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Targeting Phosphodiesterases in Anti-platelet Therapy

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Abstract

There are two primary modes of platelet inhibition: blockade of membrane receptors or neutralization of intracellular pathways. Both means of inhibition have proven benefits in the prevention and resolution of atherothrombotic events. With regard to intracellular inhibition, phosphodiesterases (PDEs) are fundamental for platelet function. Platelets possess several PDEs (PDE2, PDE3 and PDE5) that catalyze the hydrolysis of cyclic adenosine 3'-5'-monophosphate (cAMP) and cyclic guanosine 3'-5'-monophosphate (cGMP), thereby limiting the levels of intracellular nucleotides. PDE inhibitors, such as cilostazol and dipyridamole, dampen platelet function by increasing cAMP and cGMP levels. This review focuses on the roles of PDE inhibitors in modulating platelet function, with particular attention paid to drugs that have anti-platelet clinical indications.

Keywords

Phosphodiesterase; Platelet; Thrombosis; Anti-Platelet Agents; Cilostazol; Dipyridamole

1 Introduction

Two prominent phases of atherothrombosis exist: a prolonged luminal narrowing and an acute thrombotic stage. Inhibition of platelet function has demonstrated benefits in both stages, especially in the prevention and treatment of acute thrombosis. There are several different classes of pharmaceutical agents with demonstrated efficacy for the prevention and treatment of atherothrombosis. For example, some anti-platelet agents target surface receptors that play a key role in platelet aggregation (e.g., the ADP receptor or integrin $\alpha_{IIb}\beta_3$). Other anti-platelet agents function by suppressing intracellular signaling pathways to either block the production of pro-aggregating factors or to increase the activity of platelet inhibitors. Aspirin, for example, reduces platelet aggregation by neutralizing cyclooxygenase-1 (COX-1) and preventing thromboxane A_2 synthesis. Phosphodiesterase (PDE) inhibitors attenuate platelet activity by increasing cAMP and/or cGMP. Elevation of cAMP and cGMP levels subsequently dampens cytoskeletal rearrangement in platelets, activation of integrin $\alpha_{IIb}\beta_3$, and platelet secretion.

In this chapter, we will review the effects of PDE inhibitors on platelet function. In doing so, we will discuss the expression and function of PDEs in platelets focusing on anti-PDE drugs

that are used clinically. We conclude with a brief summary of additional functions of PDE inhibitors that may have pleiotropic effects for the prevention and treatment of cardiovascular disease.

2 Platelet Phosphodiesterases

cAMP and cGMP are critical intracellular second messengers that modulate platelet functions (Haslam et al. 1999; Colman et al. 2004; Gresele et al. 2011). Arguably, interest in cyclic nucleotides began over 40 years ago when prostaglandin E₁ (PGE₁) was shown to inhibit platelet responses through cAMP-dependent mechanisms (Haslam et al. 1978, 1999). The critical role of cGMP in regulating platelet function was subsequently elucidated when nitrovasodilators, such as nitroprusside, were shown to inhibit platelet aggregation and, in parallel, increase cGMP levels (Haslam et al. 1978, 1999). It is now known that platelets have the ability to synthesize cGMP in response to nitric oxide released by nitrovasodilators.

Because of their key roles in regulating cellular signaling and function, it is critical for cells to limit the formation and activity of cyclic nucleotides. PDEs are essential in this process because they catalyze the hydrolysis of cAMP and cGMP to inactive 5'-AMP and 5'-GMP, respectively (Francis et al. 2011). In mammalian tissues, 11 families of PDEs (PDE1-11) have been described. The molecular mechanisms of the physiologic functions of these PDEs have been discussed in detail recently and the reader is referred to here for a more comprehensive review on the subject (Bender et al. 2006). In the soluble fraction of platelet extracts, three distinct PDEs have been isolated: PDE2, PDE3, and PDE5 (Hidaka et al. 1976). PDE2 and PDE3 hydrolyze cAMP and cGMP while PDE5 prefers cGMP as a substrate (Table 1). Together, these three isozymes account for the majority (more than 90 %) of platelet PDE activity.

2.1 Phosphodiesterase 2

PDE2, a dual substrate enzyme that hydrolyzes cAMP and cGMP equally well, contains two GAF domains (GAF-A and GAF-B) critical for normal physiologic functions (Fig. 1) (Haslam et al. 1999). GAFs are found in a variety of proteins, but the acronym is derived from the names of the first three classes of proteins recognized to contain this domain: cGMP-binding PDEs, *Anabaena* Adenylal cyclases, and *Escherichia coli* FhlA (Zoraghi et al. 2004; Aravind et al. 1997). The GAF domain structure has several functions in PDEs, including cGMP binding and dimerization of PDE monomers. The GAF-A domain mediates dimerization of PDE2 while the GAF-B domain binds cGMP (Zoraghi et al. 2004). Upon binding of cGMP to GAF-B, PDE2 is stimulated, resulting in a conformational change in the protein and an increase in enzyme activity in platelets (Haslam et al. 1999; Bender et al. 2006; Zoraghi et al. 2004). Thus, elevated concentrations of cGMP stimulate PDE2 (Bender et al. 2006).

The highest levels of PDE2 are observed in the brain, but PDE2 activity is also found in cardiac muscle, endothelial cells, and platelets. Purification of the enzyme from platelets demonstrates that PDE2 hydrolyzes cAMP and cGMP at similar rates (Grant et al. 1990). There are three known splice variants of PDE2: PDE2A1, PDE2A2, and PDE2A3 (Beavo et al. 1995). Nevertheless, there are no demonstrated differences in the kinetic behavior of the splice variants and all are involved in subcellular targeting (Yang et al. 1994). In platelets, these splice variants have not been well characterized and some investigators have suggested that the PDE2 isoform in platelets is unlikely to be PDE2A2—which is soluble—as the PDE2 isozyme in platelets localizes to the cell membrane (Haslam et al. 1999; Yang et al. 1994; Russwurm et al. 2009). However, this premise may deserve reconsideration given the recent data from our group which used next-generation RNA-sequencing to show that PDE2A2 is the major isoform of PDE2 in human platelets (Rowley et al. 2011).

No known diseases are associated with PDE2 dysfunction and as deletion of PDE2A in mice is embryonically lethal (Stephenson et al. 2009), in vivo studies to characterize PDE2-driven platelet responses have been limited by this technical obstacle. Investigators have thus largely relied on pharmacological blockade of PDE2 to determine the molecular pathways by which PDE2 regulates platelet functions. The PDE2 inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) potentiates the inhibitory effects of nitroprusside on thrombin-induced aggregation (Dickinson et al. 1997), but this may be explained by concomitant neutralization of adenosine deaminase (Gresele et al. 2011). A new series of natural PDE2 inhibitors (purified from safrole obtained from *Ocotea pretiosa*) have recently been shown to block arachidonic acid and collagen-induced aggregation (Brito et al. 2010; Lima et al. 1999). Like EHNA, the effect of these inhibitors is enhanced in the presence of sodium nitroprusside (Brito et al. 2010).

PDE2 inhibitors are primarily used as research tools and have been studied far more extensively in other cells. There are no registered PDE2 inhibitors for clinical use (Table 1). Novel inhibitors that are more selective for PDE2, however, have recently been developed (i.e., 9-(6-phenyl-2-oxohex-3-yl)-2-(3,4-dimethoxybenzyl)-purin-6-one [PDP]) but not yet tested on platelets (Gresele et al. 2011; Diebold et al. 2009).

2.2 Phosphodiesterase 3

Like PDE2, isoforms of PDE3 hydrolyze cAMP and cGMP with a preference for cAMP (Table 1). One unique feature of PDE3 is that hydrolysis of cAMP is blocked by cGMP, leading to the alternative name of PDE3: the cGMP-inhibited PDE (Bender et al. 2006). In fact, there is evidence that cGMP exerts most of its antiplatelet effects by acting as a competitive inhibitor of PDE3A (Maurice et al. 1990).

PDE3 contains two regions [NH₂-terminal hydrophobic regions (NHR1 and NHR 2)] that provide for membrane association (Francis et al. 2011) (Fig. 1). Two PDE3 genes have been identified: PDE3A and PDE3B (Bender et al. 2006). Both PDE3A and PDE3B have been found in vascular smooth muscle cells (Palmer et al. 2000) while PDE3B is unique to adipocytes, β cells, macrophages, and T-lymphocytes (Shakur et al. 2001). PDE3A is distinct to oocytes and platelets (Shakur et al. 2001). Three variants of PDE3A have been identified (PDE3A1/2/3) (Choi et al. 2001; Wechsler et al. 2002). PDE3A1 encodes the full-length PDE3A that possesses both the NHR1 and NHR2 domains and the catalytic domain (Fig. 1) (Omori et al. 2007). PDE3A2 encodes a protein containing the NHR2 and catalytic domain while placental PDE3A3 encodes a protein that has the catalytic domain only (Wechsler et al. 2002; Kasuya et al. 1995). Platelets primarily express the transcript for PDE3A1 (Rowley et al. 2011).

PDE3A, which was originally purified from outdated banked platelets, has a very low K_m for cAMP (Colman et al. 2004; Grant et al. 1984). Levels of PDE3A in human platelets treated with PGE₁ and PGI₂ increase and parallel the increase seen in cAMP levels (Colman et al. 2004). Several groups have shown that increases in PDE3A activity are mediated by protein kinase A and C (Grant et al. 1988; Macphee et al. 1988; Hunter et al. 2009).

Unlike PDE2, gene disruption of PDE3 in mice is not embryonic lethal. However, PDE3A deficient female mice are infertile as their oocytes contain higher levels of cAMP and fail to undergo spontaneous maturation (Masciarelli et al. 2004). Subtype-selective knockout mice studies demonstrate that PDE3A is the primary PDE3 responsible for regulating platelet function (Sun et al. 2007). Compared to wild-type, littermate controls, resting cAMP levels in platelets are twice as high in PDE3A knockout mice and platelets from PDE3A, but not PDE3B, knockout mice fail to respond to PDE3 inhibitors (Sun et al. 2007). The functional significance of PDE3A has also been demonstrated in studies showing that knockout mice

are protected against collagen/epinephrine-induced pulmonary thrombosis and death (Sun et al. 2007).

PDE3A inhibitors reduce platelet aggregation in response to most agonists (Tani et al. 1992; Muggli et al. 1985). Thus, they are attractive targets for antiplatelet therapy in human diseases (Colman et al. 2004; Gresele et al. 2011). Three specific PDE3A inhibitors are currently approved for clinical use and others are in development (Gresele et al. 2011) (Table 1). A thorough discussion of the pharmacological features and clinical trial data with these agents is beyond the scope of this chapter but key data are briefly summarized in the following paragraph.

Milrinone, which increases intraplatelet cAMP levels and thus inhibits platelet aggregation in whole blood and platelet-rich plasma, is currently used clinically for the treatment of congestive heart failure (Gresele et al. 2011; Colucci et al. 1991). Anagrelide is a potent inhibitor of platelet aggregation and a platelet-reducing agent used to treat thrombocythemia (e.g., secondary to myeloproliferative disorders) (Seiler et al. 1987; Silverstein et al. 1988). Cilastazol, which is described in more detail below, is approved for the treatment of intermittent claudication (Faxon et al. 2004).

2.3 Phosphodiesterase 5

Historically, PDE5 was described as the cGMP-specific or cGMP-binding PDE (Bender et al. 2006). The structural basis for PDE5 to selectively hydrolyze cGMP was solved when it was determined that PDE5 has two GAF domains and, that in contrast to PDE2, cGMP binds the GAF-A domain (Fig. 1). Cyclic nucleotide binding to the GAF-A domain is over 100-fold more selective for cGMP over cAMP (Zoraghi et al. 2005). Binding of cGMP to the GAF-A domain significantly increases enzymatic activity (~10-fold) (Bender et al. 2006) and binding is stabilized by phosphorylation events (Bender et al. 2006; Francis et al. 2002).

PDE5 was first found in platelets and then in vascular smooth muscle and endothelial cells, with high expression levels subsequently reported in the corpus cavernosum and lung (Lin et al. 2006). PDE5 has three major isoforms (PDE5A1/2/3). However, these isoforms differ only in the initial portion of exon 1 and there are no obvious functional differences in PDE5A1, PDE5A2, and PDE5A3 (Kass et al. 2007). Nevertheless, the variants may allow for differential control of PDE5A gene expression in various cells (Bender et al. 2006). Next-gen RNA-sequencing suggests that all three PDE5A isoforms are expressed by platelets (Rowley et al. 2011).

Although gene targeted mice lacking PDE5A have not been studied, PDE5A is well recognized to regulate vascular smooth contraction through regulation of cGMP (Bender et al. 2006). In addition, PDE5 is an important regulator of platelet function. Inhibition of PDE5 increases platelet cGMP levels and amplifies the ability of nitric oxide to inhibit platelet aggregation and secretion (Ito et al. 1996; Dunkern et al. 2005). As alluded to above, part of the effect of PDE5 occurs through cGMP-mediated inhibition of PDE3 (Bender et al. 2006).

PDE5A inhibitors used in the clinic to treat erectile dysfunction include sildenafil (Viagra), vardenafil (Levitate), and tadalafil (Cialis). Similar to other PDE inhibitors, sildenafil potentiates the effect of nitroprusside on platelets (Wallis et al. 1999). Sildenafil also inhibits collagen-induced aggregation, ADP-stimulated activation of $\alpha_{IIb}\beta_3$, prolongs bleeding time in healthy volunteers after acute administration (Berkels et al. 2001; Halcox et al. 2002). Vardenafil reduces calcium influx in thrombin-stimulated platelets while tadalafil reduces the expression of surface P-selectin (De Bon et al. 2010).

Another PDE5A inhibitor, dipyridamole, is used in combination with aspirin for the prevention of secondary stroke. In addition to inhibiting PDE5, dipyridamole blocks the reuptake of adenosine by red blood cells and inhibits other PDEs (Ann et al. 1989). The anti-thrombotic properties of dipyridamole are described in more detail below.

3 PDE Inhibitors as Anti-platelet Agents

Although there are several preclinical and registered PDE inhibitors, cilostazol and dipyridamole have more established anti-thrombotic actions. As briefly mentioned above, cilostazol and dipyridamole inhibit platelet PDEs (Fig. 2). Their effect on platelet function and value in the clinic is described below.

3.1 Cilostazol

Cilostazol is a potent anti-platelet agent registered in the United States since 1999 and in many other countries, including Japan since 1988, for the treatment of intermittent claudication in patients with peripheral vascular disease (usual dose 100 mg twice daily; BID) (Okuda et al. 1993). Intermittent claudication, a pain or ache in the lower extremity muscle groups that occurs with walking and resolved with rest, is a debilitating condition that may severely restrict a person's ability to ambulate and is associated with arterial occlusive disease (Criqui et al. 1985). Cilostazol has also been tested in randomized controlled trials (RCTs) for the secondary prevention of stroke (Shinohara et al. 2010), cardiovascular disease (Han et al. 2009; Suh et al. 2011), and post-stent stenosis (Ge et al. 2005; Yamagami et al. 2012; Jennings et al. 2010). Although differences in study design and patient characteristics exist, many of the results were encouraging, particularly in trials compared to placebo. Nevertheless, as cilostazol is primarily registered for the treatment of claudication and other uses may be off-label or investigational, the remainder of this discussion will focus on the evidence supporting the use of cilostazol in patients with intermittent claudication.

The mechanism by which cilostazol improves claudication is not entirely understood but likely involves one or more processes. As discussed briefly above, cilostazol prevents primary and secondary platelet aggregation by selectively and specifically inhibiting PDE3 in platelets ($IC_{50} = 0.2 \mu\text{M}$) (Schorr et al. 2002). Cilostazol also inhibits adenosine uptake in cells leading to increased adenosine levels, which consequently increases intracellular cAMP levels (Schorr et al. 2002). Cilostazol has also been shown to prevent platelet activation through shear stress during exercise, which may be particularly critical at foci of arterial bifurcation in patients with peripheral artery disease (Dobesh et al. 2009). Additionally, cilostazol inhibits platelet surface P-selectin expression, thromboxane B_2 production, platelet factor 4 (PF4) release (Schorr et al. 2002; Kariyazono et al. 2001; Igawa et al. 1990), and may also cause increases in high-density lipoprotein and a decrease in triglyceride levels (Thompson et al. 2002). Cilostazol does not prevent synthesis of the vasodilator prostacyclin (Dobesh et al. 2009).

Cilostazol is quickly absorbed when taken orally (time-to-peak plasma concentration 2.4 h) with a half-life of approximately 10 h. Metabolism occurs primarily through the CYP3A5 and CYP2C19 pathways (two isozymes of the cytochrome P450 system) and <1 % of orally administered drug is excreted in the urine without being metabolized (Schorr et al. 2002; Akiyama et al. 1985). The metabolism of cilostazol is significantly affected by polymorphisms in the CYP3A5 and CYP2C19 pathways, which are common and result in an estimated coefficient of variation of 40–60 % (Yoo et al. 2010; Bramer et al. 1999). As a result, the dose of cilostazol should be reduced in patients who are taking CYP3A5 or CYP2C19 inhibitors concomitantly.

The clinical efficacy of cilostazol has been studied in multiple, prospective clinical trials. Although study designs differed, most patients were stable (e.g., patients with limb-threatening ischemia, ulcerations, and gangrene were excluded) with an ability to walk at least 30–60 m. In a published meta-analysis of 8 RCTs, cilostazol was superior to placebo in improving walking distances (50 % improvement for maximum distance and 67 % for pain-free distance) (Thompson et al. 2002). In a head-to-head prospective trial of cilostazol versus pentoxifylline (a methylxanthine derivative also used for the treatment of claudication), cilostazol treatment significantly increased walking distances (Dawson et al. 2000). Initial improvements in walking distances with cilostazol are often apparent within 2–4 weeks of treatment initiation (Dawson et al. 2000) but patients may see continued progress for up to 6 months.

Despite its potent antiplatelet effects, cilostazol does not eliminate the need for aspirin or clopidogrel in patients with intermittent claudication. In prospective, clinical trials studying patients on aspirin and cilostazol versus aspirin plus placebo (doses 75–325 mg/day), no significant increases in bleeding events were seen (Thompson et al. 2002). Similarly, the combination of clopidogrel and aspirin has not been demonstrated to significantly increase bleeding events in postmarketing studies, although more data are needed (Hiatt et al. 2005).

In clinical trials, the most common reported side effects of cilostazol include headache (34 %), loose stools (15 %), and diarrhea (19 %). Headache was the most common reason for drug discontinuation and appears to be dose dependent (1.3 % for 50 mg BID and 3.7 % for 100 mg BID) (Pratt et al. 2001). Cilostazol is contraindicated in heart failure as these patients are often on other PDE3 inhibitors (e.g., milrinone) which also increase cAMP levels. Nevertheless, clinical trial data have not demonstrated an increased risk of cardiovascular mortality in patients with heart failure.

3.2 Dipyridamole

Dipyridamole, initially used as a coronary vasodilator, has several anti-platelet effects that have recently been reviewed (Gresele et al. 2011; Eisert 2007). As shown in Fig. 2, one of these includes inhibition of PDE5 that in turn increases cGMP levels (Eisert 2007; Aktas et al. 2003). Dipyridamole also stimulates prostacyclin production and blocks RBC-mediated uptake of the vasodilator adenosine (Klabunde et al. 1983; Neri Serneri et al. 1981). Adenosine-reuptake blockade leads to inhibition of platelet aggregation in whole blood (Gresele et al. 1983, 1986). By scavenging free radicals that inactivate cyclooxygenase, dipyridamole has key antioxidant properties that potentiate the inhibition of platelet activation and thrombin generation (Chakrabarti et al. 2008). In addition to affecting platelet function, dipyridamole has proven anti-thrombotic properties. Dipyridamole inhibits the activation of platelets as they contact extracellular matrix (Eldor et al. 1986). It also reduces thrombus formation and fibrinogen accumulation at the surface of damaged arteries (van Ryn et al. 2003; Venkatesh et al. 2010).

Oral ingestion of dipyridamole requires low pH (~4) for absorption, which needs to be taken into consideration in order to achieve relevant plasma levels (Eisert 2007). Dipyridamole is indicated as an adjunct to coumarin anti-coagulants in the prevention of post-operative thromboembolic complications associated with cardiac valve replacement (Stein et al. 1998; Chesebro et al. 1985). Dipyridamole is also given in combination with low-dose aspirin to reduce the rate of recurrent strokes (Eisert 2007). In several prospective trials and in meta-analysis data, patients randomized to dipyridamole had significantly greater reductions in the risk of stroke (Diener et al. 1996; Halkes et al. 2006; Verro et al. 2008). This efficacy is reflected in the current guidelines from the American College of Chest Physicians recommending that patients with stroke or TIA should be prescribed dual therapy with extended-release dipyridamole (200 mg BID) plus aspirin over aspirin alone (Adams et al.

2008). A recent large clinical trial showed that the rates of recurrent stroke were similar in patients receiving aspirin and dipyridamole combination therapy or clopidogrel (Sacco et al. 2008). Like cilostazol, the most common side effect of dipyridamole is headache at the onset of therapy (Theisetal. 1999).

4 Summary

PDEs are appealing targets for anti-platelet therapy. Indeed, drugs that target PDEs are in development and cilostazol and dipyridamole have established anti-platelet effects that are mediated, in part, via inhibition of PDEs in platelets. Because PDEs are expressed by a variety of cells, it is not surprising that PDE inhibitors may have other benefits in the treatment of cardiovascular disease beyond their direct effect on platelets. As an example, dipyridamole blocks de novo synthesis of monocyte chemotactic protein-1 (MCP-1) and matrix metalloproteinase-9 (MMP-9) by platelet-leukocyte aggregates (Franks et al. 2010; Weyrich et al. 2005, 2006). Similarly, cilostazol inhibits the expression of MCP-1 in endothelial cells by mechanisms that involve increased cAMP (Nishio et al. 1997). Both cilostazol and dipyridamole inhibit inflammatory responses mediated by NF- κ B (Weyrich et al. 2005; Wang et al. 2008; Jung et al. 2010). Although not rigorously tested, it is possible that the combinatorial anti-platelet and anti-inflammatory properties of cilostazol and dipyridamole could intervene in atherosclerosis disease progression. At a minimum, a deeper understanding of PDE inhibition in platelets and other cells is warranted as we search for effective agents in the treatment of acute and chronic cardiovascular disease.

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Knowledge Gaps

- A deeper understanding of the role of PDEs in human platelets
- The development of a therapeutic strategy that specifically inhibits PDEs in platelets but not other cells
- The potential clinical benefit of combining lipid-lowering drugs with cilostazol or dipyridamole to prevent atherosclerosis disease progression

Key Messages

- PDE inhibitors, such as cilostazol and dipyridamole, inhibit platelet function by increasing cyclic adenosine 3'-5'-monophosphate (cAMP) and cyclic guanosine 3'-5'-monophosphate (cGMP) levels and are effective clinically.
- Cilostazol inhibits phosphodiesterase (PDE) 3 and has demonstrated clinical efficacy in reducing symptoms of claudication.
- Dipyridamole (a PDE5 inhibitor), given in combination with low-dose aspirin, reduces the rates of recurrent stroke and is recommended in patients with a history of stroke or transient ischemic attack (TIA).

Fig. 1.
The domain organization of platelet PDEs

Fig. 2. Schematic representation of the three isoforms of PDEs in platelets. The anti-platelet effects of cilostazol and dipyridamole are discussed in detail

Table 1

Phosphodiesterases (PDEs) in platelets

Family	Substrate	Clinically approved inhibitors
PDE2	cGMP = cAMP	None
PDE3	cAMP > cGMP	Cilostazol, milrinone, anagrelide
PDE5	cGMP	Dipyridamole, sildenafil, vardenafil, tadalafil