spots" can be targeted by small molecules [48, 49].

M. Houslay was one of the first to realise that selectively enhancing local cAMP by displacement of specific PDE4 isoforms may protect against the unwanted side effects of global PDE4 inhibition. One approach is using dominant negative versions of a PDE4. They are catalytically inactive and upon overexpression displace the endogenous PDE4 from its binding partner. The consequence would be a local rise of cAMP and an increased local PKA activity. For example, the catalytically inactive PDE4D5-D556A prevents the isoproterenol-induced binding of the endogenous PDE4D5 to the β_2-adrenoceptor complex. This is associated with an activation of ERK1/2 and thus caused by a local rise in cAMP, PKA activation and G protein switching from G_s to G_i (see 2.2) [33]. The dominant negative approach has for example also elucidated that PDE4D3 controls a cAMP microdomain at the β_1-adrenoceptor in cardiac myocytes [50] and that PDE4D3 and PDE4C2 control AKAP-

anchored PKA activity in the perinuclear region [51]. The dominant negative approach can reveal relevant physiological mechanisms, and contribute to validate a target.

The peptide array technology has provided the means for fine mapping of protein-protein interaction sites (see 2.3). Synthetic peptides can be derived from the binding regions of the interacting partners and rendered membrane-permeant by coupling to lipids such as stearate or a myristoyl group. Such peptides are valuable tools for targeting an intracellular protein-protein interaction. Peptide spots representing the entire sequence of Lis1 were incubated with full length PDE4D3. This revealed two binding sites for the PDE, one of which between amino acid residues 226-250. A peptide encompassing this region was synthesised and rendered membrane-permeant by coupling to stearic acid. The peptide prevented the interaction of LIS1–GFP and PDE4D3–VSV in HEK293 cells expressing [52]. Another example is a peptide encompassed amino acids 6–29 of β-arrestin1, which inhibits the interaction of β-arrestin1 with MEK1 in HEK293 cells [47].

A recent example convincingly showing the merit of this concept relates to heat shock protein, HSP20. HSP20 protects the heart against sepsis- and endotoxin-induced cardiac dysfunction in mice [53] and overexpression of HSP20 reduced apoptosis upon ischemia/reperfusion (I/R) injury [54]. The cardioprotective effect of HSP20 is due to a phosphorylation of serine 16 by PKA. Overexpression of a phosphomimic (HSP20-S16D), i.e. constitutively phosphorylated HSP20, in adult cardiac myocytes inhibited apoptosis [54, 55]. HSP20 interacts directly with PDE4D5. The interaction site on PDE4D5 was mapped to amino acid residues V466–L490 in the catalytic domain by spot synthesizing the entire sequence of PDE4D5 and overlaying the peptides with recombinant HSP20. In a second step a peptide representing amino acid residues 468-492 of PDE4D5 was synthesized as a stearate-coupled and thus membrane-permeant version. This peptide inhibited the interaction of HSP20 and PDE4D5 in HEK293 cells and increased the serine 16 phosphorylation. Moreover, in neonatal cardiac myocytes the peptide inhibited the isoproterenol-induced hypertrophy, it inhibited the isoproterenol-induced rise of the hypertrophy marker natriuretic peptide (ANP) by 50 % [56]. Moreover, *in vivo* in mice the peptides protected against cardiac remodelling after aortic banding, an animal model for heart failure [57, 58]. Thus the use of peptides suggests the interaction as a putative drug target for the treatment of heart failure, as cardiac hypertrophy represents an intermediate stage *en route* to heart failure. Alanine scans of the PDE4D5 region interacting with HSP20 using peptide spots revealed critical amino acids whose substitution with alanine abolished the interaction (H470, H471 K477, E481, E482 and D485) [56]. These amino acids may be considered hot spots and thus could be the starting point for the development of small molecules blocking the interaction. In addition to COPD and psoriasis for whose treatment PDE4 inhibitors are already approved (see above) there are several further major diseases associated with deregulation of PDE4 enzymes. Amongst

others cognitive enhancers [59] and asthma drugs [60] are currently being develop. They all target enzyme activities. However, there are disease-relevant PDE4 interactions in a number of instances. Examples are the interaction of PDE4A with p75NTR in fibrosis [31] or the interaction of DISC1 with PDE4B in schizophrenia [61].

Concluding remarks

The data discussed here show that specific functions have been ascribed to defined protein-protein interactions of PDE4 family enzymes which suggest that they are potential drug targets in a variety of major diseases, including cardiac and inflammatory diseases as well as neurological disorders. Developing small molecule inhibitors of these interactions would provide unique opportunities for pharmacological intervention and may overcome the dose-limiting side effects, in particular nausea and emesis, of the small molecule inhibitors that target the catalytic activity of PDE4 enzymes. With hot spots mapped, for example, in the HSP20 interacting domain of PDE4D5 [56] the grounds are laid for such developments. For a detailed understanding of PDE4-dependent protein-protein interactions structural analyses are needed. The structural analysis of PDE4 enzymes has so far mostly provided information about the catalytic units. 3D structures of PDE4 enzymes with their interacting partners would facilitate rational design of inhibitors. M. Houslay has had and still has (e.g.[62]) a great share in the elucidation of functions of PDE4 family enzymes and their protein-protein interaction.

Acknowledgements

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