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Chemoproteomic Discovery of AADACL1 as a Novel Regulator of Human Platelet Activation

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Abstract

A comprehensive knowledge of the platelet proteome is necessary for understanding thrombosis and for conceiving novel antiplatelet therapies. To discover new biochemical pathways in human platelets, we screened platelets with a carbamate library designed to interrogate the serine hydrolase subproteome and used competitive activity-based protein profiling to map the targets of active carbamates. We identified an inhibitor that targets arylacetamide deacetylase-like 1 (AADACL1), a lipid deacetylase originally identified in invasive cancers. Using this compound, along with highly selective second-generation inhibitors of AADACL1, metabolomics and RNA interference, we show that AADACL1 regulates platelet aggregation, thrombus growth, RAP1 and PKC activation, lipid metabolism and fibrinogen binding to platelets and megakaryocytes. These data provide the first evidence that AADACL1 regulates platelet and megakaryocyte activation and highlight the value of this chemoproteomic strategy for target discovery in platelets.

Human platelets are essential mediators of both hemostasis and thrombosis. Endogenous signaling molecules and pathways have been identified that directly contribute to essential platelet functions including activation of the αIIbβ3 integrin, cytoskeletal reorganization during shape change, spreading, secretion of intracellular granules and homotypic cell-cell interactions (Shattil et al., 2010). Recurrent themes have emerged from the elucidation of these signaling pathways, especially those that utilize G protein-coupled receptors (GPCRs) such as thrombin and ADP receptors or immunoglobulin-like receptors such as glycoprotein

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VI (GPVI). Signals emanating from these receptors that lead to the activation of αIIbβ3 are termed “inside-out” (Watson et al., 2005; Coughlin, 2005). These pathways share common molecular components, second messengers and integrin binding proteins and exhibit multiple overlapping nodes and feedback loops.

Knowledge of these platelet signaling pathways has slowly facilitated the development of modern antiplatelet therapies intended to prevent thrombus formation, but to more rapidly realize the potential of the platelet proteome, techniques that survey not only the expression, but also the activity of multiple proteins are required. One such proteomic technique called activity-based protein profiling (ABPP) can simultaneously detect large numbers of enzyme activities generally within the same enzyme family (Barglow and Cravatt, 2007; Cravatt et al., 2008; Paulick and Bogyo, 2008). A powerful extension of ABPP known as competitive ABPP can actually identify enzymes from complex cell or tissue proteomes and enable development of selective inhibitors for these enzymes *in vitro* and *in vivo*, even if substrate specificities are unknown. Competitive ABPP can also be coupled to libraries of small molecules (*e.g.* carbamates) to facilitate inhibitor or lead compound discovery (Li et al., 2007).

Competitive ABPP is usually applied to cell-free proteomes but has never been applied to platelets in any capacity for any enzyme family. This approach is especially suitable for broadly examining biochemical pathways in the anucleate platelet, a cell type that is resistant to many genomic approaches. Here we screened live human platelets via a combination of carbamate profiling and competitive ABPP with the goal of discovering novel enzymes that positively influence signaling pathways leading to αIIbβ3 activation. To maximize our chances of success, we targeted the serine hydrolase superfamily because of its large size, estimated to represent 1% of the proteome, and its sensitivity to well-characterized carbamate probes. We identified a chlorinated phenyl carbamate (WWL91) that blocks fibrinogen binding to activated platelets and identified its endogenous enzyme targets as arylacetamide deacetylase-like 1 (AADACL1, also known as KIAA1363 or NCEH1), a microsomal lipid hydrolase and acylpeptide hydrolase (APEH), a recently described cytosolic hydrolase that cleaves acetylated peptides in T cells (Adibekian et al., 2012; Jessani et al., 2002; Sekiya et al., 2009). The inhibitory effects of WWL91 in platelets could not be attributed to APEH, but instead depended on AADACL1. Inhibition of AADACL1 activity with a variety of agents blocked platelet aggregation in response to multiple agonists, impaired inside-out signaling to αIIbβ3 in both human platelets and their precursors, megakaryocytes, abolished synthesis of certain ether lipids and blocked activation of the small GTPase RAP1 and protein kinase C (PKC). Furthermore, inhibition of AADACL1 by RNA interference downregulated inside-out signaling to αIIbβ3 in a differentiated megakaryocytic cell line. These data represent the first report of AADACL1 protein expression in platelets and megakaryocytes and the first demonstration of AADACL1-dependent regulation of these cell types.

Results

Carbamate screening of platelets

Gel-purified human platelets were profiled with a 120-member carbamate library (Li et al., 2007) to identify compounds that antagonized αIIbβ3 activation. Platelets were pretreated separately with each carbamate before agonist addition and analyzed by flow cytometry for fibrinogen (Fg) binding as a measure of αIIbβ3 activation and for surface-exposed P-selectin as a measure of α-granule secretion. Of the entire library, 12 carbamates strongly inhibited Fg binding and P-selectin exposure in response to thrombin receptor activating peptide (TRAP, Figure 1A). No inhibitors selectively blocked Fg binding without also blocking surface accumulation of P-selectin. Inhibition ranged from 61-81% and was not caused by

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membrane permeability or cell lysis as measured by lactate dehydrogenase (LDH) activity in supernatants from platelets treated with carbamates or DMSO (Figure S1).

We next evaluated the selectivity profiles of the five most efficacious carbamates via gel-based competitive ABPP using fluorophosphonate-rhodamine (FP-Rh) to visualize serine hydrolase activities. Only WWL91 visibly inhibited serine hydrolases by gel fluorography (data not shown) and for this reason, we focused on WWL91 for subsequent experiments. To confirm that WWL91 inhibition of platelet aggregation was due to specific blockade of serine hydrolase activity, we synthesized a non-hydrolyzable derivative of WWL91 (WWL91-urea) by substituting the carbamate reactive group with a urea group, rendering this compound resistant to nucleophilic attack by serine residues (Figure 1B – C, S1A). As expected, WWL91-urea was inert at concentrations of WWL91 that almost completely blocked platelet aggregation, which provided a rationale for the pursuit of the endogenous platelet target(s) of WWL91 using more sensitive mass spectrometry (MS)-based methods.

Platelet targets of carbamate WWL91

Since carbamate profiling had revealed WWL91 as a relatively selective serine hydrolase inhibitor in platelets, we sought to identify its endogenous target(s) using FP-biotin and competitive ABPP-MudPIT, a more sensitive, solution phase variation of the gel-based method mentioned above (Jessani et al., 2005). Following WWL91 pretreatment of intact platelets, platelets were lysed and reacted with FP-biotin to enrich for active serine hydrolases not inhibited by WWL91; hydrolases bound to FP-biotin were identified and quantitated by tandem MS. At least 14 serine hydrolases were reproducibly detected by this method in human platelets, some of which have never been previously described as endogenous platelet proteins. Figure 1D shows a core list of serine hydrolases detected in DMSO- or WWL91-treated human platelets in descending order of peptide count. WWL91 competed with FP-biotin and reduced spectral counts for two enzymes: arylacetamide deacetylase-like 1 (AADACL1) and acylpeptide hydrolase (APEH). Since mRNA transcripts of both enzymes have been reported in human platelets and megakaryocytes (Rowley et al. 2011), we confirmed protein and mRNA expression of AADACL1 in these cells by Western blotting and next-generation RNA sequencing (Figure S3A, C). Interestingly, neither AADACL1 protein nor mRNA was detected in mouse platelets, which precludes traditional mouse model systems for studying the role of AADACL1 in thrombosis (Figure S3B and Rowley et al., 2011). This expression data coupled with the inhibition of platelet functions observed with WWL91 formed the basis of our initial hypothesis that one or both of these enzymes are unique and important regulators of human platelet activation.

AADACL1 is important for platelet aggregation

We first assessed the role of AADACL1 in human platelet function since selective carbamate inhibitors that block the catalytic activity of AADACL1 have been developed (Chang et al., 2011; Chiang et al., 2006). We pretreated platelets with the most selective and potent inhibitor of AADACL1 currently developed, JW480 (Chang et al., 2011), and stimulated human platelets with threshold concentrations of various agonists to calculate IC_{50} values for JW480, which allowed us to evaluate the relative sensitivities of platelet signaling pathways to AADACL1 inhibition. JW480 dose-dependently inhibited aggregation in response to collagen (500 ng/mL) with an IC_{50} of 2.2 μM, having effects at concentrations as low as 1 μM (Figure 2A). The two parent carbamates used to synthesize JW480, JW147 and JW148, also inhibited aggregation in response to collagen, but with slightly less potency (data not shown). Since collagen can stimulate the release of secondary agonists such as thromboxane A_2 (TXA_2) and ADP, we also tested the ability of JW480 to block platelet aggregation downstream of a stable TXA_2 mimetic, U46619. JW480 blocked

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aggregation in response to U46619 with an IC_{50} of 5.6 μM (Figure 2B). Although not statistically different from the inhibition of collagen-stimulated aggregation, JW480 appeared to inhibit U46619-mediated aggregation less effectively. JW480 also blocked ADP-dependent aggregation, but less potently than either collagen- or U46619-mediated aggregation (Figure 2C, IC_{50} 30 μM). JW480 was much less effective and variable in its ability to inhibit aggregation stimulated by the more powerful agonist, γ-thrombin. JW480 inhibited aggregation of platelets from two donors that responded to a low threshold concentration of γ-thrombin (24 nM, Figure 2D). However, platelets from four other donors only responded to higher concentrations of γ-thrombin (≥ 47 nM) and were not inhibited by JW480 with one exception (47 nM γ-thrombin with 20 μM JW480). This relatively weak effect on thrombin signaling prevented accurate calculation of IC_{50} values, but nevertheless suggests that AADACL1 regulates a common molecular pathway used by multiple platelet agonists including collagen, TXA_2, ADP and possibly thrombin.

In addition to AADACL1, JW480 has been reported to block murine carboxylesterase 1 in brain tissue extracts (Chang et al., 2011). Therefore, to assess carboxylesterases as potential targets of JW480 in human platelets, we pretreated platelets with benzil, an inhibitor of both hCES1 and hiCE, the two main isoforms of human carboxylesterase (Hyatt et al., 2006). At concentrations at or below 20 μM, benzil did not block platelet aggregation (Figure S2C), whereas the same concentrations of benzil abolished partially purified hCES1 activity (Figure S2D). Similarly, benzil did not affect Fg binding to megakaryocytes (Figure 2F), demonstrating that the inhibitory effects of JW480 in platelets and megakaryocytes are most likely independent of carboxylesterases.

Since competitive ABPP implicated APEH in addition to AADACL1 as a platelet target of WWL91, we also tested a small molecule inhibitor of APEH, AA74-1, for its ability to affect platelet aggregation. Human platelets treated with AA74-1 (500 nM to 50 μM) were not significantly inhibited in their aggregation response to collagen (Figure S2A - B), which further strengthens our hypothesis that the predominant target of WWL91 identified in our initial carbamate profiling efforts is AADACL1 and that JW480 disrupts aggregation by specifically targeting this hydrolase in human platelets.

AADACL1 regulates inside-out platelet signaling

Platelet aggregation reflects a complex network of extracellular and intracellular signals, including bidirectional signals leading to and from the αIIbβ3 integrin. Signals leading to the activation of αIIbβ3 for example are termed “inside-out” (Shattil et al., 2010). Because WWL91 blocked inside-out signals leading to αIIbβ3 activation and granule release, we reasoned that JW480 might also block these events. We used γ-thrombin as an agonist instead of TRAP or collagen due to its greater potency as an activator of αIIbβ3. In the absence of JW480, γ-thrombin induced a dose-dependent increase in Fg binding to platelets (Figure 2E). In the presence of JW480, Fg binding was inhibited at all γ-thrombin concentrations tested but was maximally reduced by 55% (47 nM γ-thrombin and 20 μM JW480). As expected, γ-thrombin-induced Fg binding was inhibited more readily by JW480 than γ-thrombin-induced aggregation. This probably reflects 1) the fact that significant inhibition of platelet aggregation requires ≥ 80% blockade of fibrinogen receptor (αIIbβ3) occupancy and 2) the extent to which αIIbβ3 is activated in the two assays, with aggregation involving additional feedback loops to reinforce activation (Mascelli et al., 1998).

To determine whether AADACL1 regulates inside-out signaling in megakaryocytes, we utilized primary megakaryocytes differentiated *in vitro* from human cord blood progenitors (Foulks et al., 2009). As with platelets, we pretreated megakaryocytes with JW480 and measured Fg binding by flow cytometry. JW480 blocked Fg binding to thrombin-stimulated

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megakaryocytes by 51% (Figure 2F), suggesting that AADACL1 participates in both platelet and megakaryocyte inside-out signaling.

As an additional control for JW480, we measured Fg binding to both Aadacl1-deficient and heterozygous murine platelets, neither of which express AADACL1 (Figure S3B – C). JW480 did not inhibit γ-thrombin-stimulated Fg binding to murine platelets (Figure S6), further demonstrating JW480 selectivity. This also excludes the possibility that JW480 directly interferes with αIIbβ3 ligand binding.

AADACL1 contributes to thrombus formation ex vivo

Although our data clearly implicate AADACL1 as a novel regulator of platelet activation, its role in thrombus formation under physiological shear conditions had not been addressed. Since this enzyme is not expressed in mouse platelets, we were unable to use mouse models of thrombosis (Figure S3 and Rowley et al., 2011). Furthermore, JW480 is less active in human plasma or whole blood presumably due to decreased bioavailability resulting from binding to serum proteins such as albumin. To circumvent these issues, we treated purified human platelets with JW480 or DMSO and introduced them into platelet-depleted mouse blood to study the role of AADACL1 in thrombosis in a microfluidic flow chamber (see "Experimental Procedures"). This method prevents JW480 exposure to other cell types such as neutrophils while allowing maximal JW480-dependent inhibition of platelets. At an arterial flow rate of 1600 s^{-1}, human control platelets adhered quickly and coalesced into multiple tear-shaped thrombi beginning at approximately one minute after onset of blood flow, demonstrating the feasibility of the assay (Figure 3A). Three dimensional (3D) thrombus growth was evident by video microscopy as increased cumulative fluorescence intensity over time. In contrast, JW480 dose-dependently impaired 3D growth (Figures 3A - B). At 10 μM JW480, 3D growth was reduced but detectable as isolated islands of fluorescence that exhibited 52% less fluorescence intensity than control. At a higher dose of JW480 (25 μM), 3D growth was dramatically inhibited (77%) as illustrated by the lack of intense thrombi and the attenuated increase in fluorescence over time. The αIIbβ3 antagonist, eptifibatide (Phillips and Scarborough, 1997), was used as a positive control; eptifibatide-treated platelets did not form thrombi (87% inhibition), but adhered as a monolayer with no detectable platelet-platelet interactions. Thus, high concentrations of JW480 inhibit thrombus formation almost as effectively as a potent αIIbβ3 antagonist. Importantly, JW480 did not significantly affect platelet *adhesion* to collagen under these conditions (data not shown), which demonstrates that collagen binding to its receptors, GPVI and α2β1, was intact and that JW480 effects can be attributed to defective thrombi growth due to reduced inside-out signaling to αIIbβ3 via these receptors.

AADACL1 knockdown decreases αIIbβ3 activation in CMK11-5 cells

We sought to independently confirm the importance of AADACL1 in αIIbβ3 activation in response to collagen receptor signaling using RNA interference (RNAi) and a megakaryocytic cell line, CMK11-5. These cells efficiently perform RNAi, express αIIbβ3 and reportedly respond to agonists such as PMA and thrombin (Tadokoro et al., 2011), but not ADP, collagen or convulxin, a snake toxin that binds and potently activates GPVI. To achieve responsiveness to either collagen or convulxin, we developed a unique seven-day differentiation protocol (see "Experimental Procedures") that stimulated a dramatic increase in αIIbβ3 and GPVI surface levels (Figure S4; Chen and Kahn, 2003). While these more differentiated CMK11-5 cells still responded poorly to collagen, they responded strongly to convulxin, with an 11-fold activation of αIIbβ3-mediated Fg binding (Figure 4C). Lack of collagen sensitivity may be due to the complex interplay between the collagen receptors GPVI and α2β1, inadequate surface density of these receptors or lack of appropriate

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environmental cues. Nevertheless, this is the first description of convulxin-mediated activation in CMK11-5 cells.

During the differentiation process, we transfected CMK11-5 cells with AADACL1-specific small interfering RNA (siRNA) and reduced AADACL1 protein levels by at least 90% compared to two distinct control siRNAs (Figure 4A). Depletion of AADACL1 did not block differentiation of CMK11-5 cells as judged by αIIbβ3 surface expression (Figure 4B), but did reduce Fg binding in response to convulxin by 41% compared to control siRNA-transfected cells (Figure 4C). Fg binding to these cells was αIIbβ3-dependent because 1 mM RGD peptide or 9 μM eptifibatide blocked Fg binding by 96% and 77% respectively (Figure 4C). These data provide key genetic evidence that AADACL1 regulates inside-out signals leading to αIIbβ3 activation.

Treatment of CMK11-5 cells with JW480 (10 μM) also reduced Fg binding in response to convulxin by 50% (Figure 4D). This inhibition was similar to the observed effects of JW480 on Fg binding to primary megakaryocytes, again indicating that AADACL1-dependent inside-out signaling pathways are preserved in CMK11-5 cells and that AADACL1 is required for maximal output of these pathways in response to certain agonists in megakaryocytes and platelets.

AADACL1 regulates RAP1 and PKC activation

Having established that AADACL1 regulates αIIbβ3 using complementary pharmacological and genetic methods, we searched for a molecular mechanism by probing the two major signaling nodes essential for αIIbβ3 activation in platelets, RAP1 and PKC. We treated platelets with AADACL1 inhibitors and first examined the activation status of RAP1, a small molecular weight GTPase crucial for inside-out signaling downstream of collagen receptor signaling (Chrzanowska-Wodnicka et al., 2005; Wang et al., 2009). Increasing concentrations of either WWL91 (Figure 5A) or JW480 (Figure 5B) potently blocked RAP1 activation in response to collagen at concentrations similar to the IC_{50} for JW480-dependent inhibition of aggregation.

PKC activation is the second major node responsible for αIIbβ3 activation in platelets (Cifuni et al., 2008; Stefanini et al., 2009). As with RAP1, JW480 blocked PKC activation in response to collagen (Figure 5C). Phosphorylation of PKCα itself was strongly reduced by JW480 at concentrations as low as 5 μM and the PKC substrate pleckstrin also showed reduced phosphorylation in JW480-treated platelets (Figure 5D). Interestingly, the phosphorylation of other PKC substrates was only partially diminished by JW480, suggesting that not all PKC isoforms are sensitive to JW480 or that PKC activity was only partially inhibited. Thus, JW480 inhibits both RAP1and PKC activation downstream of collagen receptors, which correlates with the aggregation defects noted earlier.

AADACL1 regulates ether lipid metabolism in platelets

AADACL1 hydrolyzes the ether lipid substrate, 1-O-hexadecyl-2-O-acetyl-sn-glycerol or HAG (general term is 2-acetyl monoalkylglycerol ether or 2-acetyl MAGE), in human cancer cells (Chiang et al., 2006). To further understand the mechanism of AADACL1 action in human platelets, we asked whether platelets possess the same HAG hydrolase activity attributed to AADACL1 in cancer cells. We found that human platelets contain a comparable level of HAG hydrolase activity compared to mouse brain extracts, which served as a positive control (Figure 6A). Pretreatment of resting platelets with JW480 dramatically inhibited HAG hydrolase activity at concentrations that block HAG hydrolysis in brain extracts, which demonstrates that AADACL1 is the predominant HAG metabolizing enzyme in these cells. We next measured endogenous levels of MAGE lipids – known

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products of 2-acetyl MAGE hydrolysis in resting and collagen-stimulated platelets. JW480-treated platelets showed a 68-80% decrease in the amounts of C16, C18 and C18:1 MAGE lipids relative to DMSO treated controls (Figure 6B). Collagen stimulated the production of C16 MAGE, which could be blocked by JW480 (Figure 6C). Thus, platelet AADACL1 exhibits a similar, if not identical, hydrolase activity found in other tissues including invasive human cancer cells. Moreover, the levels of endogenous MAGE lipids appear to be generated primarily by AADACL1 hydrolase activity in the presence and absence of the platelet agonist collagen.

Ether lipids inhibit platelet aggregation

The AADACL1 substrate, HAG, is known to exist in platelets and can exert potent biological activities on other hematopoietic cells via direct inhibition of PKC isoforms through competition with diacylglycerol (DAG) cofactors (Lee et al., 1990; Cabot and Jaken, 1984; Mandal et al., 1997; Daniel et al., 1988). Therefore, we reasoned that blockade of HAG hydrolysis by inhibiting AADACL1 may result in accumulation or stabilization of lipid substrates like HAG that inhibit PKC activity and subsequent platelet activation. To test this hypothesis, we analyzed the ability of HAG to inhibit collagen-induced platelet aggregation. HAG inhibited platelet aggregation in a dose-dependent manner with an IC_{50} of 12 μM with a maximum inhibition of 88% at 50 μM (Figure 6D). Interestingly, addition of MAGE to platelets treated with JW480 did not rescue platelet aggregation (Figure S5), suggesting that AADACL1 substrates and not their obligatory products are potential causative agents of platelet inhibition. These data are consistent with a role for AADACL1 in metabolizing ether lipid substrates that act as endogenous, naturally occurring inhibitors of platelet activation.

Discussion

We used a chemoproteomic approach combining carbamate profiling and competitive ABPP to screen human platelets and identify novel enzyme targets. This approach identified two serine hydrolases, AADACL1 and APEH, which have never before been linked to human platelets. Using additional carbamates that block AADACL1, a triazole urea compound specific for APEH, and RNA interference, we show that AADACL1 is the pharmacologically relevant target of carbamate profiling in stimulated platelets. Moreover, we propose that AADACL1 is a potential drug target for antiplatelet therapy based on reasons outlined below.

AADACL1 regulates important prothrombotic signaling pathways downstream of the physiological platelet agonists, collagen, thrombin and TXA_2. These agonists are clinically relevant, since collagen is a major component of atherosclerotic plaques that triggers thrombosis, thrombin is essential for hemostasis, and TXA_2 production is blocked by aspirin. Inhibition of these agonist-induced pathways is reversible since increasing concentrations of agonist can completely overcome the effects of the AADACL1-specific carbamate JW480 and restore platelet aggregation. Thus, like other platelet regulators such as RAP1 and its exchange factor CALDAG GEFI, AADACL1 promotes agonist signaling predominantly at low agonist concentrations when signal amplification is required to activate platelets.

While our data clearly point to a role for AADACL1 in normal platelet activation, a more pathologically relevant situation is the regulation of thrombus formation. In an *ex vivo* thrombus formation assay using an arterial shear rate, inhibition of AADACL1 with a relatively low dose of JW480 significantly reduced the number of platelets incorporating into thrombi without altering adhesion to immobilized collagen. The presence of thrombi under these conditions demonstrates that as with aggregation, inhibition of AADACL1 does

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not completely block collagen signaling and implies that higher amounts of collagen would result in relatively normal thrombi. A higher concentration of JW480, however, blocked thrombus formation more completely, resulting in a platelet monolayer that resembled the phenotype observed with eptifibatide. That inhibition of AADACL1 mimics blockade of αIIbβ3 by eptifibatide, an FDA-approved antithrombotic drug, validates AADACL1 as a new candidate target for antiplatelet therapy.

Carbamates such as JW480 inhibit the lipid deacetylase activity of AADACL1, which removes acetyl groups from ether lipids including 1-O-hexadecyl-2-O-acetyl-sn-glycerol (HAG) (Chiang et al., 2006). Our studies with JW480 demonstrate that AADACL1 also hydrolyzes HAG to its corresponding monoalkylglycerol ether (C16 MAGE) in human platelets, providing the first evidence that AADACL1 regulates ether lipid metabolism in platelets. HAG and the related ether lipid 1-O-hexadecyl-2-O-methyl-glycerol (HMG) exert a variety of effects on other hematopoietic cell types including inhibition of growth, reduction of anti-microbial processes and promotion of differentiation (Kramer et al., 1989; McNamara et al., 1984). Multiple investigators have also shown that HAG directly inhibits PKC activity by competing with diacylglycerol (DAG) binding to the N-terminal C1 domain (Daniel et al., 1988; Mandal et al., 1997; Tao et al., 2009). PKC inhibition requires an alkyl linkage at the sn-1 position of the lipid, as various hydrocarbon chain lengths, degrees of saturation or types of linkages at the sn-2 position do not affect the ability of these ether lipids to block DAG-mediated PKC activation. Consistent with this, we show that PKC activity is reduced but not completely absent in platelets treated with JW480 and that exogenous HAG dose-dependently inhibits platelet aggregation to collagen. These data suggest that HAG or related ether lipids metabolized by AADACL1 act as endogenous inhibitors of signal transduction pathways and may represent a physiologically relevant mechanism to limit platelet activation in response to agonists such as collagen (Figure 7).

In addition to HAG, cholesterol esters (CE) have been reported to be AADACL1 substrates in mouse macrophages, but this substrate assignment is controversial and complicated by the expression of several potentially redundant CE hydrolases in macrophages (Buchebner et al., 2010; Sekiya et al., 2009). AADACL1 does not hydrolyze abundant phospholipids such as phosphatidylcholine or the bioactive ether lipid platelet activating factor (Chiang et al., 2006). Therefore, 2-acetyl MAGE is the only undisputed physiological substrate for AADACL1 identified to date, but even so, it may not totally account for the inhibitory effects of WWL91 or JW480 in platelets.

Significance

Human platelets are crucial mediators of the physiological balance between hemostatic processes that control blood loss and thrombotic diseases such as heart attack and stroke. In this study, we employed a chemoproteomic approach consisting of phenotypic screening and competitive ABPP to rapidly identify inhibitory carbamate compounds and their respective molecular targets in human platelets. We identified several inhibitors of human platelet activation including WWL91, a chlorinated phenyl carbamate which may represent a lead compound for the future development of more selective and potent antiplatelet agents. We also identified the major pharmacological target of WWL91 as AADACL1, a fundamental discovery that will likely drive future investigations. We showed for the first time that AADACL1 is an ether lipid deacetylase in platelets required for signaling to the αIIbβ3 integrin. Since this enzyme appears to superimpose a novel mechanism of regulation downstream of many but not all platelet agonists, AADACL1 represents a potential drug target. An effective anti-AADACL1 drug would limit thrombosis by blocking distinct signaling pathways not involved in thrombin-dependent hemostasis and thus minimize the risk of spontaneous bleeding. In conclusion, our discovery of AADACL1 in human platelets

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highlights the multilayered complexity of platelet signaling networks and affords an opportunity to explore the therapeutic potential of this enzyme in thrombosis. More generally, our combination of carbamate and activity-based protein profiling offers a powerful strategy for identifying small molecule inhibitors and their corresponding endogenous targets that may be applicable to a range of critical cellular and biochemical processes.

Experimental Procedures

Carbamate profiling

A small library of 120 carbamates (Li et al., 2007), JW480 (Chang et al., 2011) or DMSO vehicle control was incubated in duplicate with quiescent platelets (1×10^6) in HEPES-Tyrode's buffer containing 1 mM $CaCl_2$ for 30 min. Platelets were then treated with an agonist cocktail containing 15 μg/mL Alexa Fluor 647-Fg, 20 μg/mL FITC-P-selectin antibody (clone AK-4) and various agonists including 10 μM thrombin receptor activating peptide (TRAP) or 95 nM - 1 μM γ-thrombin for 30 min. Fg binding and P-selectin staining are shown as mean fluorescence intensities normalized to the positive control (i.e. no carbamate) set at 100%. Normalization was performed by dividing experimental fluorescence values by positive control fluorescence. For Fg binding assays with JW480, Fg and AK-4 were added separately. No agonist and DMSO vehicle controls containing Fg and AK-4 were included to measure background fluorescence. All reactions were performed at room temperature (RT). Reactions were stopped with 5-10-fold dilutions in HEPES-Tyrode's buffer and immediately subjected to two-color flow cytometry on a BD FACSCanto dual laser flow cytometer. Spectral overlap was automatically compensated before data acquisition and data was evaluated using mean fluorescence intensities (MFI) from duplicate samples.

Competitive ABPP

Competitive ABPP was performed with FP-biotin as described previously (Chang et al., 2011). Briefly, platelets were treated with 0.25% DMSO or 25 μM carbamate for 30 min at RT and snap-frozen. Pellets were resuspended in PBS, lysed and 1 mg of human platelet lysate was treated with 5 μM FP-biotin for 1 hr at RT. Unreacted probe was removed with a PD10 column and the lysate was treated with 0.5% SDS and heated. FP-biotin binding proteins were enriched using avidin affinity chromatography, reduced with TCEP, alkylated with iodoacetamide and digested with trypsin overnight. The resulting peptides were analyzed by high resolution MudPIT featuring a Thermo Scientific LTQ Orbitrap Velos mass spectrometer. Enzymes were identified by searching public databases with the SEQUEST algorithm.

Differentiation and RNA interference of CMK11-5 cells

Human megakaryocyte-like CMK11-5 cells were purchased from the Health Sciences Research Resources Bank in Japan and grown in RPMI, 10% FBS (growth media) at 37° C at 5% CO_2. Differentiation of CMK11-5 cells was improved by a two-step process. First, cells were plated at a density of 2×10^6 cells/well in 6-well plates in growth media plus 160 nM phorbol myristate acetate (PMA) for 48 hr. Second, nonadherent cells were removed by aspiration and media was changed to IMDM, 10% FBS plus PMA for five days to achieve improved agonist responsiveness and high levels of αIIbβ3.

RNAi-mediated AADACL1 knockdown was achieved during CMK11-5 differentiation with 50 nM siRNA. Control siRNAs were derived from a mouse R-Ras coding sequence or a non-targeting control sequence (Dharmacon). Knockdown of AADACL1 was routinely greater than 90% as detected by Western blotting with the monoclonal antibody 16C8.

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Loading was confirmed with an anti-protein phosphatase 2A antibody (PP2A, clone 46). Band intensities were quantitated with ImageJ software.

Flow chamber assay

Washed human platelets were treated with the indicated concentrations of JW480, labeled with calcein green (2.5 μg/mL 10 min), diluted to ≤ 3×10^8 platelets/mL and mixed with mouse blood that was made thrombocytopenic by i.v. injection of antibodies against the human IL-4 receptor (hIL4Rα) into transgenic mice (Kanaji et al., 2002). Reconstituted blood was immediately perfused over collagen-coated chambers (200 μg/mL) at a shear rate of 1600 s^{-1} using a continuous flow syringe pump and adhesion of human platelets was visualized on a Nikon TE300 microscope equipped with a QImaging Retiga Exi CCD camera. Images were acquired and analyzed with Slidebook software. At each timepoint, fluorescence volume intensities were normalized to the maximum value observed for the DMSO control.

Platelet isolation and aggregation assays

All human and mouse blood was obtained using approved protocols from appropriate committees at the University of North Carolina at Chapel Hill (IRB and IACUC respectively). Platelet experiments were performed as described in “Supplemental Information”.

RAP1 and PKC activation assays

RAP1 activation was detected essentially as described with minor modifications (Wang et al., 2009). Gel-purified platelets were adjusted to 3×10^8/mL in HEPES-Tyrode’s buffer in the absence of calcium and pretreated with 0.25% DMSO or the indicated concentrations of carbamate for 30 min at RT in the presence of 10 μM eptifibatide to prevent outside-in signaling. Platelets were stimulated with various concentrations of agonist for 10 min and lysed with cold 2X Lysis Buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 10 mM CHAPS, 5 mM $MgCl_2$, 5% glycerol, 1 mM Na_3VO_4, 1 mM NaF and protease inhibitors). Lysates were probed with RalGDS-RBD and processed as in Wang et al. GTP-bound and total RAP1 were detected via Western blotting with a monoclonal or polyclonal antibody (clone 3, BD Biosciences or sc-65, Santa Cruz).

PKC activation was examined in the same platelet lysates used for RAP1 detection above. Phosphorylated substrates of PKC were detected with a polyclonal anti-phosphoserine PKC substrate antibody (#2261, Cell Signaling Technology). These blots were stripped and reblotted with a polyclonal anti-PKCα (#2056, Cell Signaling Technology) or pleckstrin (clone 25, BD Biosciences) antibody to verify protein loading.

Statistics

Data are presented as means ± SEM. Statistical significance was evaluated using unpaired t-tests and a 0.05 confidence limit for all experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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antibody, which was raised by Dr. Mark Kahn. This work was supported by the AHA (10GRNT3870017 to LVP) and the NIH (R01CA087660 to BFC, R01HL066277 to ASW, and R01HL094594 and R01HL106009 to WB).

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Highlights

- First application of activity-based protein profiling to platelets and thrombosis.
- A new endogenous target of the carbamate WWL91 is AADACL1 in platelets.
- AADACL1 regulates αIIbβ3 integrin activation and thrombus formation in platelets.
- AADACL1 deacetylates an ether lipid that blocks platelet signaling and aggregation.

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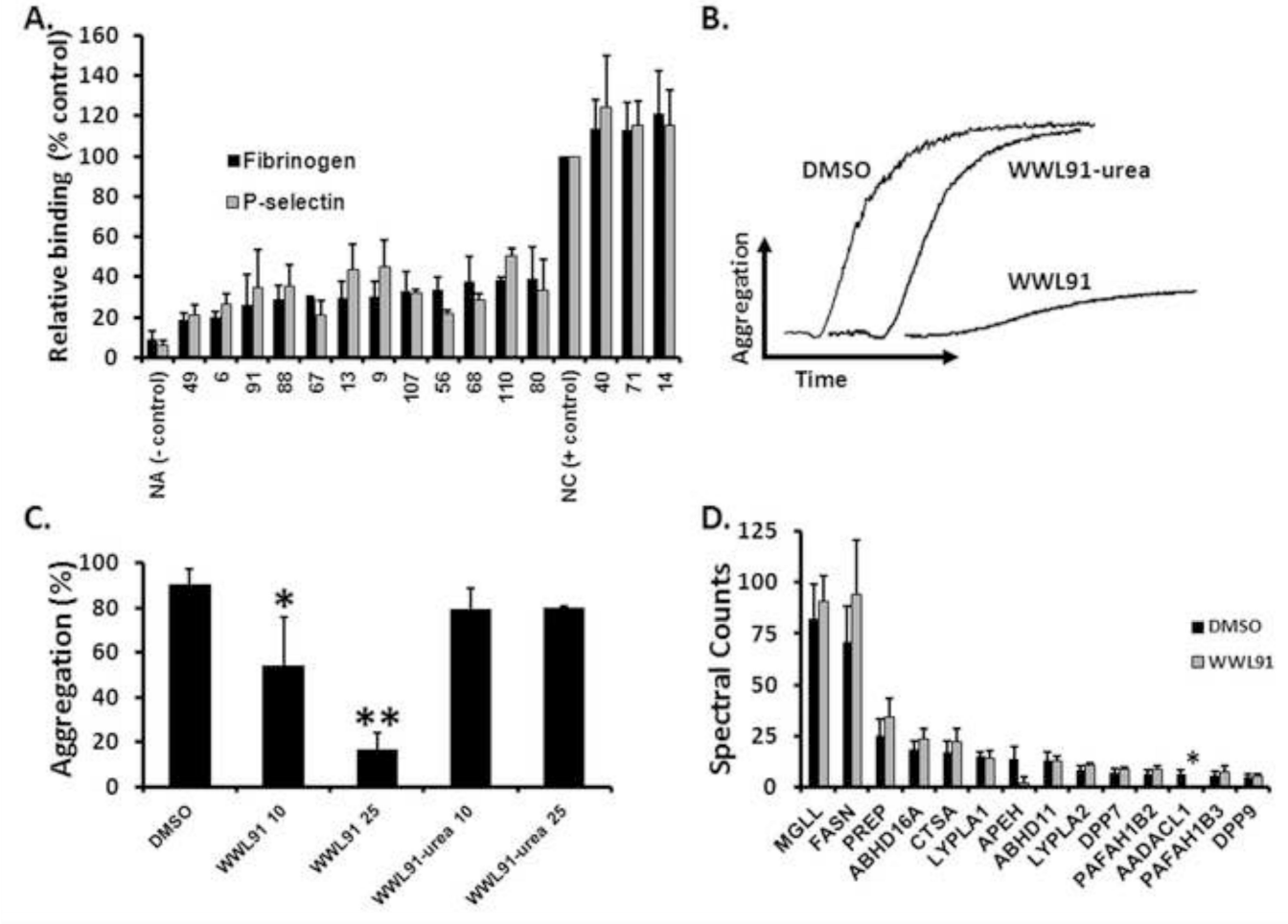

Figure 1. Inhibitor discovery via carbamate profiling of human platelets

A) Gel-filtered human platelets were treated with 120 distinct carbamates (25 μM) for 30 min before stimulation with 10 μM thrombin receptor activating peptide (TRAP) or not (NA/no agonist). Mean fluorescence intensity of Fg binding and P-selectin exposure were measured by flow cytometry and normalized to a TRAP-treated positive control set at 100% (NC is no carbamate plus 0.5% DMSO). Inhibitory carbamates are shown in decreasing potency ranging from 81 ± 3.1% to 67 ± 9.4% inhibition; 40, 71 and 14 are negative controls. Data represent the means ± SEM ($n > 2$). **B**) Platelets were pretreated with 0.5% DMSO vehicle control, 10 μM WWL91-urea or 10 μM WWL91 for 30 min before addition of 10 μg/mL type I collagen to stimulate aggregation, indicated by an upward trace (y-axis) over time (x-axis). Similar results were obtained with TRAP. **C**) Aggregation traces represented in panel B were averaged and presented as means ± SEM. WWL91-urea was statistically indistinguishable from vehicle control, but WWL91 inhibited aggregation by 82 ± 8.2% (25 μM WWL91, * $p < 0.02$, ** $p < 0.001$, $n = 5$). **D**) Platelets were treated with 0.25% DMSO or 25 μM WWL91 for 30 min and prepared for competitive ABPP as described in “Experimental Procedures”. Shown are spectral counts (means ± SEM) of serine hydrolases in decreasing order of abundance. WWL91 treatment significantly reduced spectral counts for AADACL1 only (* $p < 0.002$ for AADACL1, $p = 0.06$ for APEH, $n = 2$). See also Figure S1.

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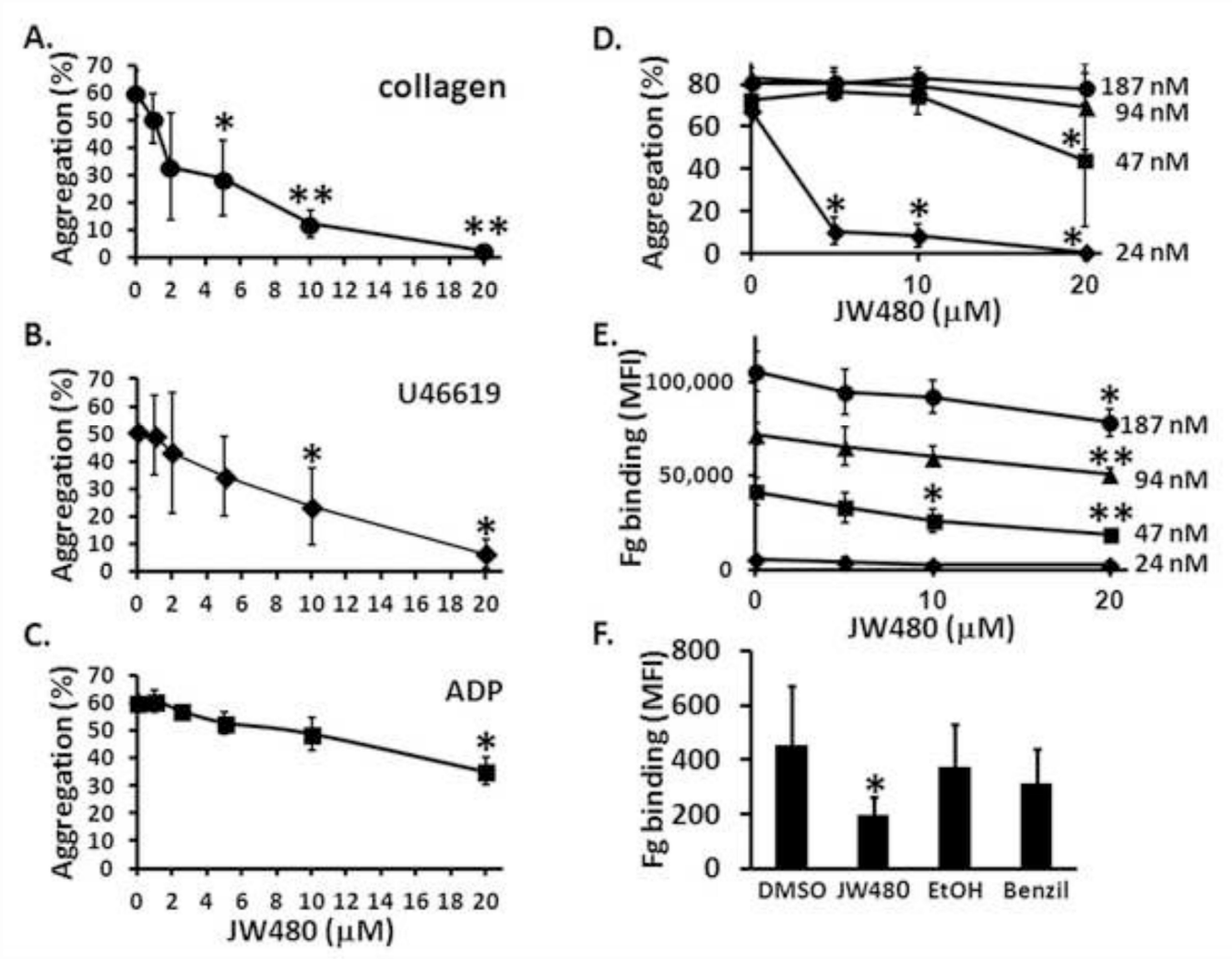

Figure 2. The selective AADACL1 inhibitor, JW480, blocks platelet and megakaryocyte functions

A - D) Human platelets were pretreated with varying concentrations of JW480 or DMSO (0.5% for all experiments) for 30 min and warmed to 37° C before initiation of aggregation with 500 ng/mL collagen (**A**), 400 nM U46619 (**B**), 10 μM ADP (**C**) or 24 – 187 nM γ-thrombin (**D**). JW480 inhibited collagen-dependent aggregation with an IC_{50} of 2.2 μM (* $p < 0.004$ for 5 μM JW480, ** $p << 0.001$ for 10 – 20 μM JW480 all vs. DMSO, n = 7) and an IC_{50} of 5.6 μM for U46619-dependent aggregation (* $p < 0.01$ for 20 μM JW480 vs. DMSO, n = 6). JW480 also blocked ADP-dependent aggregation (* $p < 0.01$ for 20 μM JW480 vs. DMSO, n = 3). **D**) Platelets were isolated using a wash protocol prior to treatment with 0, 5, 10 or 20 μM JW480 and stimulated with 24, 47, 94 or 187 nM γ-thrombin. JW480 significantly inhibited γ-thrombin-dependent aggregation (* $p < 0.02$ for 5 – 20 μM JW480 at 24 nM γ-thrombin and for 20 μM JW480 at 47 nM γ-thrombin). Data are means from six experiments (47 – 94 nM γ-thrombin), three experiments (187 nM) and two experiments for 24 nM due to the lack of response from some donors at this low concentration. **E**) Platelets were treated as in panel **D** before measurement of Fg binding by flow cytometry. JW480 significantly inhibited Fg binding to activated platelets by 55 ± 7.2% at 20 μM vs. DMSO at 47 nM γ-thrombin (* $p < 0.05$, ** $p < 0.004$, n = 4). **F**) Primary megakaryocytes (Foulks et al., 2009) were pretreated with 5 μM JW480, 50 μM benzil or

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vehicle controls prior to stimulation with thrombin (0.01 units/mL) and Fg binding to integrin αIIb-expressing cells was detected via flow cytometry. JW480 significantly inhibited Fg binding by 51 ± 13% (* $p < 0.05$, $n = 4$). All data represent means ± SEM. See also Figure S2 and Figure S6.

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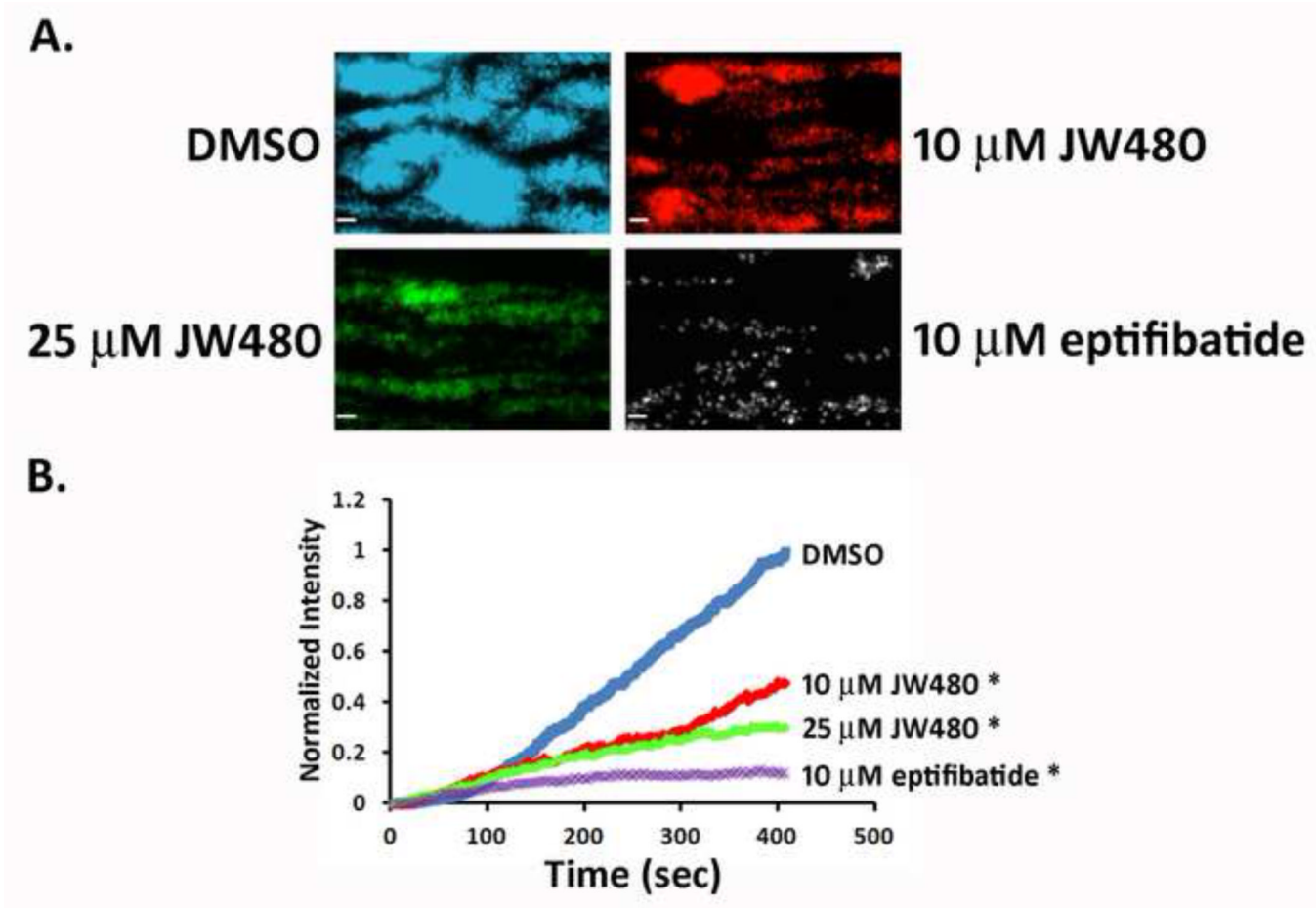

Figure 3. AADACL1 inhibition attenuates thrombus formation

Washed human platelets were treated with 10 μM or 25 μM JW480, 10 μM eptifibatide or 0.25% DMSO and mixed with platelet-depleted mouse blood (see "Experimental Procedures"). Blood was perfused over collagen at a flow rate of 1600 s^{-1} and thrombus formation was monitored by video microscopy at one second intervals. **A**) Representative images of thrombi are shown. Note the teardrop morphology of rapidly-forming thrombi in DMSO-treated controls and the lack of thrombus growth in platelets treated with JW480 or eptifibatide. Scale bar is 10 microns and images were extracted from movies at five (DMSO and 25 μM JW480) or seven (10 μM JW480 and eptifibatide) min. **B**) Kinetics of thrombus growth are shown for the conditions depicted in panel **A**. Data are presented as normalized fluorescence intensity over time. Pretreatment with JW480 or eptifibatide significantly reduced thrombus growth compared to DMSO (52 ± 16% for 10 μM JW480, * $p << 0.001$; 77 ± 2.5% for 25 μM JW480, * $p << 0.001$; 87 ± 5.8% for eptifibatide, * $p << 0.001$; means ± SEM, n = 4). See also Figure S3.

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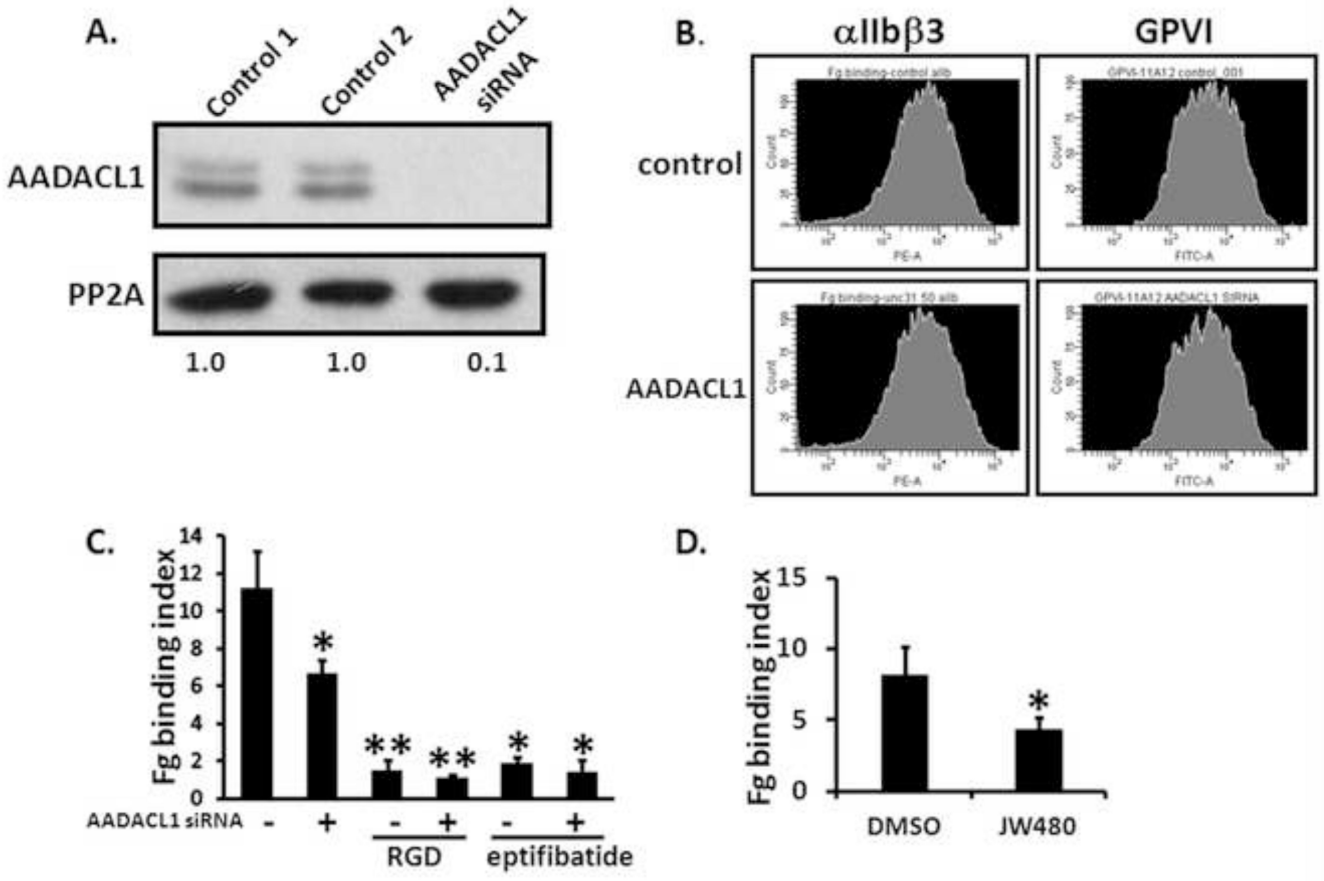

Figure 4. Genetic disruption of AADACL1 in CMK11-5 cells decreases αIIbβ3 activation

CMK11-5 cells were transfected with 50 nM siRNA for 24 hr and differentiated with PMA as described in "Experimental Procedures". **A**) AADACL1 expression was genetically reduced (knockdown) as detected by a monoclonal antibody as described (Chiang et al., 2006). Numbers indicate relative expression levels of AADACL1 normalized to a PP2A loading control across four experiments (94 ± 1% knockdown of AADACL1). **B**) Surface expression of αIIbβ3 and GPVI did not change upon siRNA treatment. Shown are representative histograms of αIIbβ3 and GPVI as detected by flow cytometry on differentiated CMK11-5 cells transfected with control or anti-AADACL1 siRNA. **C**) AADACL1 protein levels were reduced as in panel **A** and Fg binding to differentiated CMK11-5 cells was measured (see "Experimental Procedures"). AADACL1 knockdown significantly decreased Fg binding to αIIbβ3 (* $p = 0.03$, means ± SEM, $n = 5$) in response to 5 μg/mL convulxin. RGD (1 mM) and eptifibatide (10 μM) blocked Fg binding by 82 - 86% and 79 - 86% respectively, demonstrating that Fg binding is αIIbβ3-dependent (* $p < 0.03$ for eptifibatide, ** $p < 0.003$ for RGD). Knockdown was evaluated from the same batch of cells used for every Fg binding experiment. **D**) Differentiated CMK11-5 cells were treated with 10 μM JW480 for 24 hr before measurement of Fg binding as described above. JW480 significantly inhibited Fg binding by nearly 50% (* $p < 0.04$, means ± SEM, $n = 3$). See also Figure S4.

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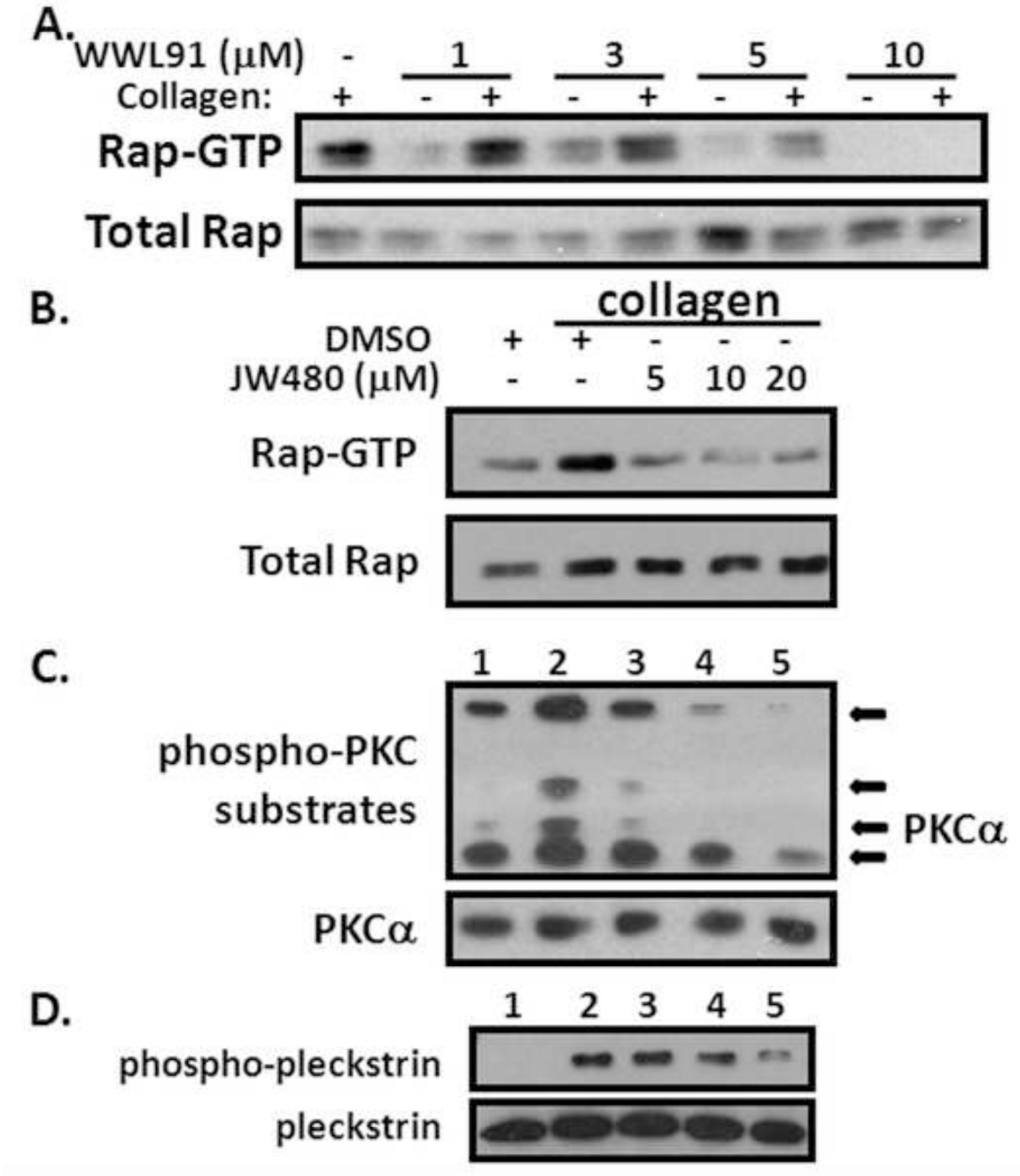

Figure 5. AADACL1 inhibitors block RAP1 and PKC activation

Platelets were pretreated with the indicated concentrations of WWL91 (**A**) or JW480 (**B**) for 30 min and then stimulated with 10 μg/mL collagen (**A**) or 2 μg/mL collagen (**B**) for 10 min before lysis and measurement of RAP1-GTP (activated RAP1). In panels **B** – **D**, 10 μM eptifibatide was also added to prevent outside-in signaling via αIIbβ3. Total RAP1 served as a loading control. **C**) Activated PKC was analyzed with a polyclonal antibody that recognizes serine phosphorylated substrates of PKC using the same conditions as in panel **B**: lane 1 (0.5% DMSO, no agonist), lane 2 (0.5% DMSO and 2 μg/mL collagen), lane 3 (5 μM JW480 plus collagen), lane 4 (10 μM JW480 plus collagen) and lane 5 (20 μM JW480 plus

collagen). Arrows indicate proteins phosphorylated on PKC-consensus sites in a collagen-dependent manner that were decreased in the presence of JW480. Blots were stripped and reblotted for PKCα to assess protein loading. **D**) Platelets from different donors were treated as in panel **C** (lanes 1 – 5). A serine-phosphorylated protein was detected at 47 kDa (upper panel) in platelet lysates with the same antibody used in panel **C**. Samples were also probed with an anti-pleckstrin antibody to confirm pleckstrin identity and to assess protein loading (bottom panel).

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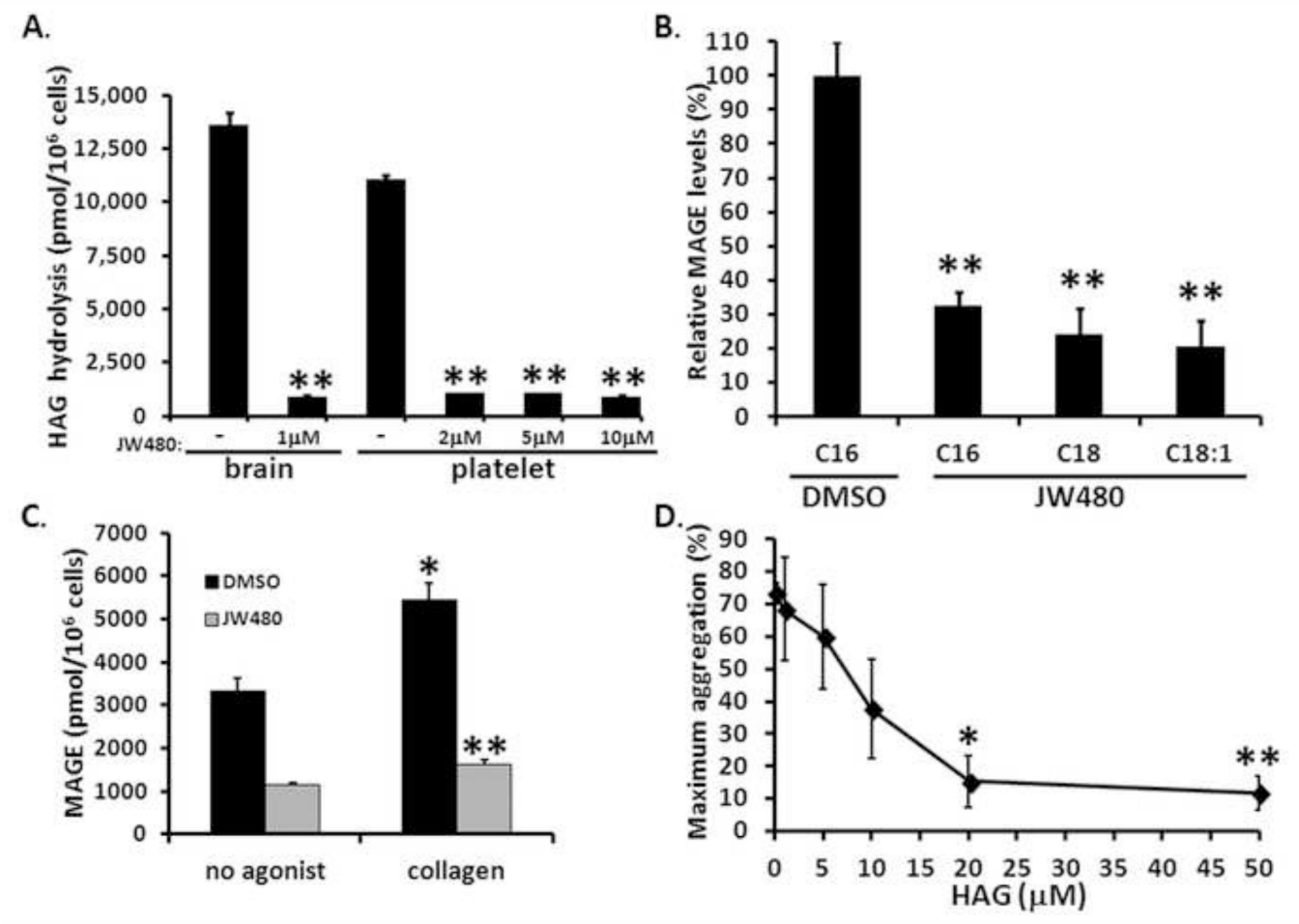

Figure 6. AADACL1 inhibition disrupts ether lipid metabolism in platelets

A) Platelets were treated with the indicated concentrations of JW480 or 0.5% DMSO for 30 min before lysis and evaluation of AADACL1 activity. HAG hydrolysis (pmol/10^6 cells) was measured in human platelets in parallel with mouse brain extracts using exogenous HAG substrate (Chang et al., 2011). JW480 treatment inhibited HAG hydrolysis by 90 ± 0.56% (2 μM), 90 ± 0.85% (5 μM) and 91 ± 0.69% (10 μM). Shown is a representative experiment (** $p < 0.001$, $n = 3$). **B**) Platelet C16, C18 and C18:1 MAGE lipids were assayed using a metabolomics approach in the presence or absence of 20 μM JW480. JW480 lowered levels of C16, C18, C18:1 MAGE by 67 ± 3.8%, 76 ± 7.6% and 80 ± 7.6% respectively. MAGE levels were normalized to DMSO-treated controls: each value from JW480-treated samples was divided by its corresponding DMSO control from the same donor to generate "% control" values which are independent of other controls. These values were depicted as means ± SEM for all donors (only C16 control is shown, ** $p << 0.001$, $n = 4$). **C**) C16 MAGE levels were measured by triple quadrupole MS in DMSO- or collagen-treated platelets. Platelets were treated with 10 μM JW480 or 0.1% DMSO for 2 hr, stimulated with 10 μg/mL collagen or not and lipids were extracted for MAGE quantitation (* $p < 0.003$ for DMSO black bars, ** $p << 0.0001$ for black and grey collagen bars, means ± SEM, $n = 5$). **D**) HAG was directly incubated with platelets for 30 min before addition of 2 μg/mL collagen to induce aggregation. HAG significantly blocked platelet aggregation in a dose-dependent manner compared to ethanol vehicle (0 HAG). Maximum inhibition was

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88 ± 5.1% for 50 μM HAG (* p = 0.001, **p << 0.001, means ± SEM, n = 4). See also Figure S5.

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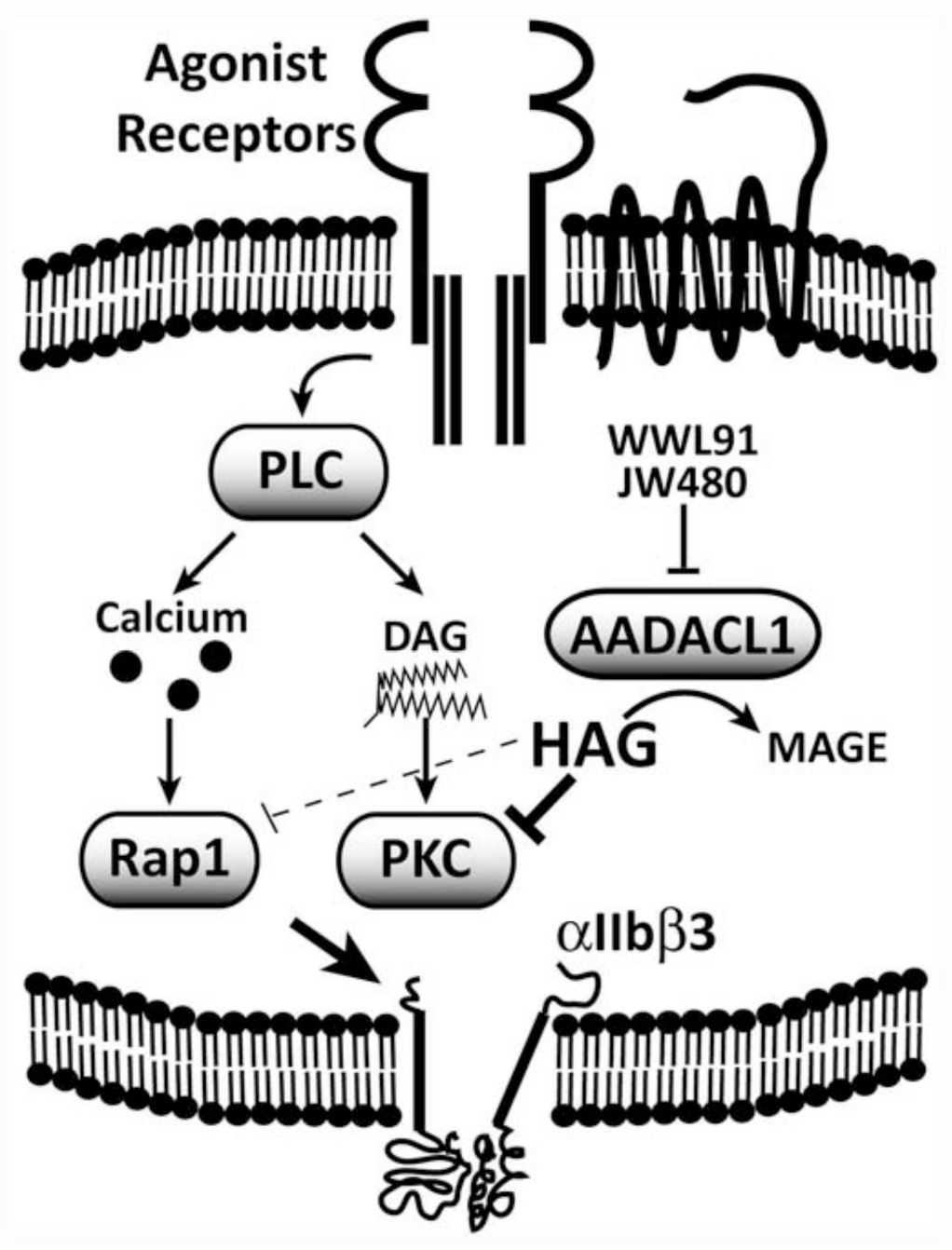

Figure 7. AADACL1 regulation of agonist signaling in human platelets

Agonist binding to immunoglobulin-like and G protein-coupled receptors stimulates intracellular calcium release and DAG production. These second messengers activate RAP1 and PKC which ultimately convert αIIbβ3 from a resting to an active conformation capable of binding Fg and promoting aggregation. AADACL1 hydrolyzes endogenous ether-linked lipids such as HAG that can compete for DAG binding to PKC, which may initiate and/or sustain PKC activation in response to agonists. Carbamates such as WWL91 or JW480 prevent AADACL1-dependent lipid hydrolysis and sensitize PKC to inhibitory ether-linked competitors of DAG. JW480 also inhibits RAP1 activation through an unknown mechanism (dashed line). Therefore, AADACL1 metabolizes inhibitory ether lipids in platelets to

maintain an optimal signaling flux, especially at low doses of agonist that require amplification through positive feedback loops.

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