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The long and short of vascular smooth muscle phosphodiesterase-4 as a putative therapeutic target.

By

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Running title:- phosphodiesterase-4, stoke and restenosis

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Abstract

In this issue Tilley and Maurice show that differentiation of vascular smooth muscle cells (VSMC) to a proliferative phenotype is associated with a profound up-regulation of specific phosphodiesterase-4 (PDE4) isoforms due to increased histone acetylation. The increased PDE4 activity is seen as preventing cAMP from inhibiting the enhanced proliferation, migration and production of intracellular matrix seen in activated VSMC. This perspective examines the proposal that selective inhibition of PDE4D1/2 could find use in adjunctive pharmacotherapy following percutaneous coronary interventions and, in addition, discusses the recent genetic evidence that PDE4D7 may provide a therapeutic target in stroke.

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There is currently great interest in attempting to deploy inhibitors specific for phosphodiesterase-4 (PDE4) to treat conditions associated with inflammation, such as asthma and chronic obstructive pulmonary disease (COPD) (Jeffery, 2005; Lipworth, 2005; Maurice et al., 2003; Spina, 2004). The development of such PDE4-selective, active site-directed inhibitors has recently been furthered by the elucidation of the 3-D structure of the PDE4 catalytic unit and that of other PDEs, which form the 11 member PDE superfamily (Card et al., 2005; Xu et al., 2000). However, not only has an increasing range of PDE4 inhibitor scaffolds become available but also the range of potential therapeutic uses for such compounds has broadened.

The article by Tilley and Maurice in this issue of *Molecular Pharmacology* (Tilley and Maurice, 2005) highlights an exciting new aspect of 'PDE4-ology', which relates to a potential role for PDE4 inhibitors in adjunctive pharmacotherapy following percutaneous coronary interventions. In this case the target cells in question are vascular smooth muscle cells (VSMC), which can undergo a phenotypic change from a 'normal', 'contractile/quiescent' state to an 'activated', 'synthetic' state. Various types of vascular injury can effect this transition including, in the case of percutaneous coronary intervention, both inflation of the intracoronary balloon and the metal of the stent itself. The activation of proliferative capacities of VSMC causes vessel wall remodelling associated with reduced contractile capacity and thickening of vessels. Inappropriate migration of VSMC into the intimal layer and the generation of extracellular matrix disrupts normal vessel functioning are characteristic of atherosclerosis and can promote post-angioplasty 'in-stent' restenosis. Provision of a means of addressing these clinically important problems would thus have considerable therapeutic potential. Pertinent to this, a number of investigators have shown that increasing cAMP levels in activated VSMC can profoundly inhibit their proliferation and migration as well as the synthesis of extracellular matrix proteins (Hayashi et al., 2000; Koyama et al., 2001; Kronemann et al., 1999; Pelletier et al., 2005; Vadiveloo et al., 1997). On this basis one might expect activated smooth muscle cells to deploy a mechanism that would protect them from potential inhibitory effects of cAMP. In their study, Tilley and Maurice (Tilley and Maurice, 2005) identify just such a mechanism, namely the induction of specific PDE4

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isoforms, which serve to desensitize the susceptibility of activated VSMC to the inhibitory action of cAMP.

PDE4 enzymes specifically hydrolyse cAMP, compared to cGMP, and are regulated through phosphorylation by both protein kinase A (PKA) (Alvarez et al., 1995; MacKenzie et al., 2002) and ERK (Baillie et al., 2001; Baillie et al., 2000; Hoffmann et al., 1999; MacKenzie et al., 2000). PDE4s also provide a key component that underpins compartmentalised cAMP signalling in cells (Mongillo et al., 2004) due to their ability to target to specific intracellular sites and to interact with various signalling scaffold proteins (Conti et al., 2003; Houslay and Adams, 2003). Four genes (A/B/C/D) encode some 20 different PDE4 isoforms, each of which is characterised by a unique N-terminal region whose key role is to confer intracellular targeting to specific sites and signalling complexes (Conti et al., 2003; Houslay and Adams, 2003). The isoforms encoded by each PDE4 gene are separated into long, short and super-short groupings based upon the presence / absence of upstream conserved (UCR) domains located between the isoform-specific N-terminal region and the catalytic unit (Fig. 1). Long isoforms have such domains, called UCR1 and UCR2, whilst short isoforms have only UCR2 and super-short isoforms merely a truncated UCR2. These UCR domains exert a regulatory role, with UCR1 providing a site for stimulatory phosphorylation by PKA (MacKenzie et al., 2002; Sette and Conti, 1996). Additionally, upon ERK phosphorylation of the PDE4 catalytic unit, the paired UCR1/UCR2 module orchestrates an inhibitory action in long forms, whilst the lone UCR2 orchestrates a stimulatory action in short forms, with little discernible effect seen in super-short forms (Baillie et al., 2000; MacKenzie et al., 2000).

The distinct modes of intracellular targeting and recruitment to scaffold complexes coupled with differences in regulation by PKA and ERK, means that the pattern of PDE4 isoform expression in a cell can be expected to have profound effects on both compartmentalised cAMP signalling and cross-talk with the ERK pathway (Baillie and Houslay, 2005; Houslay and Adams, 2003; Houslay and Kolch, 2000). In this regard, Tilley and Maurice (Tilley and Maurice, 2005) have made the unusual observation that the short PDE4D1 and super-short PDE4D2 isoforms are specifically induced in activated VSMC. Such a remodelling of the PDE4 profile has interesting consequences for the cross-talk with the growth promoting ERK signalling pathway (Fig. 1), which is

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activated in proliferating VSMC and because the short PDE4D1 isoform will be activated by proliferative signals involving ERK (Baillie et al., 2000). This latter mechanism will serve to further depress potentially inhibitory levels of cAMP and, in so doing, further promote the proliferative response. Interestingly, a cognate remodelling has been described upon monocyte to macrophage differentiation (Shepherd et al., 2004). In monocytes ERK-inhibited long PDE4D isoforms predominate, whilst in macrophages ERK-activated short PDE4B2 isoform predominates. Thus, in macrophages, activation of ERK by pro-inflammatory agents serves to depress inhibitory cAMP levels whilst, in monocytes a transient, inhibitory increase in cAMP occurs due to an initial ERK-mediated inhibition of PDE4 long isoforms, which generates a rise in cAMP that causes PKA to subsequently phosphorylate and activate the long isoform (Fig 1) (Baillie et al., 2001; Baillie et al., 2000; MacKenzie et al., 2000).

PDE4D1/2 isoforms have a very restricted pattern of expression, making Tilley and Maurice's observations of particular interest. The more so as they identified increased histone-3 (H3)-acetylation, which applies to histones associated with smooth muscle specific promoters upon differentiation (Cao et al., 2005), as playing a key role in regulating the functioning of the PDE4D1/2 intronic promoters in VSMC. Intriguingly, a decrease in histone deacetylase activity has been observed in COPD (Ito et al., 2005), a pathological condition where airway remodelling occurs and PDE4 inhibitors have therapeutic potential (Jeffery, 2005; Lipworth, 2005). Nothing is known, however, about changes in PDE4 expression profile in pulmonary VSMC of COPD patients, so it would seem pertinent to address this, especially as a change in the macrophage PDE4 profile of COPD patients has been noted (Barber et al., 2004).

A hallmark of atherosclerosis is the proliferation and migration of VSMC. In this regard the provocative proposal has been made (Gretarsdottir et al., 2003) that the *PDE4D* gene may be linked to large vessel (e.g. carotid) occlusive stroke but not to strokes that occur via occlusion of small vessels. PDE4 genes are extremely large and encompass about 50kb of sequence, making them difficult to analyse: 7 exons encode the catalytic unit, 8 exons encode UCR1/2 together with their linker regions and individual 5' exons encode the extreme N-terminal regions unique to each isoform (Houslay and Adams, 2003). The first linkage study, which was done on a cohort of patients from

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Iceland, identified association of stroke with a non-coding region of the PDE4D gene (Gretarsdottir et al., 2003). This is well upstream from the intronic PDE4D1/2 promoter (Vicini and Conti, 1997) and has been suggested (Gretarsdottir et al., 2003; Gulcher et al., 2005) to be associated with a putative promoter for the long PDE4D7 isoform. The mutations associated with stroke predisposition were proposed to lead to enhanced PDE4D7 expression (Gretarsdottir et al., 2003). If PDE4D7 were to be up-regulated in VSMC the prediction would be that enhanced VSMC proliferation and migration into the intima with concomitant lesion formation would ensue. Indeed, RhoA activity is intimately linked with cell migration (Nobes and Hall, 1999) and increased RhoA activity is invariably seen in various models of hypertension, including VSMC from spontaneously stroke prone rats (Moriki et al., 2004). As PDE4 inhibition and increased cAMP levels have been shown to inhibit Rho activity and cell migration (Fleming et al., 2004; Netherton and Maurice, 2005) this may indicate a further link. If PDE4D7 was up-regulated in macrophages then the resultant reduction in cAMP levels might be predicted to enhance the susceptibility of macrophages to activation in atherosclerotic plaques, influencing atherogenesis and plaque stability.

Intronic promoters for a number of specific PDE4 isoforms (Le Jeune et al., 2002; Rena et al., 2001; Vicini and Conti, 1997; Wallace et al., 2005), including PDE4D1 (Vicini and Conti, 1997), have been identified and defined by first cloning, in frame, 1500bp or so of sequence located immediately 5' to the ATG start codon. A similar strategy applied to PDE4D7, so as to encompass the presumed association sites, would allow rigorous evaluation of mutations suggested to either predispose or protect from large vessel and cardiogenic stroke. However, being the most 5' isoform encoded by the *PDE4D* gene this will not be done quite so readily for PDE4D7. This is because 3 exons, rather than 1, encode the unique N-terminal region of PDE4D7. The additional complexity in splicing associated with PDE4D7 may therefore underpin a purported susceptibility to altered expression as a consequence of mutations identified in the Icelandic study. A functional promoter analysis of PDE4D7 becomes all the more pressing in view of recent studies on European populations (Bevan et al., 2005; Lohmussaar et al., 2005) that have failed to identify such a clear cut connection between

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the PDE4D7 markers and stroke, as identified in the Icelandic study (Gretarsdottir et al., 2003).

One of these new studies (Bevan et al., 2005), however, did record the possibility of an association of *PDE4D* with cardioembolic stroke and, whilst failing to show that PDE4D was a major risk factor for ischemic stroke, could not exclude a connection. It is possible that the strong correlation seen in the Icelandic study (Gretarsdottir et al., 2003) may result from a founder effect within the restricted gene pool of this small population. Notwithstanding this, such studies and those of Tilley and Maurice (Tilley and Maurice, 2005) suggest an urgent need for promoter analyses to be performed on PDE4D7 and PDE4D1/2 isoforms in VSMC and macrophages so as to appreciate regulation both in resting cells and also those activated by various challenges. These are an essential prerequisite to any further evaluation involving transgenic analyses. It should also be noted that Maurice and co-workers have demonstrated that PDE4D3 expression can be regulated by ERK action through changes in message stability (Liu et al., 2000). These data suggest the possibility that mutations identified in the Icelandic study could lead to either direct alterations in PDE4D7 transcript stability or exert indirect effects by changing regulatory processes associated with transcript stability.

It will be particularly interesting to delineate the functional consequences of up-regulating PDE4D1, PDE4D2 and PDE4D7 in activated VSMC. Given that PDE4 enzymes provide a major component that underpins compartmentalised cAMP signalling in cells, such an evaluation should probe for a role associated with the key differences between activated and resting VSMC. These features include proliferation, for which there is a wealth of data indicating various potential points for inhibitory intervention by cAMP (Balmanno et al., 2003); migration, where PDE4 inhibition and elevation of cAMP levels in a number of cell types appears to exert its action by an inhibitory effect on Rho-regulated processes (Fleming et al., 2004; Netherton and Maurice, 2005); cell shape, where cAMP-mediated inhibition of Rac plays a role (Pelletier et al., 2005) and, finally, regarding extracellular matrix generation, where PDE4 inhibitors exert an inhibitory effect (Maurice et al., 2003). Little, however, is known about the functional role of these isoforms.

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In order to undertake these studies it will be necessary to inactivate them selectively, which has not been done to date. However, the identical catalytic unit of isoforms from any one PDE4 sub-family, coupled with the similarity of catalytic units between sub-families, has meant that no highly specific, isoform discriminatory inhibitors are available.

Up until recently the sole way of addressing the functional significance of specific PDE4 isoforms has been to over-express them in cells. However, our understanding of the role of specific PDE4 sub-families and isoforms has dramatically changed recently with the advent of gene knockout approaches (Ariga et al., 2004; Hansen et al., 2000; Jin and Conti, 2002) and, crucially, the development of both dominant negative (Baillie et al., 2003; McCahill et al., 2005) and siRNA-mediated knockdown strategies[§] to discern the functional role of individual PDE4 isoforms. Thus siRNA-mediated knockdown can be used to target either entire PDE4 sub-families or specific isoforms, dependent upon probe design[§], whilst dominant negative approaches exploit the targeting of specific isoforms by overexpressing a catalytic inactive isoform so as to displace the tethered endogenous active species from the functionally relevant site in the cell (Baillie and Houslay, 2005; Baillie et al., 2003).

Is it feasible to try to employ such strategies in order to dissect out the role of PDE4 isoforms in activated VSMC and thereby to determine their potential as a specific target for selective therapeutic intervention? PDE4D7 is a long isoform that contains both UCR1 and UCR2 together with a unique 91 amino acid N-terminal region (Fig. 1). Thus, as used to assess PDE4D5 and PDE4D3 functioning (Baillie and Houslay, 2005; Baillie et al., 2003), the unique 5' region of PDE4D7 makes it eminently suitable for siRNA intervention and its N-terminal and UCR targeting regions indicate potential for a dominant negative approach. PDE4D1 is a classic short form, lacking UCR1 but having both the 80 amino acid UCR2 together with a unique 45 amino acid N-terminal region, again making it suitable for both approaches (Fig. 1). PDE4D2 is a super-short isoform that has just a truncated UCR2; namely one lacking the N-terminal first 34 amino acids of full length UCR2 (Fig. 2). However, it is curious in that, unlike 'classic' super-short forms such as PDE4A1 (Sullivan et al., 1998), PDE4D2 lacks any unique N-terminal region. This means that there is no possibility of using a siRNA-mediated approach to

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target PDE4D2 specifically and because a dominant negative approach may well interfere with other isoforms, assessment of the role of PDE4D2 will be extremely challenging.

The ability of changes in cAMP to alter VSMC and monocyte / macrophage functioning, the alterations in PDE4 occurring in such cells upon activation / differentiation and proven action of PDE4-selective inhibitors all argue for further analysis of the role of PDE4 isoforms in these cell systems. This will indicate whether PDE4 can be exploited either diagnostically or therapeutically in certain forms of stroke. Here Tilley and Maurice (Tilley and Maurice, 2005) propose a novel therapeutic use of PDE4 inhibitors that might be further refined by targeting action to the PDE4D1/2 short isoforms. Exploiting dominant negative and siRNA technologies may provide the required proof of principle to test this notion prior to progressing along the rocky road of attempting to develop a PDE4D1/2-selective inhibitor. It is worth noting, however, that the Ca^{2+}-activated PDE1C isoform has been shown to be up-regulated in proliferating VSMC (Rybalkin et al., 2002) and that a PDE1-selective inhibitor can attenuate VSMC proliferation (Phillips et al., 2005). As PDE1 and PDE4 invariably control different pools of cAMP in cells then a comparative evaluation of PDE1C with PDE4D1, PDE4D2 and PDE4D7 may prove highly informative in terms of identifying regulatory mechanisms as well as defining appropriate therapeutic targets and strategies and, possibly, diagnostic indicators.

Footnote. [§] Lynch et al., ms submitted

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Figure Legends

Figure 1. The PDE4D gene, the long PDE4D7 and super-short PDE4D1/2 products plus putative networks linking them to functions in VSMC.

(a) Schematic of the *PDE4D* gene. Exons are numbered so as to indicate both those coding the common core PDE4D plus the unique 5' exons encoding the N-terminal regions of particular splice variants (Houslay and Adams, 2003). Note that single exons encode the N-terminal regions of individual isoforms except for the 'first' isoform from each PDE4 sub-family whose N-terminal region is encoded by multiple exons located at the extreme 5' region of the gene. The figure is a schematic and for simplicity does not indicate relative distances separating these exons to scale. The 3 arrows indicate the start of the PDE4D7, PDE4D1 and PDE4D2 coding regions, in order.

(b) Schematic of the domains of the indicated PDE4D isoforms. PDE4D7 is a long isoform with both UCR1 and UCR2, PDE4D1 is short isoforms with only UCR2 and PDE4D2 is a super-short form with a truncated UCR2. PDE4D1 and PDE4D7 both have unique N-terminal regions whilst PDE4D2 has not. Shown (arrows) are the phosphorylation sites for PKA on UCR1 and the phosphorylation site for ERK on the catalytic unit.

(c) Schematic to show stimulatory (arrows) and inhibitory (dashed lines + circle) connections linking PDE4 long and short isoforms in VSMC. The 'inhibitory' effect on cAMP exerted by PDE4 is through cAMP degradation. Whilst the major effector of the intracellular actions of cAMP is PKA (Tasken and Aandahl, 2004), actions may also be exerted by EPAC (Bos, 2003) and cyclic nucleotide gated ion channels (Zagotta and Siegelbaum, 1996). PKA is known to mediate the actions shown here on PDE4, RhoA, Raf-1 and various other actions that attenuate proliferation. Cross-talk between the ERK and cAMP pathways can occur at the level of Raf, with Raf1 providing a point of inhibition by cAMP and B-Raf a point of activation (Houslay and Kolch, 2000).