CHAPTER 1

cAMP-Specific Phosphodiesterases: Modulation, Inhibition, and Activation

R. T. CAMERON and GEORGE S. BAILLIE

Molecular Pharmacology Group, Institute of Neuroscience and Psychology, College of Medical, Veterinary and Life Sciences, Wolfson Building, University of Glasgow, Glasgow, UK

1.1 INTRODUCTION

Cell surface, 7-span, transmembrane receptors recognize various environmental stimuli and transform them into intracellular signals via associated G proteins. This allows cells, tissues, and organs to alter specific aspects of their homeostasis in response to physical or chemical challenges. As such, cellular signals propagated in this way must be highly regulated so that their amplitude and timing produce a measured, appropriate response. The signal must be strong enough to produce the desired effect but also be transient so that the cell can easily prepare for other potential challenges. Additionally, the signal must also be targeted to the correct functional machinery, which often resides in discrete intracellular locations; hence signaling must be compartmentalized. To achieve all of these goals, cells have developed signaling molecules known as *second messengers* to convey complex information from receptors, temporally and in three dimensions, into the cell to signaling nodes where functional decisions are made. Although it is known that second messengers can take the form of lipids, gasses, ions, or nucleotides, discoveries around one such messenger, cyclic adenosine monophosphate (cAMP), provided the conceptual framework on which the second messenger concept was based [1]. Soon after its discovery in 1958 [2], it was realised that cAMP was synthesized at the membrane by adenylate cyclase in response to hormones and degraded to 5′-AMP by the action of cyclic nucleotide phosphodiesterases in the cytoplasm (reviewed in Ref. 1). One decade later, the discovery of the first cAMP effector molecule, Protein kinase A (PKA), was made and the cAMP signaling pathway was taking shape [3]. In the early 1980s, compartmentation of cyclic

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nucleotides was proposed by Brunton and colleagues when they noticed that stimulation of two different cardiac receptors (PGE receptor and the β-adrenergic receptor) both resulted in increases in cAMP and PKA activity, but only the β-agonist activated glycogen phosphorylase [4]. These different functional outcomes were underpinned by the activation of distinct PKA isoforms that were restricted to specific intracellular compartments [5].

Today, the notion of compartmentation within cell signaling pathways is widely accepted and there are many examples of *signaling nodes* where several key protein intermediates are anchored at discrete locations within cells. This is particularly true for the cAMP signaling pathway, where scaffolds, such as AKAPs, sequester PKA, phosphatases, phosphodiesterases, and PKA substrates to compartmentalize and orchestrate signals emitting from membrane-bound adenylate cyclase isoforms [6]. As cAMP positively transduces signals into the cell via PKA and the cAMP GEF EPAC [7], phosphatases and phosphodiesterases play the opposite role by dephosphorylating PKA substrates and hydrolizing cyclic nucleotides, respectively [8]. As hydrolysis by cAMP phosphodiesterases is the only route by which cAMP can be eliminated, these enzymes are poised to play a crucial role in intracellular signaling and as such represent excellent therapeutic targets [9].

Here we aim to review current knowledge on cAMP-specific phosphodiesterases and will describe their properties, distribution, and regulation and what is currently known about their inhibition by conventional-active-site directed compounds, novel allosteric inhibitor classes, and novel peptide disruptors. We will not discuss either cGMP-specific phosphodiesterases or dual cAMP-cGMP phosphodiesterses, as many more recent reviews have appraised current developments in those areas [10–16].

1.2 GENERAL CHARACTERISTICS OF PHOSPHODIESTERASES SPECIFIC FOR CYCLIC ADENOSINE MONOPHOSPHATE

1.2.1 Modular Structure of cAMP-Specific PDEs

Phosphodiesterases are divided into 11 families (reviewed in Ref. 17); PDE4, PDE7, and 8PDE are cAMP-specific, whereas PDE3, PDE6, and PDE9 are cGMP-specific, and the other 5 (PDEs PDE1, PDE2, PDE3, PDE10, and PDE11) have dual specificity with differing affinities for both types of cyclic nucleotide. The differing cyclic nucleotide specificity of specific PDE families is caused by a structural switch whereby a conserved glutamine residue within the catalytic unit is either free to rotate at will (dual specificity) or is locked into one of two positions by neighboring residues (one position = cAMP specificity; the other = cGMP specificity). As multiple genes with alternate splicing sites encode the various PDE families, the number of transcripts is large, and this results in the expression of a highly diverse collection of enzymes with divergent functional roles [18].

The modular structure of cAMP-specific PDEs (PDE4, PDE7, and PDE8) is represented in Figure 1.1.

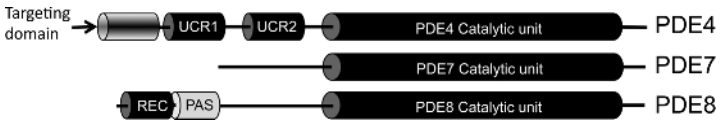

FIGURE 1.1 Modular structure of cAMP-specific phosphodiesterases. Schematic representation of the structure of cAMP-specific phosphodiesterase families, PDE4, PDE7, and PDE8. Each family has a conserved catalytic domain and a variety of unique regulatory domains, including UCR (upstream conserved region), *N*-terminal targeting sequence, PAS (period, arnt, sim) domains, and REC (receiver) domains, which are discussed in the text.

PDE4 has the most complex framework consisting of a subfamily specific *C*-terminal domain and dual regulatory domains, upstream conserved region 1 (UCR1) and upstream conserved region 2 (UCR2), together with an isoform-specific *N*-terminal region [19]. PDE8 is characterized by its *period*, *arnt*, *sim* domain (PAS), and all three families have a conserved catalytic domain that acts to hydrolyze cAMP. Alignment of the amino acid sequence of PDE4, PDE7, and PDE8 shows that 11 of the 17 conserved residues seen in all PDEs are located within the catalytic pocket of these enzymes. The cAMP hydrolyzing machinery of all three PDE families have a similar structure containing 16 compact alpha helices neatly orientated into three subdomains [17]. Current knowledge of the potential of each cAMP-specific PDE family as therapeutic targets will be presented in separate sections.

1.2.2 PDE4s: Characterization and Regulation

Diversity of Isoforms Pioneering studies on PDE4 family characterisation were done on the *Drosophila* dunce PDE locus that corresponds to the human PDE4D gene [20]. The fly PDE gene produced many transcripts, which corresponded to multiple distinct protein types, and this property is conserved in the mammalian PDE4D ortholog that results in the expression of 11 variants (PDE4D 1–11) [21,22]. PDE4s are encoded by four genes (A,B,C,D), and these give rise to at least 25 different proteins (6 PDE4A forms, 5 PDE4B forms, 3 PDE4C forms, and 11 PDE4D forms) via mRNA splicing and promoter diversity [19]. The fact that all PDE4 enzymes have been highly conserved over evolution suggests that they play an important role in cAMP homeostasis, and it is now thought that each isoform has nonredundant functional roles in underpinning the compartmentalization of cAMP signaling [23]. As all PDE4 isoforms have similar K_m and V_{max} values for cAMP hydrolysis, their functional role is determined largely by their cellular location, interaction with other signaling proteins, and posttranslational modification. Discrete intracellular targeting of individual PDE4 isoforms is most often directed by a *postcode* sequence within their unique *N*-terminal domain (see Fig. 1.2) [24]. This region is responsible for promoting many of the protein–protein interactions and one protein–lipid interaction that act to anchor PDE4s to signaling nodes in subcellular compartments.

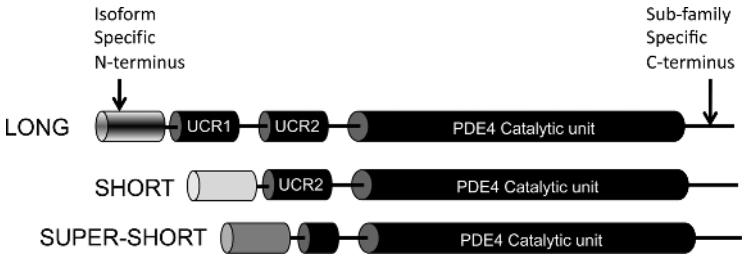

FIGURE 1.2 Modular structure of long and short PDE4 isoforms. PDE4 genes encode a variety of PDE4 isoforms that are categorized as long, short, and supershort. Alternate mRNA splicing allows expression of PDE4s with different combinations of UCR1 and UCR2 regulatory modules. Longforms contain UCR1 and UCR2, shortforms only UCR2, whereas supershortforms contain a truncated version of UCR2.The N-terminal region contains the targeting sequence and is unique to specific isoforms, the C-terminal region is sub-family specific.

PDE4 enzymes can be broadly subdivided into four categories according to their sequence length and differential expression of regulatory modules (Fig. 1.2) [25]. Long isoforms contain UCR1 and UCR2, shortforms lack UCR1, supershortforms lack UCR1 and express a truncated UCR2, and dead shortforms lack UCR1 and UCR2 [26]. As stated previously, each isoform has a conserved catalytic unit and a unique *N*-terminal region. Additionally, all PDE4s have a *C*-terminal region that extends past the catalytic unit, and this is sub-family-specific [17]. The intricacy and complexity of each of the components described above is now becoming clear and will be discussed in the following review; however, it is obvious that the domain structure of PDE4s allow the cell to "dial up" bespoke PDE4s to fit immediate requirements for cAMP hydrolysis in a diverse range of cell types and tissues [27]. Such tailored expression allows precise targeting and regulation of PDE4s to control and shape local cAMP pools.

N-Terminal "Anchor" The unique *N*-terminals of PDE4s are encoded by 5′ exons that are preceded by isoform-specific promoters. Many studies on the compartmentation of PDE4 enzymes have concluded that the *N*-terminal region directs their distribution by promoting the formation of complexes with scaffolds, regulators, or lipids. These include the scaffold proteins RACK1, AKAP18 [28], and β-arrestin [29–31], SRC family tyrosine kinases [32–34], immunophillin XAP2 [31], mAKAP [6], dynein complex member Nudel [35], and the β_1-adrenergic receptor [36]. PDE4A1 is unique in that it is the only PDE4 discovered so far that is anchored to membranes by its *N*-terminal [37] and, as such, has provided the paradigm for elucidation of the *N*-terminal of PDE4s as tethers [38]. PDE4A1 is entirely membrane-bound locating to the Golgi apparatus and Golgi vesicles; however, if the unique 25mer *N*-terminal is removed, the PDE becomes cytosolic and more active [39]. Moreover, cytosolic proteins such as GFP or chloramphenicol acetyltransferase can be rendered membrane-associated by simply engineering the addition of the PDE4A1 25mer [40] and a 25mer peptide corresponding to the 4A1

N-terminal sequence inserts into lipid bilayers in <10 ms, as evidenced by stop-flow analysis [41]. Subsequent work showed that the membrane association of PDE4A1 was triggered by calcium [41]. These studies demonstrate that all the information required for intracellular targeting is encompassed within the *N*-terminal of PDE4A1 and this observation paved the way for the general consensus that the *N*-terminal region of PDE4s is essential for intracellular localization of PDE4s [38].

Further proof that confirmed the role of the unique regions of PDE4A subfamily members in conferring their cellular localisation was the observation that the *N*-terminal region of PDE4A4 and its rat homolog PDE4A5 contain a number of SH3 binding domains that direct binding to a number of members of the SRC-tyrosyl kinase family [33]. This association alters the conformation of PDE4s such that they exhibit an increase in sensitivity to rolipram in addition to directing the intracellular targeting of the enzyme. Additional experimentation also showed that one of the PDE4D enzymes, PDE4D4, also had the ability to associate with src, lyn, and fyn via its *N* terminal [34].

Another first for the PDE4 field was the realization that *N*-terminal targeting could result in the dynamic redistribution of PDE4s following receptor ligation. Thus, although PDE4s were anchored, that did not mean they were static but instead could be recruited by other proteins to sites of high cAMP concentrations within the cell [42]. This paradigm was based on studies of the PDE4-β-arrestin complex [43] that is translocated to activated β_2-adrenergic receptors following agonist occupancy. Thus, an active pool of PDE4D5 is recruited to the site of cAMP synthesis at the membrane to kickstart the desensitization process [44]. Functionally, the dynamic movement of PDE4 was shown to downregulate PKA phosphorylation of the β_2-adrenergic receptor by the AKAP79-PKA complex [45]. This, in turn, promoted switching of β_2-adrenergic receptor signaling via adenylate cyclase activation through G_s, to inhibition via G_i and subsequent activation of the ERK MAP kinase pathway [46]. Although β-arrestin was shown to bind all PDE4s via a docking region on the catalytic domain of the enzyme, an extra binding site within its *N*-terminal sequence conferred preference for PDE4D5 [30,31]. The involvement of PDE4D5 in desensitising the β_2-adrenergic response represented the first time a function had been ascribed to a single PDE4 species and hatched the idea that each isoform serves a nonredundant regulatory role [42]. This notion has been supported by a diverse body of research utilizing a variety of techniques such as yeast 2 hybrid [31], cAMP reporters [47,48], small interfering RNA (siRNA) RNA [46,48], peptide array [30], PDE4D5 dominant negative constructs [45], PDE4D knockout mice [49], and peptide disruption molecules [50].

The concept of intracellular targeting and assembly of PDE4s, into signaling complexes via *N*-terminal postcodes, does not mean that single isoforms undertake only one function. PDE4D5, for example, is known to form high-affinity complexes with both arrestin and RACK1 by virtue of interactions mediated by the PDE's unique *N*-terminal [30]. As both scaffolds bind to a small, defined area within the *N*-terminal of PDE4D5, albeit via overlapping but different amino acids, binding of the two scaffolds is mutually exclusive [50]. Rather than binding concomitantly, RACK1 and arrestin compete to sequester defined "pools" of PDE4D5. More

recently, a function for the RACK1 sequestered subpopulation of PDE4D5 was discovered in the field of cancer biology. In association with focal adhesion kinase, the RACK-PDE4D5 complex forms a *direction sensing module* that regulates cancer cell polarity and spreading [51]. Alterations in RACK1 expression may influence β_2-adrenergic signalling via changes of the amount PDE4D5 associated with β-arrestin scaffolds and conversely β-arrestin expression could similarly affect the manner in which cancer cells sense directional cues.

UCR Regions The key functional role of the UCR regions is in regulating PDE4 activity changes triggered by phosphorylation of the enzyme by ERK MAP kinase and PKA. Longform PDE4s contain the UCR1 module, which houses a PKA site to allow activation of the enzyme by the kinase [52]. This important modification allows feedback regulation by increasing the capacity for cAMP hydrolysis at times when the nucleotide is in high concentration and constitutes an essential part of the signal desensitization process. The catalytic unit of all PDE4s, save those from the PDE4A subfamily, have an ERK consensus motif near their *C*-terminal end [53]. The functional outcome of ERK phosphorylation is determined by discerning which UCR modules are expressed by the enzyme [54]. UCR1 regions orchestrate inhibition of long isoforms, and its lack in short isoforms promotes activation [55]. The activity of supershortforms is unaffected by ERK phosphorylation. Changes in expression of long and short isoforms can therefore elicit a range of cellular responses to hormones such as isoprenaline that raise cAMP or EGF that promote ERK activation. Indeed, transitory rises in cAMP can be educed by ERK activation if long isoform PDE4s are in ascendance, as the inhibition by ERK will be countered by activating PKA phosphorylation triggered by rising cAMP levels [55]. Another level of regulation is conferred by an as yet unknown kinase activated by reactive-oxygen species. Although this kinase phosphorylates PDE4s at a site within the *N*-terminal part of the catalytic domain, it switches the effect of the phosphorylation by ERK on PDE4 long isoforms from inhibition to activation [30]. Presumably, this phosphorylation attenuates interactions between the UCR1 and UCR2 modules and the catalytic unit to reprogram the functional consequences of ERK phosphorylation. The UCR regions also have a role in protein docking and have been indentified as binding sites for (SUMO) E3 ligase UBC9 [56]. Dimerization of PDE4 long isoforms is also mediated by the UCR regions [57], and such oligomerization is essential for the maintenance of UCR regulatory properties and inhibitor sensitivities [58]. Mutants of PDE4 long isoforms that do not dimerize are not activated by PKA phosphorylation in UCR1 and show decreased sensitivity to the PDE4 inhibitor rolipram [58].

C-Terminal Site The *C* terminal of PDE4s is conserved within subfamilies and until 2010, there had been a paucity of information surrounding the role of this region. A more recent report on inhibition of the phosphatase, calcineurin by the immunosuppressant cyclosporine A (CsA), reported two novel sites in the *C* terminal of PDE4D isoforms that directed sub-family-specific regulation [59]. First, the sequence directly abutting the end of the catalytic domain in PDE4D was identified

as a phosphodegron motif. Specifically, the sequence D-pS-G-X_{2-4}-pS (where pS is phosphorylated serine and X is any amino acid) is known to direct polyubiquitin-mediated degradation of phosphoproteins via the Skp-cullin-Fbox (SCF) E3 ubiquitin ligase complex and in this case, the *C*-terminal site was dually phosphorylated by GSK3β and casein kinase 1 (CK1) to direct degradation of the PDE4 [59]. Interestingly, calcineurin opposed the activity of the kinases by maintaining PDE4D isoforms in the unphosphorylated state, and a typical calcineurin docking motif or PXIXIT domain was the second new site to be uncovered by this report. The *C*-terminal region of PDE4A5 has also been implicated in its interaction with the p75 neurotrophin receptor [60]. Intruigingly, *C*-terminal targeting of the PDE enhances cAMP degradation in the vicinity of the receptor to trigger extracellular proteolytic activity that serves to promote matrix remodeling that is essential for fibrin clearance and tissue repair. Discovery of the p75–PDE4A5 interaction has provided a crucial step into the understanding of the molecular mechanisms that regulate fibrin deposition in a variety of diseases, including stroke, atherosclerosis, and pulmonary disease and offers a novel therapeutic route via development of PDE4A selective inhibitors [61].

Regulation of PDE4 Function by Posttranslation Modifications Other than Phosphorylation It has been established that both ubiquitin and SUMO modifiers can regulate PDE4s selectively [56,62]. In the case of ubiquitin, the E3 ligase, Mdm2, is sequestered by β-arrestin to allow rapid and transient ubiquitination of PDE4D5 in response to β-adrenergic stimuli [62]. Critical for this is the ubiquitin-interacting motif (UIM) found in the PDE4D subfamily specific *C*-terminal region. Ubiquitination of PDE4D5 occurs at three lysine residues in the unique *N*-terminal of PDE4D5 and also at a single lysine in the UCR1 region. Analysis revealed that monoubiquitination at the UCR1 site primes the enzyme for subsequent polyubiquitination at the other sites, thus changing the conformation of the protein, increasing the fidelity of its interaction with β-arrestin. The net effect of the ubiquitin modification is to increase the pool of PDE4D5 associated with arrestin molecules while concomitantly decreasing the fraction sequestered by other PDE4D5 scaffolds. Functionally, this promotes a more efficient desensitisation of the β_2-adrenergic receptor.

Unlike ubiquitination, which can occur on any surface associated lysine, SUMO modification of many proteins takes place at a consensus motif (hydrophobic-K-any amino acid-E) [63]. Isoforms from the PDE4A and PDE4D subfamilies contain a single SUMO motif within their catalytic domains that can be selectively modified by SUMO [56]. This reaction is catalyzed by the SUMO E3 ligase PIASy, which binds to PDE4s via two sites, one in the catalytic domain and one in the UCR1 region. SUMO modification of PDE4D long isoforms serves to augment their activation by PKA phosphorylation and repress their inhibition by ERK phosphorylation and represents another means whereby cells can achieve the selective modulation of the activity of cAMP-specific PDE enzymes.

Another regulatory modification specific to PDE4A5 is cleavage by the protease caspase 3 during apoptosis [64]. Sequences within the unique region of this enzyme

contain caspase 3-specific cleavage motifs that serve to remove a 10-kDa(kilodalton) segment from the *N*-terminal. This region confers an ability to bind SH3 domains of the tyrosyl kinase, LYN, and its removal alters the activity of PDE4, its sensitivity to inhibitors and its localization to perinuclear anchor points. The importance of the localization of PDE4A isoforms in modulation of the apoptotic pathway was realized when it was discovered that full-length PDE4A5 protects cells from staurosporine-induced apoptosis whereas redistributed, cleaved PDE4A5 does not.

Long PDE4 isoforms have been shown to have binding sites for the lipid phosphatidic acid (PA) [65], and direct association of PA with PDE4s activates the enzyme by increasing the V_{max} without affecting the enzyme's K_m. For PDE4D3, the PA binding site is known to reside within a sequence of the *N*-terminal region rich in basic and hydrophobic residues [66]. These observations suggest that an accumulation of cellular PA during mitogenic stimulation of thymocytes concomitantly decreases cAMP levels by directly activating PDE4 longforms [67].

1.2.3 Inhibition of PDE4 as a Therapeutic Strategy

Pharmacologic Inhibition of the PDE4 Active Site The development of selective PDE4 inhibitors initially interested pharmaceutical companies for two reasons: (1) elevated cAMP within immune cells was found to be antiinflammatory and (2) PDE4 isoforms made up the largest percentage of cAMP hydrolyzing PDEs in immune cells [68]. PDE4 inhibition by compounds that targeted the active site of the enzyme bestowed therapeutic advantages in animal models of chronic obstructive pulmonary disease (COPD) [69,70], rheumatoid arthritis [71], inflammatory bowel disease [72], and psoriasis [73]. PDE4 inhibitors have also been shown effective in promoting memory function [74], treating depression [75,76], protecting against some aspects of Alzheimer's disease [77], and reversing age-associated memory deficits [78]. There is also a growing body of evidence suggesting that PDE4 inhibition may also be effective for the treatment of certain cancers [79]. So, in theory, PDE4 inhibitors have great potential, however, in practice, their clinical utility is compromised by mechanism-associated side effects that limit maximally tolerated doses (reviewed in Refs. 80 and 81). Headache, nausea, emesis, and diarrhea are the most commonly reported side effects, and these stem from the inhibition of PDE4s in nontarget tissues. Specifically, PDE4D expression is high in an area of the brain known to trigger nausea called the *area postrema* [82,83], and PDE4 inhibition may also act directly on the gastrointestinal tract [83]. Other possible side effects that have been suggested on the basis of genetic ablation in animal studies include the development of immunosuppression [70], heart failure, and arrythmia [84], although these have not been noted in clinical trials of PDE4 inhibitors that have passed phase III clinical trials. Indeed, such studies lead to loss of protein and complete cessation of the PDE4 subfamily through development into adulthood. This is very different for the use of a competitive inhibitor in a person.

One strategy that has been proposed to reduce the systemic side effects of isoform-nonspecific PDE4 inhibitors has been the development of compounds that potentially

had a degree of selectivity for non-PDE4D types [85]. As the catalytic units of all PDE4 isoforms show a high degree of similarity in structure and sequence, synthesis of isoform or sub-family-specific inhibitors has been difficult [86]. However, more recent analysis of the many crystal structures available has pointed to a region called the *M loop* that differs slightly between PDE4D and PDE4B isoforms, and this region can be targeted pharmacologically. Indeed, a PDE4 inhibitor developed in 2009 illustrated the potential of this approach with a 100-fold selectivity of PDE4B versus PDE4D, potent antiinflammatory effects *in vivo*, and little emetic side effects [87]. Despite the drawbacks of general PDE4 inhibitors, one such compound has been approved by the European Commission for the treatment of severe chronic obstructive pulmonary disease (COPD). Roflumilast (reviewed by Fabbri et al. [88]). This has a range of antiinflammmatory properties and shows efficacy in animal models of inflammatory diseases of the airways [89]. Roflumilast has been licensed as an *add-on* treatment to be administered with longacting bronchodilators, and this strategy has decreased symptoms of COPD and causes a reduction in disease exacerbations [90] via reductions in pulmonary inflammation [28].

Novel Allosteric PDE4 Inhibitors All of the PDE4 inhibitors developed by pharmaceutical companies to treat diseases such as schizophrenia, asthma, prostate cancer, and osteoporosis are competitive inhibitors of cAMP hydrolysis by PDE4s. When in high concentrations, these compounds prevent cAMP from accessing the catalytic pocket of the enzyme and in so doing, effectively raise cAMP above normal, physiological concentrations. Such inhibitors tend to have no or little selectivity for subfamilies, as the catalytic domains of all PDE4s show a high degree of structural similarity. The elevation of cAMP to supraphysiological levels in such a nonspecific manner has resulted in serious side effects that have rendered most of the current crop of PDE4 inhibitors unsuitable for the clinic. More recently, however, a novel class of PDE4 inhibitors has been designed using detailed knowledge about the structural regulation of the enzyme [91]. It has been known for some time that the UCR2 region is autoinhibitory for the catalytic machinery of PDE4 [92] and that PKA phosphorylation releases this inhibition [93], although the structural complexity of this inhibition/activation switch had remained a mystery. Novel structures showing the UCR2 region's association with the catalytic pocket suggested that the inhibitory function of UCR2 was dependent on its ability to fold across the catalytic site to occlude cAMP and gate its access [91]. Analysis of such structures from the catalytic domains of PDE4D and PDE4B and subsequent mutation of key residues informed the design of allosteric inhibitors that bind preferentially to residues in UCR2 to change its conformation to ensure that the PDE4 would be effectively "capped" by UCR2. Interestingly, as the amino acid sequences in the UCR2 gating sequence differ slightly between subfamilies, the novel compounds exhibited high selectivity for PDE4D isoforms without evoking the expected emetic effects. The lack of side effects is thought to be due to the novel action of these inhibitors in controlling access to the active site without fully inhibiting enzymatic activity, and this strategy may lead to the development of a novel, safer class of PDE4 inhibitor [94].

Alternative Strategies for Inhibition of Localized PDE4 Pools As stated earlier in this chapter, cAMP is the archetypal second messenger produced in response to a variety of stimuli in many cell types to regulate specialized processes such as growth, differentiation, movement, and heartbeat [1]. PDE4s play a pivotal role in underpinning compartmentalized cAMP responses by creating gradients that are sampled and acted on by appropriately positioned subpopulations of cyclic nucleotide-gated channels [95], PKA [96], and EPAC [7]. Evidence suggests that in many situations, targeted PDE4 enzymes control the magnitude and duration of cAMP-dependent events (reviewed in Ref. 44), and only relatively recently have the nonredundant functions of different PDE4 isoforms been investigated. Critical to this has been the development of novel technologies that have helped define the role of targeted subpopulations of PDE4. The real breakthrough in this regard, came with the invention of optical probes that were able to define spatiotemporal flux of cAMP in living cells [97]. Visualization of discrete cAMP microdomains in cardiac myocytes in reponse to β-agonists provided groundbreaking evidence that cAMP was not free to diffuse at will, but was restricted to the vicinity of the sarcoplasmic membrane by phosphodiesterase activity [98]. Further studies using these FRET probes showed that the targeting of PDE4s shaped cAMP gradients resulting from prostaglandin E treatment of HEK293 cells [99] or β_2-adrenergic stimulation of cardiac myocytes [100]. The conclusion of this work was that cAMP gradients were not formed by enzymatic barriers of PDE4, but rather, that in areas of high PDE4 expression, a sink would be formed to locally drain cAMP [23]. Such targeting of PDE4 isoforms would allow multiple gradients of cAMP to be created simultaneously in response to one receptor-mediated event and form hotspots where cAMP concentrations could be increased sufficiently above the threshold of activation for cAMP effectors such as PKA and EPAC. The notion that PDE4 hotspots act as cAMP sinks has also been supported by studies that have used PDE4 isoforms themselves as cAMP reporters [99,101].

As the utilization of specific inhibitors to ablate PDE4 activity affects all PDE4 isoforms to the same degree, compounds such as rolipram have been ideal for pinpointing general functions of the PDE4 family but have not been able to facilitate understanding of the unique, nonredundant roles that PDE4 isoforms play in shaping compartmentalized cAMP cell signaling [42]. Such analysis is now possible using novel technologies [24] such as siRNA knockdown [46], dominant negative approaches [102], peptide disruption [50] (see Fig. 1.3), and using knockout mice [103].

The Dominant Negative Approach The dominant negative approach relies on ectopically expressed forms of PDE4 that have been engineered, by a single point mutation in their catalytic site, to be incapable of hydrolyzing cAMP [45,102]. The construction of these mutants became possible only after publication of the crystal structure of the PDE4B2 catalytic unit [104]. This study identified seven residues that were conserved in all PDE4s and essential for catalysis. Mutants, which contained any of these residues converted to alanine, were completely inactive, and overexpression did not change global PDE4 activity of the cell [24]. Such overexpressed,

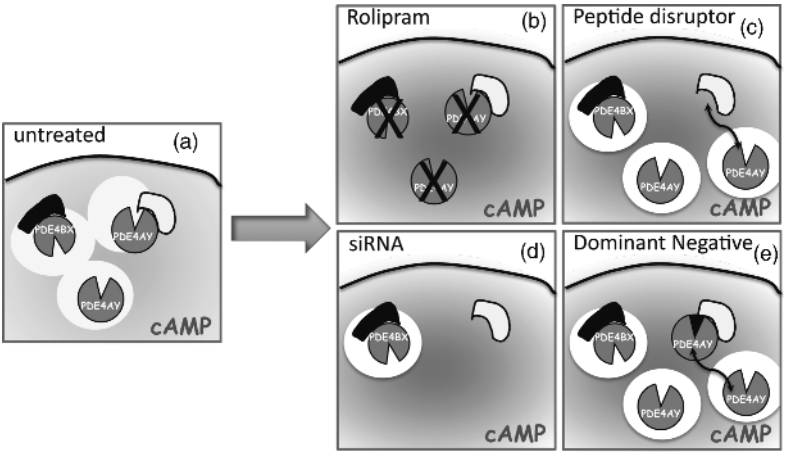

FIGURE 1.3 Alternative strategies for the inhibition of compartmentalized PDE4 pools. Areas of high PDE4 expression act as cAMP sinks to form discrete cAMP gradients in response to the activation of G-coupled-receptors. (a) Under basal conditions, compartmentalized PDE4 isoforms form sinks to keep cAMP (pink) levels low in order to prevent inappropriate phosphorylation of PKA substrates (black- and white-shapes) under basal conditions. (b) Specific PDE4 inhibitors attenuate equally, the activity of different compartmentalized PDE4 isoforms, allowing global cAMP levels to rise throughout the cell following G-coupled-receptor activation. (c) Peptide disruption strategies allow selective interruption of a single PDE4 isoform at one site within the cell to allow cAMP increases in a defined cellular microdomain. Other compartmentalized pools of the same isoform remain unaffected and maintain normal cAMP metabolism in those locations. (d) Selective genetic silencing of a specific PDE4 isoform increases cAMP concentrations in all cellular locations where that isoform is expressed. (e) Displacement of an endogenously expressed, active PDE4 isoform by an ectopically expressed, catalytically dead, cognate enzyme, increases cAMP concentrations in all cellular locations where that isoform is expressed. *(See insert for color representation of this figure.)*

exogenous forms have been shown to displace the cognate endogenous species from their site of anchor within cells to enable detection of functional significance of the replaced species [102]. Experimentation using dominant negative constructs have helped reveal the importance of PDE4D5 in the desensitization of signals resulting from activated β_2-adrenergic receptors [45,47], PDE4D3 and PDE4C2 to gate activation of AKAP450-anchored PKA type II in the perinuclear region under basal conditions [102], tethered PDE4D3 isoforms in regulation of basal cAMP dynamics in a subplasmalemmal compartment [47], PDE4D5 in the shaping of dynamic cAMP events mediated by prostanoid receptor activation [47], PDE4D5 in influencing glucose-induced glucose-like peptide 1 release [105], PDE4D8 in control of cAMP signals following β_1-adrenergic receptor activation [36], and PDE4D5 in the formation of nascent adhesions, cell polarization, and cellular spreading of cancer cells [51]. Although the dominant negative approach is clearly a powerful technique for elucidating the functions of anchored PDE4 isoforms, it is limited by the fact that it relies on ectopic overexpression, making functional analysis in primary cells

difficult. Another drawback relates to differently targeted subpopulations of the same isoform, which will be displaced simultaneously Fig. 1.3e. This may render investigation of functional outputs problematic for isoforms such as PDE4D5, which have multiple nonredundant functions within the same cell or tissue.

PDE4 Silencing via siRNA Another technique that can selectively attenuate the local activity of individual PDE4 isoforms is genetic silencing using siRNA. Selective silencing was first used to identify the functional importance of PDE4D5 in controlling the PKA/AKAP79-mediated switching of the β_2-adrenergic receptor to the ERK MAP kinase pathway [46]. Subsequent development of PDE4B-directed siRNA sheds light on the ability of PDE4B enzymes to control the activity and cellular location of DNAPK, a critical kinase that acts to repair double-stranded breaks in damaged DNA and to phosphorylate the cell survival kinase, PKB/Akt [106]. Cyclic AMP breakdown in the nucleus was compromised following PDE4B knockdown, allowing activation of nuclear EPAC that triggered Rap2-mediated DNA-PK nuclear exit and to the activation of DNAPK. The functional selectivity of PDE4B was revealed when it was discovered that neither siRNA silencing of PDE4D nor treatment of cells with the pan PDE4 inhibitor rolipram could recaplitulate these actions [106]. PDE4B siRNA has also been used with good effect to define the role of PDE4B2 in the degradation of agonist-induced renovascular cAMP in smooth muscle cells derived from spontaneously hypertensive rats [77]. Selective silencing of PDE4A and PDE4D isoforms have also implicated these enzymes in the regulation of epithelial–mesenchymal transition induced by TGF-β_1 [107] and repression of PDE4D or PDE4B isoform expression inhibited interleakin 2 (IL2) release following CD3/CD28 stimulation of primary human T cells [108]. Finally, knockdown of lesser-studied PDE4C isoforms significantly enhanced glucose-dependent insulin secretion in rat insulinoma-derived cells [109]. However, although the siRNA approach can provide essential clues regarding the function of specific PDE4 isoforms and highlight the potential therapeutic benefits of isoform-specific inhibitors, the siRNA approach does not easily translate to the clinic [24]. It also suffers from the same lack of specificity as the dominant negative strategy as it cannot target differently located subpopulations of the same isoform (Fig. 1.3d).

Peptide Disruptors of Localized PDE4 Pools As targeting and compartmentalization are fundamental to the functioning of PDE4s, PDE4 inhibitors generate unpleasant side effects as a result of the universal inhibition of all isoforms [8,21]. As stressed above, dominant negative and siRNA approaches are also limited by the fact that they are not directed against discretely localized subpools of individual PDE4 isoforms. In this regard, future PDE4 isoform-selective inhibitors should be aimed at the cellular targeting of the enzymes rather than their catalytic activity. One such approach has been facilitated by the introduction of peptide array technology, a technique that allows rapid determination of the molecular nature of protein–protein interactions [110]. The technique uses the sequence of one of the interacting partners to generate a library of spotted, immobilized peptides

(overlapping 25mers, each shifted by five amino acids) that is probed by a purified, recombinant form of the other protein partner and detected using standard, western blotting techniques protocol. Positive spots contain putative binding sequences that can then be used to inform mutagenesis studies to map interaction sites in cellular proteins. The identified sequences can also be transferred into powerful small peptide agents that have the potential to interfere with protein interactions in vivo and help link targets with phenotypes [110] (Fig. 1.3c). This approach has been used extensively in the cAMP signaling field to map interactions between PDE4 enzymes and the signaling proteins DISC1 [111], β-arrestin [50,94], RACK1 [30], EPAC [112], Ndel [35], and the SUMO E3 ligase PIASy [56]. A modified version of the technique whereby activated kinases or ubiquitin/SUMO ligases are overlayed onto PDE4 peptide arrays has also been used to study the sites of posttranslational modification of phosphodiesterase complexes by PKA phosphorylation, MDM2-directed ubiquitination, and UBC9-directed SUMOylation [56].

A pioneering study investigating the protein scaffolding of PDE4D5 pinpointed the peptide array as being a rapid and accurate method of predicting anchor sites on PDE4s [30]. Conventional studies utilizing yeast 2 hybrid, NMR, and mutagenesis mapped interaction sites for association of the scaffold protein, RACK1 within the [113] *N*-terminal region of PDE4D5 [29,31,50]. Reassuringly, peptide array analysis identified the same sequence and delineated a second, previously unknown, RACK1 interaction site within the catalytic unit of PDE4D5 [30]. Two known sites on PDE4D5 were also confirmed for the binding of another important signaling scaffold, β_2-arrestin. As the *N*-terminal site for RACK1 association overlapped with one of the β-arrestin sites, simultaneous overlay of the PDE4D5 array with both proteins revealed that RACK1 and β-arrestin compete for the same docking site [30]. Subsequent alanine scanning peptide array analysis revealed that, although both proteins bound to the same linear stretch of sequence, many of the actual amino acids involved in docking of each protein were distinct. With respect to protein complex formation, this meant that association with PDE4D5 was mutually exclusive for RACK1 and β-arrestin, due to lack of availability of the docking site. However, for the design of peptide disruptor molecules, differences in key interacting residues for each partner meant that it was possible to design peptide agents that could disrupt PDE4D5's interaction with RACK1 or β-arrestin or both at the same time [50]. Subsequent evaluation of cell permeable versions of these peptide disruptors proved that the disturbance of targeted pools of the same isoform (in this case PDE4D5) could lead to very different functional outcomes. The β-arrestin-PDE4D5 disruptor, for example, attenuated recruitment of PDE4D5 the β_2-adrenergic receptor leading to a hyper-phosphorylation of the receptor after stimulation [50], whereas the RACK1-PDE4D5 disruptor was effective in preventing the formation of spreading initiation centers in cancer cells [51]. Clearly, both peptides have potential as therapeutics, the former for the treatment of asthma, where PDE4D5 is upregulated following chronic bronchodilator use [114] and is a key regulator of β_2-adrenergic(β_2Ar)-induced cAMP turnover within human smooth muscle [48]; the latter is an agent for preventing polarization and metastasis of cancer cells [51].

PDE4 Knockout Mice Gene targeting by homologous recombination in mouse embryonic stem cells and injection into early-stage mouse embryos to produce germline chimeras has been established as a means of silencing specific loci in the mouse genome. This method has been used to study function of PDE4B, D subfamily genes in PDE4 knockout mice [115]. This approach has been valuable for the identification of PDE4D isoforms in the pathophysiology of asthma where PDE4Ds have a crucial role in the contraction of airway smooth muscle [25], muscarinic receptor signaling [49], and recruitment of neutrophils to the lung [116]. Studies using PDE4D knockout mice or cells derived from them have also supported the role of this subfamily in the desensitization of the β_2-adrenergic receptor [117,118] and excitation–contraction coupling in heart muscle [84]. Isoforms from the PDE4B subfamily have also been implicated in asthma, but in contrast to PDE4D knockout animals, PDE4B silencing is necessary for immune signaling via toll-like receptors in monocytes and macrophage [113,119], and its expression is required for the development of allergen-induced airway hyperresponsiveness and T(h)2-driven inflammatory responses [120]. Studies using PDE4 knockout mice have undoubtedly provided proof of concept that PDE4 inhibitors with selectivity for subfamilies or isoforms may be useful in the treatment of asthma.

1.2.4 PDE7

Characterization PDE7 was first isolated from a cDNA complementation screen in *Saccharomyces cerevisiae*. One of the gene products originally termed *high-affinity cAMP-specific PDE* (HCP1) had a predicted size of 498 amino acids, and it hydrolyzed cAMP with a low K_m, while cGMP had no effect, even at relatively high concentrations. HCP1 was shown to be insensitive to both the PDE3 inhibitor milrinone and PDE4 inhibitor rolipram, and despite sequence similarities with other cAMP-specific PDEs, represented a novel class of PDE designated PDE VII [121]. The isolated cDNA clone of PDE7 from human glioblastoma cells was designated PDE7A1. An alternative splice variant was isolated subsequently from mouse skeletal muscle using the full PDE7A1 ORF as a probe. The splice variant, PDE7A2, ORF encoded a 456 amino acid protein with a predicted molecular weight of 52.4 kDa. PDE7A2 shared 90% identity with PDE7A1, although the 5′ end of this new variant was divergent from PDE7A1 and was shown to be more hydrophobic. A recombinant baculovirus expression system in sf9 cells demonstrated that the biochemical and pharmacological characteristics of the PDE7A2 splice variant were almost identical to those of PDE7A1 expression in *Saccharomyces cerevisiae* [122].

Further studies on the new cAMP-specific PDE identified PDE7 activity in T-cell lines [123]. The pharmacological characteristics matched those of both PDE7 variants [122] and the presence of a 55-kDa band in a human T-cell line matched the predicted 498–amino acid PDE7A1 cDNA reported previously. PDE7A1 expression was also assessed in a number of B-cell lines, but despite comparable mRNA expression levels to T cells, the activity of PDE7 in B cells was undetectable,

suggesting posttranslational regulation of PDE7 within these cell types [122]. Both isozymes of PDE7 have also been cloned from human skeletal muscle [124], confirming PDE7A2 as a novel 5′ splice variant of PDE7A with a hydrophobic *N*-terminal end encompassing 20 residues. The localization of PDE7A2 to particulate fractions was suggested as the reason why it had previously escaped detection in PDE activity assays, due to use of only soluble fractions in the previous studies. Experimentation into PDE7A2 activity found only marginal differences in the kinetics of cAMP hydrolysis compared with PDE7A1, although the expression of PDE7A2 is the more prevalent of the two isozymes found in human tissue [125].

A further splice variant of PDE7A was later isolated from $CD4^+$ T cells, PDE7A3. This novel isozyme was undetectable in resting T cells, but both mRNA and protein levels were strongly induced following T-cell stimulation. PDE7A3 has an almost identical sequence in the catalytic domain as PDE7A1; however, PDE7A3 had no measurable activity when the recombinant protein was expressed in sf9 cells. Using the known crystal structure of PDE4B2B, the sequences of PDE7A1, 3 were aligned in order to compare the catalytic domains of the splice variants. PDE7A1 was shown to contain all the homologous helices surrounding the catalytic domain, whereas PDE7A3 was missing half of helix 15 and all of helix 16. Although these structures are not thought to have an immediate role in the proteins' catalytic properties, this may give rise to PDE7A3's intrinsic inactivity [126].

A second gene encoding a PDE7 isozyme was discovered using an EST database search [127]. An unknown gene with homology to PDE7A was found in an EST derived from mouse mammary gland. The ORF from the clone predicted a 446–amino acid protein with a catalytic domain with 30–40% homology to other PDEs. The homology with PDE7A in this region was found to be around 70% and was the basis of its classification as a novel PDE7 gene. The pharmacological characteristics of PDE7B were similar to those of PDE7A with respect to inhibitor specificity; it was, however, noted that the IC_{50} of several inhibitors tested were markedly different, which could potentially lead to isozyme-specific selective inhibitors for the PDE7 family [127]. The human variant of PDE7B was cloned using similar EST searching techniques in two separate studies and shown to have a predicted protein size of 450 amino acids. The characteristics were only slightly different from those of 7B isolated in mouse. The human 7B hydrolyzed cAMP with a slightly higher K_m, while the IC_{50} of IBMX was almost doubled [128,129]. Various splice variants of PDE7B have been found in rats [130]; however, no such variants of the human gene, which maps to chromosome 6q23–24 [129], have not yet been isolated.

Expression The expression profiles of the two PDE7 gene variants are similar. PDE7A is highly expressed in human skeletal muscle and B-and T-cell lines and detected in heart, fetal, and brain tissue [121,122,124,131–133]. While PDE7B is most abundantly expressed in the pancreas, brain, heart, thyroid, and skeletal muscle, it is also detectable in the eye, liver, and various other tissues [127,129]. The ubiquitous expression of PDE7A in proinflammatory and immune cells [134] drew attention to PDE7A as a potential target for therapeutic intervention in a variety of immunological diseases. The promoter sequence of PDE7A, for example, has been

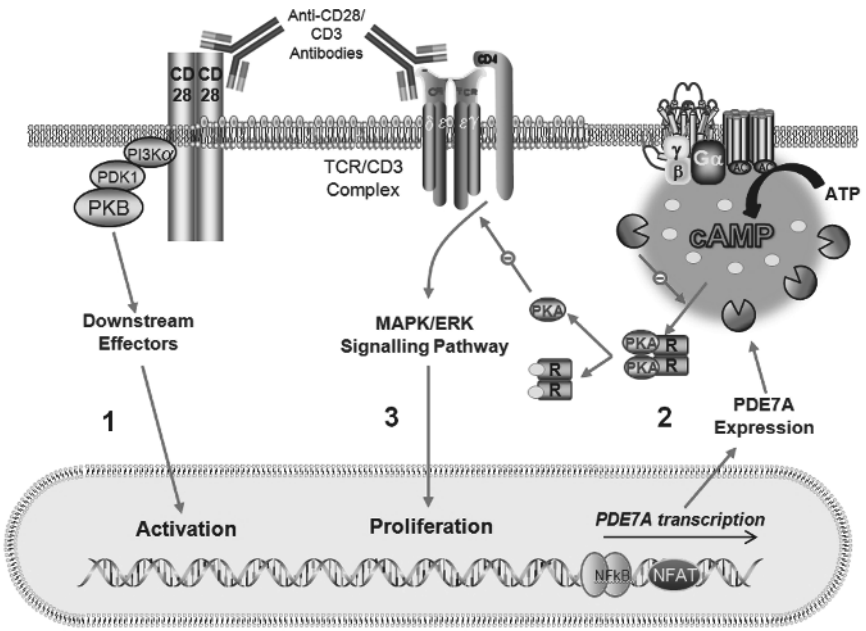

FIGURE 1.4 TCR/CD-28 costimulation induces rapid PDE7 upregulation leading to activation and proliferation of $CD4^+$ T cells. Antibody targeting of the CD28 and CD3 ligands, in resting T cells, is a method for activating T cells and has been used extensively to study T-cell proliferation *in vitro*. Following TCR/CD28 costimulation phosphoinositide 3 kinase, (PI3K) becomes activated, resulting in phosphorylationof various downstream signaling kinases, including PDK1 and PKB **(1)**. These kinases, in turn, phosphorylate various effector proteins transcription factors such as NFκB and NFAT. The regulatory sequence of the PDE7A gene contains consensus for NFκB and NFATb. The *N*-terminal region binding sites and these transcription factors are thought to regulate PDE7A expression. Once T cells are activated, PDE7 transcription is induced, rapidly leading to increased protein levels that are detectable within 1 h and reach maximum after 8 h. High basal levels of cAMP are known to have an inhibitory effect on T-cell proliferation through PKA activity. PKA prevents $CD4^+$ T-cell proliferation and IL2 production by inhibiting the MAPK/ERK signaling pathway. Increasing levels of PDE7A leads to decreasing levels of cAMP and subsequent deactivation of PKA in subcellular compartment contcontaining the T-cell receptor (TCR)/CD3 signaling complex **(2)**. Once the inhibitory effect of PKA is removed, MAPK/ERK signaling can progress, leading to the induction of various genes involved in T-cell proliferation **(3)**. *(See insert for color representation of this figure.)*

characterized and found to have strong activity in Jurkat T cells, where it contains binding sites for transcription factors such as Ets-2, NFAT1, and NFκB, which are all assumed to be involved in PDE7A1 transcriptional regulation [135] (Fig. 1.4). One of the functional roles in immune disease ascribed to PDE7A is in the onset of chronic lymphocytic leukemia (CLL) [132], where the clinical effects of a broad-spectrum PDE inhibitor theophylline have been demonstrated in patients with advanced CLL. As PDE7A expression is upregulated by IBMX and theophylline and also by

forskolin and dbcAMP, it has been suggested that PDE7A is part of a feedback loop that compensates for increases in intracellular cAMP (Fig. 1.4). It is worth noting that effects of theophylline are unlikely to be evoked solely by the inhibition of PDE7A alone, as theophylline is a relatively weak inhibitor ($IC_{50} = 343.5\,\mu M$) for this enzyme.

Another facet of PDE7A signaling in T cells revealed that costimulation of CD3 and CD28 receptors leading to T-cell activation results in heightened PDE7A activity and accordingly, silencing of the PDE7A gene through antisense oligonucleotides, leads to a blockade in the T-cell proliferation pathway [136]. A further study [126] also showed that PDE7A1 expression was induced in $CD4^+$ cells following activation and a novel splice variant, PDE7A3 isozyme, was shown to be constitutively expressed in the human T-cell lymphoma cell line, Hut78. Other lines of investigation contradicted these findings by reporting that T cells from PDE7 knockout mice function normally with respect to proliferation and cytokine production driven by CD3 and CD28 costimulation. Therefore, some doubt has been cast on the premise that PDE7A could be the key regulator of T-cell proliferation [137] (Fig 1.4). These findings were later confirmed in human $CD4^+$ T cells, when selective inhibition of PDE7A1 had no effect on CD3/CD28-mediated stimulation. It was found that PDE7A1 expression levels were higher in resting naive $CD4^+$ cells than in memory T cells; however, PDE7A mRNA levels were shown not to be upregulated on T-cell activation [138].

More recently, a role for PDE7A was suggested when it was discovered that expression of the enzyme was shown to mediate the effects of concanavalin A (ConA)-induced liver injury in mice. Inhibition of PDE7A ameliorated degenerative changes in the mouse liver through suppression of natural killer T-cell (NKT) activation, and inhibition of PDE7A in these cells reduced FasL expression as well as reducing tumor necrosis factor alpha (TNFα) and IL4 production. IL4 production in NKT cells is thought to be the key mediator of ConA-induced liver injury in this model of viral and autoimmune hepatitis, and therefore PDE7A could be the key target for prevention of liver degeneration arising from such diseases [139]. Finally, another possible function for PDE7A in keratinocytes has been elucidated [140]. Excessive proliferation of keratinocytes is thought to be the basis of chronic skin conditions such as psoriasis. PDE7A was shown to regulate keratinocyte proliferation by selective inhibition of PDE7A in a skin inflammation model. The results further highlighted the importance of PDE7A expression in the development of various diseases and how selective targeting can prevent progression of disease. The PDE7B gene is not as ubiquitously expressed in immune cells as PDE7A. However, PDE7B has been implicated in CLL with a 23-fold increase in PDE7B mRNA (which correlated with a 10-fold increase in protein expression) found in patients with CLL when compared with expression levels in peripheral blood mononuclear cells (PBMC) from healthy adults. A lower level of cAMP in these cells is thought to be antiapoptotic, and consequently PDE7 inhibitors were shown to promote significant induction of apoptosis in CLL cells [141].

Variation of PDE7A and PDE7B expression has been characterized in postmortem studies of patients with Alzheimer's disease (AD) [142]. The study found that

PDE7 expression was altered in AD brains, and the different levels of expression correlated with different stages of disease. The expression of PDE7B has also been associated with schizophrenia [143]. In a candidate gene analysis, a genomewide, pharmacogenetic study of the response to the atypical antipsychotic drug risperidone was compared to a global transcriptome study of mRNA levels modified by risperidone treatment to the prefrontal cortex in mice. PDE7B, which is abundantly expressed in the brain [144], was identified as having strong correlation with schizophrenia. Significantly, linkage regions associated with the disease map to the same chromosomal location as the PDE7B gene, 6q23-24.

Modulation Cellular PDE7 activity can be regulated by variations in expression; however, very little is known about PDE7 regulation via posttranslational modulation. Although splice variants of PDE7B have been cloned from rats, the human orthologs have yet to be characterized [128]. The splice variants in rat PDE7B contain putative PKA sites at the amino and carboxyl ends of 7B1 and 7B3, and the carboxyl terminus of 7B2. All three proteins are substrates of PKA both *in vitro* and *in vivo*, which may constitute a novel regulatory mechanism for the control of PDE7B activity in rats [130]. PDE7A1 also contains a consensus PKA site in the *N*-terminal region of the protein in addition to two copies of a sequence (RRGAI), which modulates the binding of the enzyme to the catalytic subunits of PKA, and directly inhibits PKA kinase activity. This represents a noncatalytic action of PDE7A1 in modulating the effects of increased intracellular cAMP; however, PKA does not seem to phosphorylate PDE7A1 at the consensus RRXS site [145]. As mentioned earlier, the 20 *N*-terminal residues found in PDE7A2 are hydrophobic; however, this sequence also contain potential palmitoylation and myristoylation sites that may further enhance hydrophobicity and therefore modulate the targeting of PDE7A2 to specific subcellular compartments [124].

Inhibition The expression profile of PDE7 suggests that specific inhibitors of this enzyme family may have therapeutic relevance as immunosuppressants, and for this reason, a number of active-site-directed compounds have been synthesized. The first-ever PDE7 inhibitors were of a family of benzyl derivatives of 2,1,3-benzo- and benzothieno[3,2-a]2,2-dioxides [146]. The activities of the various derivatives were tested against human recombinant PDE7A expressed in *S. cerevisiae,* and although the two best had potency toward PDE7 ($IC_{50} = 11$ and $8\,\mu M$, respectively), they showed no significant selectivity over PDE3, 4 enzymes. The next class of inhibitors synthesised were based on guanine analogs. These were more selective for PDE7 when compared with PDE3, 4 and had an IC_{50} of $1.3\,\mu M$ [147]. Further development of PDE7 inhibitors was carried out by the Pfizer global research group [148]. They investigated the structure–activity relationship of various spiroquinazolinone derivatives and synthesized a number of compounds with nanomolar affinity for PDE7A1, which had considerable selectivity over PDE1,3,4,5. The second round of optimization produced compounds that were slightly less potent but more selective toward PDE7. More importantly, the study highlighted the *in vivo* pharmacokinetic properties of these spiroquinazolinone derivatives in rats, while also indentifying useful inhibitors for *in vitro* characterization of PDE7 [149].

One advantage of family-specific PDE inhibitors is that the functional role of any enzyme family can be examined in a physiological setting. As mentioned previously, the functional outcome of PDE7 inhibition in T lymphocytes has been the subject of some controversy due to conflicting data [136–138,150]. This seems to have been resolved using the novel PDE7-specific inhibitor ASB16165 [151]. In experiments on cytotoxic T lymphocytes (CTL) from the spleen of mice, ASB16165 was shown to inhibit $CD4^+$ and $CD8^+$ T-cell proliferation and the induction of CTL activation and cytotoxicity normally induced through the murine mixed lymphocyte reaction (MLR). Both effects were mediated by increases in intracellular cAMP linked to the inhibition of PDE7A. Additionally, a related study found that ASB16165 also blocks IL2-induced INFγ production in activated T cells, further demonstrating the role of PDE7 in the regulation of T-cell function [152]. The most recent study of PDE7 inhibition utilized a fission yeast-based growth assay to develop a high-throughput inhibitor screen [153]. This technique used a cAMP signaling pathway in *Schizosaccharomyces pombe*, which monitors extracellular glucose levels, thereby negatively regulating transcription of genes involved in gluconeogenesis and sexual development. By exchanging the cAMP PDE gene ($cgs2^+$) with PDE7A1 or PDE7B1, growth characteristics could be monitored in fission yeast with defective glucose signaling pathways. In this system, cAMP is supplied exogenously to allow for cell growth in selective media, allowing compounds that inhibit PDE7 to further increase intracellular cAMP in order to promote proliferation. The level of growth can be readily measured through standard optical density readings (absorbance at 600 nm) in a high-throughput manner. This methodology has been used to screen over 48,000 known compounds and identified several that inhibit PDE7. Novel inhibitors of PDE7 discovered in this manner include steroids, podocarpanes, and heterocyclic compounds that have no structural similarity with the other PDE7 inhibitors described already, and their mode of action is currently under investigation. The development of inhibitors to target PDE7 may have significant therapeutic potential in treating a number of immunological conditions. As described previously, PDE7 regulates major B/T-cell pathways and may play a role in the development of various lymphomas. Other diseases where PDE7 inhibition may have therapeutic relevance include psoriasis, sclerosis, insulin-dependent diabetes mellitus, multiple sclerosis, and inflammatory bowel disease [151].

1.2.5 PDE8

Characterization PDE8 was first isolated and characterized in 1998 [154] following the discovery of two ESTs with homology to the catalytic domain of PDE4 but with significant genetic variation compared with all other known PDEs. The cloned PDE8 protein consisted of 713 amino acids, which included a 283–amino acid catalytic domain that was 38.5% similar to PDE4 and 33.5% to PDE7. As similarities in this region within a particular family normally average ~85%, it was obvious that this novel clone represented a new class of PDE and was designated PDE8A1 [154]. Preliminary characterization of PDE8A1 showed that it had a high affinity for cAMP (K_m 55 nM) and low affinity for cGMP (K_m 124 μM) and that hydrolysis of cAMP

was cation-dependent (Mn^{2+}, Mg^{2+}) and unaltered by high cGMP concentrations. As expected, PDE inhibitors directed at other PDE family members (e.g., rolipram and zaprinast) had little effect on PDE8A. However, the most significant and surprising result was that the broad-range, nonspecific inhibitor IBMX had no effect on PDE8, even at a concentration of 100 μM. This property still distinguishes PDE8 from all other known PDEs [154].

A complementary but separate study also cloned the full-length cDNA of PDE8 from mouse testis [155]. Using the *basic local alignment search tool* (BLAST) to search for ESTs with homology to PDEs, they discovered an EST that represented an unknown PDE. The original clone of the unknown PDE was truncated in the 5′ region and contained only part of the catalytic domain. The sequence was extended toward the 5′ end of the gene, through subsequent cloning and "walking" the EST database through further homology searches with the new sequence data. This identified another EST tag with an in-frame stop codon following a Kozak consensus start codon. Both of the EST tags combined to encompass the full ORF of PDE8A, which encoded an 823–amino acid protein (95 kDa). Further sequence analysis of the full-length ORF of PDE8A, uncovered a highly conserved signaling motif at the *N* terminal, found across bacteria, archea, and lower eukaryotes, known as the *per–arnt–sim* (PAS) domain. PAS domains are known to direct protein–protein interactions and are likely to play a role in PDE8 modulation. The full-length enzyme was found to have a higher K_m for cAMP (150 nM), when compared to the 5′ truncated cloned by Fisher et al. [154]. Both studies were in agreement on the efficacy of other PDE inhibitors in blocking of the catalysis of cAMP by PDE8, and both found that the cGMP-specific inhibitor, dipyridamole, was a moderate inhibitor of PDE8 with an IC_{50} of either 9 or 4.5 μM, respectively [155]. Since the discovery of PDE8A, four additional splice variants of PDE8A have been identified by other groups [156]. A total of five splice variants are known (PDE8A1–5). The PDE8A gene contains 23 exons and spans 1740 base pairs (bp), with the full-length human PDE8A1 cDNA encoding a protein of 829 residues containing an *N*-terminal REC and PAS domains, and the catalytic domain at the *C*-terminal end. PDE8A2 encodes a protein of 783 residues, similar to 8A1, but lacks the PAS domain. PDE8A3 encodes a truncated, 449 residue protein, which contains only the active catalytic domain, and PDE8A4 and A5 encode an identical 582 residue, *N*-terminally truncated protein with only half of the PAS domain. The most abundantly expressed isoform is PDE8A1 [156]. Further studies revealed that two overlapping genomic clones contained the sequence of PDE8A, which spanned 167 kilobases (kb), and mapped to the chromosomal region 15q25.3–26.1 [157].

A second gene encoding a novel PDE8 protein was identified through similar EST database searches. The human gene variant shared 80% amino acid identity with PDE8A, 70% in the catalytic domain alone. The cloned gene product, designated PDE8B, encoded 659 amino acids and lead to an eight-fold increase in the PDE activity of cells that overexpressed it. All other pharmacological characteristics of PDE8B matched those of previously characterized PDE8A and therefore represented a new member of the PDE8 family [158]. The full-length clone of PDE8B and its four isoforms were characterized in 2003 [157]. PDE8B1 protein was slightly larger than

PDE8A1, with which it shared 68% overall identity. PDE8B2 is composed of 788 amino acids and lacked residues 293–389, PDE8B3 is composed of 830 amino acids and is lacking residues 456–509, while PDE8B4 consisted of 865 amino acids and was found to lack residues 113–133.

Expression Early characterization studies of PDE8 noted that it was expressed in a variety of tissues in humans, mice and rats. In human tissue, PDE8A was expressed in the testis, ovary, small intestine, and colon. There was also significant expression in the heart, brain, kidney, and pancreas [154]. In a more comprehensive tissue screen in mice, PDE8A expression was highest in the testis, eye, skeletal muscle, heart, 7-day embryo, kidney, ovary, and brain [155]. PDE8A has also been shown to be expressed in $CD4^+$ T cells, Leydig, cells, effector T cells, and ventricular myocytes [126,159–161]. The obvious abundance of PDE8A in the testis of mice, together with *in situ* hybridization techniques showing spatiotemporal regulation of PDE8 in seminiferous tubules, suggested that PDE8 may play an important role in the regulation of germ cell development. Cyclic AMP levels are thought to be important in regulating spermatogenesis in mice, and this has been demonstrated through stage-specific expression of PDE4, PKA, AKAP84, and the cAMP response element modulator CREMτ in spermatocytes. Furthermore, CREMτ knockout mice do not produce mature sperm and are sterile [155]. More recently, the function of PDE8A has been characterized in Leydig cells from the testis of mice. Leydig cells produce androgen and testosterone under the control of luteinizing hormone (LH) released from the pituitary gland. cAMP has been shown to be the main intracellular messenger for LH modulation of steroidogenesis via downstream signaling through PKA, which regulates proteins involved in testosterone biosynthesis [159]. Indisputable proof that PDE8A was a key regulator of steroidogenesis in Leydig cells was uncovered from studies utilizing PDE8A knockout (KO) mice and the broad-range PDE inhibitor IBMX. In the PDE8A KO mice, very low PDE8 expression resulted in a fourfold increase in the basal levels of testosterone production. Furthermore, a significant increase in testosterone secretion was detected when cells were incubated with LH, suggesting that PDE8A regulates cAMP pools involved in steroidogenesis. In contrast, IBMX treatment, which inhibits all known PDEs apart from PDE8, induced only a small increase in LH sensitivity in wildtype Leydig cells, suggesting that although PDE8 is a key regulator in steroidogenesis, other PDEs may have a minor role in the regulation of testosterone production.

Other than steriodogenesis, PDE8A is also thought to play an important role in immune cell signaling. For example, PDE8 has been shown to regulate chemotaxis in activated lymphocytes [162] where its inhibition resulted in the retardation of migration in both stimulated and unstimulated splenocytes. This effect was mimicked by forskolin treatment, suggesting that high levels of cellular cAMP were required to reduce the chemotactic function of leukocytes, a finding that is consistent with the recognized importance of cAMP in regulating a number of other immunological functions [162].

PDE8A is also known to be involved in T-cell activation, where, similarly to PDE7A, it has been observed that its expression is induced on costimulation of

$CD4^+$ T cells. It should be noted that basal levels of PDE8A in T cells are undetectable and rise sharply after 8 h of costimulation of the CD3 and CD28 receptors. Further evidence of PDE8A function in T cells has been demonstrated in $CD4^+$ effector T (T_{eff}) cells, where the cAMP-PKA signaling pathway regulates T-cell interaction with the vascular endothelium and controls adhesion to vascular ligands [160]. In this case, PDE8 activity was thought to be a key regulator of T-cell adhesion and was subsequently shown to play an important role in extravasation of lymphocytes, through modulation of key integrins. Interestingly, this study also found that despite high levels of PDE3 and PDE4 expression, targeting of these isozymes with selective inhibitors had no effect on T-cell adhesion, suggesting that PDE8 is solely responsible for regulating this function. These data highlight PDE8 as a novel target to suppress overrecruitment of activated T cells into tissue as part of the inflammatory response and emphasizes the importance of PDE8 inhibitors as useful therapeutic tools [160].

It has been established for some time that PDE8 is expressed in heart tissue and that PDE8A mRNA levels are comparable with important function enzymes such as PDE3A and 4B; nevertheless, the role of PDE8A in cardiomyocyte was established [161] in groundbreaking work utilizing genetically engineered mice with LacZ-neomycin cassettes containing a nuclear localization signal in place of the PDE8A catalytic domain. Immunocytochemical staining for β-galactosidase was carried out in order to visualize active PDE8A gene transcription in ventricular myocytes (VMs). The size and activity of the PDE8 protein that was immunoprecipitated from this tissue also matched those of recombinant PDE8A. As cAMP is known to be important in regulating excitation–contraction coupling in VM, the role of PDE8A in this process was examined by measuring the action potential in WT and PDE8A−/− VM loaded with the Ca^{2+} indicator Fluo4. Under nonstimulated basal conditions the amplitude of the Ca^{2+} transient was the same in WT and PDE8A−/− VM, demonstrating that a loss of PDE8A expression has minimal effect on this process. However, on β-adrenergic stimulation, using isoproterenol (ISO), the loss of PDE8A significantly potentiated the increase in amplitude of the Ca^{2+} transient found in stimulated WT VM. The levels of cAMP in WT and PDE8−/− VM were the same at basal levels; however, the increase in cAMP levels after ISO stimulation was higher in the PDE8A−/− VM, which indicates that PDE8A is an important regulator of acute Ca^{2+} transients following β-adrenergic stimulation in ventricular myocytes [161].

In contrast to the tissue distribution profile of PDE8A, PDE8B expression has been found to be more specific to the human thyroid [163]. In a study of single-nucleotide polymorphisms (SNPs) [164], it was discovered that SNPs in intron 1 of PDE8B are associated with increasing serum levels of thyroid secreting hormone (TSH) and thyroid functioning. Abnormal thyroid function can lead to diseases such as hyper- or hypothyroidism; hence, it is suggested that the SNP in the PDE8B gene could affect the intracellular levels of cAMP in this tissue, resulting in changes of T4 and T3 production, which would, in turn, affect levels of TSH secretion [164].

In addition to concentration of PDE8B expression in the thyroid, there is also notable expression in various brain tissues [158]. Analysis of PDE8B distribution in

rats revealed expression in all parts of the brain except the cerebellum. The expression of PDE8B has also been discovered in postmortem brain tissue from patients with Alzheimer's disease (AD) [142]. In fact, changes in the expression profile of all three cAMP-specific PDEs (PDE4, PDE7, PDE8) was noted in various brain segments. PDE8B expression was apparent in the entire hippocampus, and PDE8B mRNA levels in this area were increased in advanced-stage AD brains, while PDE4, PDE7, PKA, and adenylate cyclase expression was unaffected. PDE8B, along with PDE7, is thought to regulate basal levels of cAMP in neuronal cells, due to their relatively low expression profiles and high affinity for cAMP. Furthermore, the differential and overlapping expression profile of cAMP-specific PDEs in the brain is thought to play an important role in processes such as memory and learning [142]. As the advanced stages of AD are associated with increasing levels of cAMP, upregulation of PDE8B may be part of a cellular response for regulating cAMP homeostasis; however, the functional relevance of PDE8B expression in the human brain has yet to be elucidated.

Modulation The modulation of PDE8 activity is thought to be controlled through a PAS domain found at the *N* terminal of the protein. PAS domains are known to mediate homomeric and heteromeric protein–protein interactions, which can regulate subcellular localization and targeting of proteins. It can also interact with small ligands, which act in a sensory capacity, and has been shown to alter activity of proteins in response to certain stimuli such as light and oxygen [165]. Interestingly, certain PDE8A and PDE8B splice variants lack all or the majority of this domain, which suggests, a possible means of isoform specific for modulation of PDE8 activity through PAS domain function. Further evidence of PAS domain involvement in PDE8 regulation comes from comparisons of K_m values for cAMP hydrolysis between full-length and truncated versions of PDE8. Unlike other PDE families (PDE4,7,9,10 [166]), it has been shown that the K_m of the catalytic domain of PDE8A is significantly higher than that of full-length PDE8A and PDE8B. Such data suggest an allosteric affect, orchestrated by the PAS domain, to regulate the activity of the catalytic machinery of PDE8.

The receiver (REC) domain [167], found adjacent to the PDE8 PAS domain, is another region known to be involved in signal transduction. REC domains have been shown to function by receiving signals from sensory proteins in bacterial two-component systems [168] where aspartyl phosphorylation of the REC domain regulates other "effector" domains. Interestingly, the REC sequence of PDE8 contains a highly conserved aspartic acid residue, which is a putative phosphorylation site for this system, suggesting that PDE8 modulation may occur through phosphoregulation [163]. Because of its proximity, the PAS domain could possibly function as the effector domain, similar to that exhibited in bacterial two-component systems [157]. To date, however, a kinase that acts on the PDE8 remains to be isolated, although such experiments may reveal novel modes of regulation as PDE8A has been shown to contain three putative PKA sites [157] and sites for tyrosine kinase, PKC, and casein kinase phosphorylation along with other consensus sites for *N*-glycosylation, *N*-myristoylation, and amidation [156].

Inhibition The first truly selective PDE8 inhibitor has been described in literature [160]. The novel compound PF-4957325-00 has IC_{50}-values of 0.7 nM for PDE8A, <0.3 nM for PDE8B, and >1.5 μM for all other PDE isoforms, making it a selective and highly potent inhibitor. Moreover, in a model for T-cell adhesion the inhibitor was effective in suppressing T_{eff}-cell adhesion to endothelial cells during extravasation. It is envisaged that the development of similar compounds could be used in treating a number of diseases such as T-cell lymphomas inflammatory disorders, male sterility, cardiovascular disease, Alzheimer's disease, and hyperthyroidism.

1.3 CONCLUSIONS

The activity of enzymes from cAMP-specific PDEs is required for normal cell function, and aberrant cAMP signals resulting from the overexpression, inhibition, or mutation of these enzymes have been implicated in a range of disease states and pathological conditions. Clearly, such enzyme families represent ideal targets for specific inhibitors, and the development of small molecules against the respective families is ongoing. PDE4 inhibitors have proved problematic because of the associated side effects, which have limited their clinical application; however, alternative strategies that serve to target these compounds to specific tissues or enzyme pools in microcellular domains are a cause for optimism.

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