Dynamin-Related Protein-1 Controls Fusion Pore Dynamics During Platelet Granule Exocytosis


Objective—Platelet granule exocytosis serves a central role in hemostasis and thrombosis. Recently, single-cell amperometry has shown that platelet membrane fusion during granule exocytosis results in the formation of a fusion pore that subsequently expands to enable the extrusion of granule contents. However, the molecular mechanisms that control platelet fusion pore expansion and collapse are not known.

Methods and Results—We identified dynamin-related protein-1 (Drp1) in platelets and found that an inhibitor of Drp1, mdivi-1, blocked exocytosis of both platelet dense and α-granules. We used single-cell amperometry to monitor serotonin release from individual dense granules and, thereby, measured the effect of Drp1 inhibition on fusion pore dynamics. Inhibition of Drp1 increased spike width and decreased prespike foot events, indicating that Drp1 influences fusion pore formation and expansion. Platelet-mediated thrombus formation in vivo after laser-induced injury of mouse cremaster arterioles was impaired after infusion of mdivi-1.

Conclusion—These results demonstrate that inhibition of Drp1 disrupts platelet fusion pore dynamics and indicate that Drp1 can be targeted to control thrombus formation in vivo. (Arterioscler Thromb Vasc Biol. 2013;33:00–00.)

Key Words: dynamin-related protein-1 ■ exocytosis ■ fusion pore ■ platelets ■ thrombosis

Platelet granules are required for normal hemostasis and contribute to pathological thrombus formation. Platelet granule exocytosis has also been implicated in numerous other physiological functions, including inflammation, microbial host defense, angiogenesis, and wound healing. Platelet granule types include α-granules (50–80 granules/platelet), dense granules (3–6 granules/platelet), and lysosomes (0–3 granules/platelet). These granules release their contents after stimulation of receptors on the platelet surface. Although much has been learned about the proximal signal transduction mechanisms that mediate the release of granule contents after stimulation of surface receptors, considerably less is known about the distal mechanisms that facilitate granule exocytosis.

Exocytosis of platelet granule contents requires the fusion of granule membranes with plasma membranes or membranes of the open canalicular system. The release of contents likely occurs through a dynamic fusion pore. Electrophysiological techniques have been used to study the formation, expansion, and collapse of the fusion pore in neurons and neuroendocrine cells. However, the mechanisms regulating the platelet fusion pore are poorly understood, and the proteins that control platelet fusion pore dynamics have not been identified. Platelet fusion pore dynamics have been difficult to study by standard electrophysiological methods because of their small size and atypical membrane system. Single-cell amperometry, however, has sufficient temporal and spatial resolution to evaluate fusion pore dynamics in live platelets and has, recently, been used to study quantal release of serotonin from individual dense granules. Amperometric tracings of platelet dense granule release demonstrate features previously observed only in nucleated cells, indicating that stable fusion pore formation, fusion pore expansion, and kiss-and-run exocytosis occur in platelets. We have previously used amperometry of individual platelets to evaluate mechanisms of platelet membrane pore dynamics.

We now use single-cell amperometry to study the role of dynamin-related protein-1 (Drp1) in platelet granule release. Drp1 belongs to the dynamin superfamily, a group of large GTPases that act as mechanoenzymes and demonstrate oligomerization-dependent GTPase as well as membrane modeling activities. Drp1 is most widely known for its role in mediating mitochondrial fission and fusion. We demonstrate that platelets contain Drp1 and that Drp1 is phosphorylated on platelet activation. Blocking Drp1 inhibits platelet granule exocytosis and impairs fusion pore stability. Inhibition of Drp1 also interferes with platelet accumulation during thrombus formation in vivo. These studies demonstrate a heretofore unrecognized function of Drp1 in platelet physiology.
Materials and Methods

Platelet Preparation
Human and rabbit platelets were prepared as described in the Methods in the online-only Data Supplement.

Single-Cell Amperometry
Amperometry experiments were performed with an Axopatch 200B potentiostat (Molecular Devices, Inc) controlled by locally written LABVIEW software. Fabrication of carbon-fiber microelectrodes and the experimental instrumentation have been previously described.2,4,8 Rabbit platelets were used for these experiments because they have a substantially higher number of dense granules per platelet than human platelets. To begin a measurement, a drop of rabbit platelet suspension was added to the experimental chamber that contained either 10 μmol/L mdivi-1 (Tocris), or 10 μmol/L compound E (generous gift of Dr Jodi Nummari and Dr Ann Cassidy-Stone) in Tyrode’s buffer (prepared from stock solutions in dimethyl sulfoxide) or the same volume of dimethyl sulfoxide in Tyrode’s buffer for experimental and control conditions, respectively. Platelets were allowed to sediment on poly L-lysine-coated coverslips for 15 minutes before amperometry measurements. One platelet at a time was stimulated with a 3-s bolus of thrombin solution in Tyrode’s buffer (10 U/mL), and the amperometric response was recorded for 60 seconds. Data were analyzed as has been previously described4 and reported as mean±SEM; t tests were used to identify statistically significant differences compared with the control condition. Amperometric measurements were recorded from 107 platelets for control condition, 39 platelets for mdivi-1 condition, and 31 platelets for the compound E condition.

Thrombus Formation Model
Intravitral video microscopy of the cremaster muscle microcirculation was performed as previously described.1 Mouse antihuman fibrinogen II b-chain monoclonal antibody was purified over protein G-Sepharose (Invitrogen) from a 59D8 hybridoma cell line10 and labeled with Alexa Fluor 488 (Invitrogen). Digital images were captured with a Cooke Sensicam charge-coupled device camera (The Cooke Corporation) connected to a VYS-1845 Image Intensifier GEN III (Video Scope International). Injury to a cremaster arteriole (30–50 μm diameter) vessel wall was induced with a Micropoint Laser System (Photonics Instruments) focused through the microscope objective, parfocal with the focal plane and tuned to 480 nm through the dye cell containing 5 mmol/L coumarin in methanol. Data were captured digitally from 2 fluorescence channels, 488/520 nm and 647/670 nm. Data acquisition was initiated both before and after a single laser pulse for each injury. The microscope system was controlled and images were analyzed using Slidebook (Intelligent Imaging Innovations).

Image Analysis
For each thrombus generated, a rectangular mask was defined that included a portion of the vessel upstream of the site of injury. The maximum fluorescence intensity of the pixels contained in this mask was extracted for all frames (pre- and postinjury) for each thrombus. The mean value calculated from the maximal intensity values in the mask for each frame was determined and used as the background value. Finally, for each frame, the integrated fluorescence intensity was calculated as per the following equation:

\[
\text{Integrated fluorescence intensity} = \text{sum intensity of signal} - (\text{mean of the maximal background intensity} \times \text{area of the signal}).
\]

This calculation was performed for all frames in each thrombus and plotted versus time to provide the kinetics of thrombus formation. For multiple fluorescence channels, calculations of background were made independently for each channel. The data from 25 to 30 thrombi were used to determine the median value of the integrated fluorescence intensity to account for the variability of thrombus formation at any given set of experimental conditions.

Statistical Analysis
A value representing area under the curve was calculated for each curve generated by measurement of fluorescence after laser injury of an arteriole. A Mann-Whitney test was used for statistical comparison of data sets comprising the indicated number of thrombi formed under each indicated condition. P values of 0.05 or less were considered statistically significant and are indicated. Statistical analyses were performed using Prism software package (version 4; GraphPad).

Immunoblot Analysis
Immunoblot analysis of human platelet lysates was performed as described in the Methods in the online-only Data Supplement.

Immunogold Electron and Immunofluorescence Microscopy
Human platelets were analyzed using transmission electron microscopy and immunofluorescence microscopy as described in the Methods in the online-only Data Supplement.

Flow Cytometry
Flow cytometry was used to quantify P-selectin surface exposure to monitor α-granule release and measure JC-1 fluorescence to monitor mitochondrial membrane potential as described in the Methods in the online-only Data Supplement.

Detection of Adenine Nucleotide Release
A luciferin–luciferase detection system was used to measure ADP/ATP release to monitor dense granule secretion as described in the Methods in the online-only Data Supplement.

Results

DYNAMINS IN PLATELETS
Dynamins are a superfamily of large GTPases that serve a wide range of membrane-shaping functions.7 Their expression and function in platelets are not well understood. Megakaryocytes have previously been shown to express dynamin 3.11,12 However, whether other dynamins and dynamin-related proteins are present in platelets has not been assessed. We, therefore, evaluated platelet lysates for dynamin 1, dynamin 2, and Drp1. Dynamin 1 was not identified under the conditions of our assay. In contrast, dynamin 2 and Drp1 were recognized as single bands with apparent molecular weights of 100 kDa and 80 kDa, respectively (Figure 1A). No additional bands were detected in immunoblots of platelet lysates. To evaluate for the presence of Drp1 in platelet cytosol, platelets were permeabilized with streptolysin-O and subsequently pelleted. Evaluation of platelet cytosol and membranes demonstrated Drp1 in both fractions (Figure 1B), indicating that a portion of platelet Drp1 is cytosolic. Evaluation of activation-dependent phosphorylation of platelet Drp1 using phosphorylation site-specific antibodies demonstrated that Drp1 is phosphorylated at serine 616 after incubation with SFLLRN to activate PAR1 and at serine 637 after incubation with forskolin to activate adenyl cyclase (Figure 1C). Immunogold electron microscopy was performed to further define the localization of Drp1 in platelets. Electron microscopy demonstrated that Drp1 associated with granule membranes and the cytoplasmic face of the platelet plasma membrane and was present in the cytosol (Figure 1E; Figure I in the online-only Data Supplement).
Studies using a nonimmune antibody demonstrated no staining (Figure I in the online-only Data Supplement). These results show that Drp1 is found in platelets, is phosphorylated in an activation-dependent manner, and localizes to both membranes and cytosol.

**Drp1 Functions in Platelet Granule Exocytosis**

Dynamins have a well-established role in endocytosis, associating with the stalk of the maturing endocytic vesicle and catalyzing vesicle scission. More recently, dynamins have been shown to function in granule exocytosis. To evaluate whether dynamins function in platelet granule exocytosis, we tested the effect of dynamin inhibitors, dynasore and MiTMAB, on exocytosis using surface expression of P-selectin as a marker of α-granule release (Figure 2).

Dynasore blocked agonist-induced P-selectin surface expression with an IC₅₀ of ~20 μmol/L (Figure 2), close to its IC₅₀ for recombinant Drp1 in a GTPase assay. MiTMAB also inhibited P-selectin surface expression with an IC₅₀ of ~20 μmol/L (Figure 2). These results indicate a role for dynamins in platelet granule exocytosis. Dynasore and MiTMAB have been used widely to assess the role of specific dynamin subtypes in exocytosis.

Drp1 is widely known for its role in mitochondrial fission and fusion. However, a role in degranulation has also been described. To determine whether Drp1 functions in platelet granule exocytosis, we used the well-characterized Drp1 inhibitor, mitochondrial division inhibitor-1 (mdivi-1). Mdivi-1 selectively blocks Drp1, but not other dynamin isoforms, and acts outside of the GTP binding site. This inhibitor blocked PAR1-mediated P-selectin release with an IC₅₀ of ~10 μmol/L (Figure 3A), similar to its IC₅₀ for inhibition of recombinant Drp1 in a GTPase assay. One method to assess the selectivity of a small molecule for a particular protein target is to use compound analogs that vary in their ability to inhibit the target and determine whether or not their activity is replicated in the cellular system of interest. Analogs of mdivi-1 were used to assess whether Drp1 is the relevant target of mdivi-1 in platelet exocytosis. Compound D, an analog with potency equal to that of mdivi-1 against recombinant Drp1 in a GTPase assay, demonstrated similar potency to mdivi-1 in an assay of α-granule release (Figure 3A). Compound E was substantially less potent than mdivi-1 against recombinant Drp1 in a GTPase assay, demonstrating only ~45% inhibition at 50 μmol/L. Therefore, if Drp1 is the relevant target for mdivi-1 in platelet exocytosis, then compound E would demonstrate significantly less activity than mdivi-1 in the secretion assay. Compound E was significantly less active in inhibiting platelet α-granule release (Figure 3A). To determine whether Drp1 functions in dense granule release as well as α-granule release, we evaluated the role of mdivi-1 on adenine nucleoside release using a luciferase-based assay. Dense granule release was inhibited by mdivi-1 with an IC₅₀ of ~20 μmol/L.
of ≈10 μmol/L (Figure 3B). These results indicate a role for Drp1 in platelet granule exocytosis.

**Drp1 Controls Platelet Fusion Pore Dynamics**

Drp1 functions in mitochondrial dynamics that contribute to granule secretion in mast cell exocytosis, synapses, and insulin-secreting cells.\(^ {29,31,32} \)

We used single-cell amperometry to evaluate, with submillisecond resolution, the effect of mdivi-1 on the release of serotonin from rabbit platelets (Figure 4A).

Statistical analyses of multiple tracings showed that 10 μmol/L mdivi-1 had no effect on the quantal concentration of released serotonin (Figure 4B) or the number of fusion events per platelet (Figure 4C), which enabled unambiguous evaluation of the change in fusion pore behavior and kinetics of release. Results showed that although 10 μmol/L mdivi-1 did not affect the time required for transition from fusion pore to maximal release (\(T_{\text{rise}}\); Figure 4D), it changed the kinetics of the total release event (Figure 4E). The prolonged spike width in

**Figure 3.** Role of dynamin-related protein-1 (Drp1) in α-granule and dense granule exocytosis. **A**, Platelet P-selectin surface expression in response to 5 μmol/L SFLLRN was assayed in the presence of the indicated concentrations of either mdivi-1 (black circle), compound D (black square), or compound E (open circle). Data are representative of 3 experiments ± SD. **B**, Platelets were incubated with the indicated concentrations of mdivi-1 for 20 minutes before addition of 5 μmol/L SFLLRN. Release of adenine nucleosides was subsequently monitored using a luciferin–luciferase assay. Data are representative of 3 similar experiments.

**Figure 4.** Role of dynamin-related protein-1 (Drp1) in the kinetics of platelet granule release. **A**, Representative amperometric traces of rabbit platelet dense granule release in the presence and absence of mdivi-1. The heavy bar beneath the tracings indicates the time and the duration of the thrombin stimulation. Insets: The distinct spike shapes for control (top) and mdivi-1 (bottom) conditions depicted in the millisecond time scale demonstrate different serotonin release kinetics. Mdivi-1 (10 μmol/L) did not influence quantal release (B), the number of granules released per cell (C), or the time required for transition from fusion pore to full fusion (\(T_{\text{rise}}\); D). However, the total time required for the release event (\(T_{1/2}\)) was longer for platelets exposed to mdivi-1 (\(P<0.001\); E). Data represent the average+SEM of 107 control tracings and 39 tracings from platelets exposed to mdivi-1.
amperometric tracings from platelets exposed to mdivi-1 indicated that mdivi-1 reduced the efficiency of serotonin release through the fusion pore. Time of half maximal release (T_{1/2}) was 14.97±0.81 ms for control and 23.00±1.70 ms for mdivi-1 conditions (Figure 4E). This effect can be observed in Figure 4A (see inset), where representative spikes for each condition are shown above the tracings; mdivi-1–treated cells produced wider (larger T_{1/2}) and smaller amplitude spikes (because the Q values are similar) on release of serotonin. Amplitude analysis demonstrated a mean amplitude of 16.58±1.35 pA in control samples compared with 8.96±0.93 pA in samples incubated with mdivi-1 (P=0.0004). In contrast, compound E had no effect on spike amplitude or T_{1/2} (Figure II in the online-only Data Supplement ). This result demonstrates that mdivi-1 impairs efficient extrusion of serotonin from platelet dense granules.

Additional characteristics of amperometric tracings were evaluated to assess the role of Drp1 on the rate of dense granule release and on fusion pore stability (Figure 5). Cumulative frequency analyses indicate how efficiently granules are trafficked, docked, and fuse on stimulation.6 Because the percent of granules released over time does not change on mdivi-1 treatment (Figure 5B), it is unlikely that mdivi-1 influences delivery of granules to surface connected membranes by cytoskeletal transport or docking at the release site. After membrane fusion and initial pore formation, pore expansion can ensue, leading to full granule collapse and concomitant extrusion of granule contents. Initial formation of the fusion pore can be identified in amperometric spikes as a subtle increase in the current which is called a foot. Alternatively, the fusion pore may close after partial secretion in an event termed kiss-and-run exocytosis (Figure 5A). In this work, standalone spikes with an integrated area between 30 fC (≈100× the RMS noise of each tracing) and 100 fC (≈30% of the average Q value) are classified as kiss-and-run events. Release events through fusion pores followed by either reclosure of the fusion pore or full release after foot process formation were monitored. The total %kiss-and-run events did not differ significantly between the 2 conditions (Figure 5C). However, there was a significant difference between the fusion events completed by full fusion through a foot process in control platelets versus platelets exposed to mdivi-1 (Figure 5D; % foot is 17.35±1.37 for control and 9.46±2.01 for mdivi-1 conditions; P<0.01). In contrast to the effects of mdivi-1, compound E had no effect on platelet pore formation (Figure II in the online-only Data Supplement). These results indicate that inhibition of Drp1 impairs fusion pore stability.

Drp1 Antagonism Inhibits Thrombus Formation

Inhibition of Drp1 after infusion of mdivi-1 has previously been demonstrated to block reperfusion injury in cardiac, renal, and retinal ischemia models.33-35 The protective effect has been attributed to prevention of mitochondrial...
fragmentation because Drp1 is known to function in mitochondrial fission. 26,27 However, antiplatelet compounds can also confer protection against reperfusion injury, and platelet function was not evaluated in previous studies. Because inhibition of Drp1 inhibits platelet exocytosis, we determined whether pharmacological inhibition of Drp1 could control thrombus formation after vascular injury. Platelet accumulation (Figure 6A, red) and fibrin formation (Figure 6A, green) were monitored after laser-induced injury of cremaster arteries before (Figure 6A; Movie 1 in the online-only Data Supplement) and after infusion of mdivi-1 (Figure 6B; Movie II in the online-only Data Supplement). A 59% reduction in platelet accumulation (P < 0.001, as determined by AUC) was observed after infusion of mdivi-1 (Figure 6C; Figure III in the online-only Data Supplement). In contrast, there was no significant effect of mdivi-1 on fibrin generation after laser-induced vascular injury (Figure 6D; Figure III in the online-only Data Supplement), consistent with other interventions specifically targeting platelet function in this model. 30

Discussion

These experiments evaluate the role of Drp1 in platelet function using small molecules. The indication that Drp1 functions in granule exocytosis was originally derived from a chemical genetic screen of >300,000 compounds to identify inhibitors of platelet granule secretion. 37 This unbiased forward screen identified a compound, ML160, which also demonstrated activity in an assay designed to identify inhibitors of the product of yeast Dnm1, the yeast ortholog of Drp1. After the identification of ML160 as an inhibitor of platelet exocytosis, we used 2 well-characterized dynamin family inhibitors, dynasore and MiTMAB, which also blocked platelet granule secretion (Figure 2). This result implicates dynamin family proteins in platelet granule release. To evaluate the role of Drp1 in granule release, we used mdivi-1, which is selective for Drp1 and has proven to be a useful compound in its study. 30,34,35,38,39 Mdivi-1 inhibits exocytosis in the same concentration range that it inhibits the recombinant Drp1 GTPase activity. The fact that the inhibitory activity of various mdivi-1 analogs in the exocytosis assay mirror their activity against the recombinant Drp1 GTPase (Figure 3) further supports the premise that Drp1 is the relevant target of mdivi-1 in the exocytosis assay.

Studies demonstrating a role for Drp1 in granule secretion prompted us to evaluate the effect of mdivi-1 on serotonin release from individual granules using single-cell amperometry. Experiments were conducted with a low concentration of mdivi-1 (10 μmol/L) so as not to impair total quantal release of serotonin [Q(fC)] and to enable direct comparison of tracings with untreated controls. Lower antagonist concentrations also decrease the likelihood of off-target effects. These studies demonstrated that mdivi-1 has a significant effect on spike width and the percent of foot processes associated with spikes (Figures 4 and 5). Figure 4 shows representative traces from both control and mdivi-1–exposed platelets. Unlike the control platelets, mdivi-1–treated platelets show an extended release behavior for each fused granule. This result implies that mdivi-1 impairs the rate of expansion of the fusion pore, and thus, slows the time course for serotonin release from individual granules. Moreover, foot process formation was inefficient when platelets were exposed to mdivi-1, demonstrating that inhibition of Drp1 causes fusion pore instability.

There are several potential mechanisms by which Drp1 may control fusion pore dynamics. Drp1 colocalizes with mitochondria in platelets (Figure IV in the online-only Data Supplement) and could participate in granule secretion via effects on mitochondria. In mast cells, either incubation with mdivi-1 or treatment with Drp1 small interfering RNA blocks mitochondrial translocation required for activation-induced tumor necrosis factor-α and β-hexoseaminidase release. 29 Mitochondria play a prominent role in insulin secretion by generating required ATP and metabolites that stimulate secretory processes. 40 Silencing Drp1 impairs insulin secretion in the hypothalamus. 35 Mitochondria also contribute to synaptic vesicle release, and Drp1 has been shown to function in this capacity. 11 Mdivi-1 impairs mitochondrial permeability transition pore formation and reduces ischemia-mediated
death in cardiomyocytes.\textsuperscript{33} However, under the conditions of our assay, we found no effect of mdivi-1 on mitochondrial membrane potential (Figure V in the online-only Data Supplement). Similarly, mdivi-1 had no effect on platelet apoptosis as measured by annexin V binding (data not shown). In contrast, antimycin A, an inhibitor of mitochondrial respiration, impaired mitochondrial membrane potential without blocking granule secretion (Figure V in the online-only Data Supplement). Thus, impairment of mitochondrial membrane potential is not a likely explanation for the effects of mdivi-1 on granule secretion. An alternative possibility is that Drp1 acts to control fusion pore formation at the site of stalk formation. Unlike classical dynamins, Drp1 lacks a pleckstrin homology domain or SH3 domain and is not known to act at sites of membrane fusion. Nonetheless, Drp1 interacts with membranes and could potentially influence pore expansion directly. Further studies will be required to determine the mechanism by which Drp1 controls platelet fusion pore dynamics.

Inhibition of Drp1 blocked platelet accumulation during thrombus formation after vascular injury. In contrast, fibrin generation was unaffected (Figure 6). This observation is consistent with the fact that fibrin generation after laser-induced injury of the cremaster arteriole is independent of platelet accumulation.\textsuperscript{36} Infusion of mdivi-1 has previously been shown to decrease infarct size in murine ventricles in an in vivo model of cardiac ischemia-reperfusion injury.\textsuperscript{37} Systemic infusion of 50 mg/kg of mdivi-1 has also been used to reverse ischemic renal injury and tubular apoptosis induced by reperfusion after renal ischemia.\textsuperscript{38} The effect was attributed to suppression of ischemia-induced mitochondrial fragmentation. Similarly, mdivi-1 was shown to inhibit early neurodegenerative events and increase retinal ganglion cell survival after acute retinal ischemia.\textsuperscript{39} Our results using the same mdivi-1 concentration demonstrate that mdivi-1 is an antiplatelet agent in the setting of injury-induced thrombus formation. A limitation of our studies is that we cannot restrict the activity of mdivi-1 to platelets after systemic infusion. We cannot rule out an effect of mdivi-1 on endothelial cells or leukocytes in our assay. Nonetheless, because antiplatelet therapy interferes with platelet secretion, effects on platelet function must also be considered when assessing the role of Drp1 in reperfusion injury. The combination of maintaining vascular patency and inhibiting apoptosis may be useful in the treatment of ischemic injury.

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Disclosures

None.

References


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Supplemental Materials

Supplementary Methods

Platelet preparation

For human platelets, blood was obtained from healthy donors who had not ingested aspirin or NSAIDs in the 2 weeks prior to donation. Platelets were isolated by centrifugation followed by isolation of washed platelets from platelet-rich plasma. For single cell amperometry experiments, blood was drawn from the midear artery of New Zealand rabbits (Bakkom Rabbitry) according to approved University of Minnesota IACUC protocol #0802A27063. Isolation of washed rabbit platelets in Tyrode’s buffer (NaCl, 137 mM; KCl, 2.6 mM; MgCl₂, 1.0 mM; D-glucose, 5.6 mM; N-2-hydroxyethylpiperazine- N'-2-ethanesulfonic acid (HEPES) 5.0 mM; and NaHCO₃, 12.1 mM with pH adjusted to 7.3) was previously described. ¹

Immunogold Electron Microscopy

For preparation of cryosections, isolated human platelets were fixed with 4% paraformaldehyde in 0.1 M Na phosphate buffer, pH 7.4. After 2 hours of fixation at room temperature, the cell pellets were washed with PBS containing 0.2 M glycine. Before freezing in liquid nitrogen, cell pellets were infiltrated with 2.3 M sucrose in PBS for 15 minutes. Frozen samples were sectioned at 120°C, and the sections were transferred to formvar-carbon coated copper grids and floated on PBS until the immunogold labeling was carried out. The gold labeling was carried out at room temperature on a piece of parafilm. Anti-Drp1 antibody (Abcam) and Protein A gold were diluted with 1% BSA. The anti-Drp1 antibody recognized a single band of the appropriate molecular weight in immunoblot staining of platelet lysates (Fig. 1A,B). Grids were floated on drops of 1% BSA for 10 minutes to block for nonspecific labeling, transferred to primary antibody, and incubated for 30 minutes. The grids were then washed, transferred to Protein A gold for 20 minutes, and washed in PBS followed by double distilled water. Contrasting/embedding of the labeled grids was carried out on ice in 0.3% uranyl acetate in 2%
methyl cellulose for 10 minutes. The grids were examined in a Tecnai G2 Spirit BioTWIN transmission electron microscope (Hillsboro, OR) at 18 500 magnification at an accelerating voltage of 80 kV. Images were recorded with an AMT 2k CCD camera.² Control samples using a non-immune antibody demonstrated no staining.

**Immunoblot analysis**

Platelet lysates were prepared by lysis of human platelets (2 x 10⁹/ml) in sample buffer. To prepare cytosol and membranes, platelets were incubated with 15 U/mL streptolysin-O (Sigma, MO) overnight and subsequently pelleted at 1000g for 15 minutes. The cytosol fraction was collected and the pellet was washed in PIPES/EGTA/KCl buffer (25 mM PIPES, 2 mM EGTA, 137 mM KCl, 4 mM NaCl, 0.1% glucose, pH 6.4). Proteins in cytosol and membranes were then solubilized in sample buffer and separated by SDS-PAGE. Immunoblotting was performed using antibodies directed against dynamin 1, dynamin 2, Drp1 (Abcam), phospho-Drp1 serine 616 (Cell Signaling Technology), and phospho-Drp1 serine 637 (Cell Signaling Technology) and FITC-labeled or HRP-labeled secondary antibodies (Jackson Immunorearch Laboratories). FITC-labeled or HRP-labeled secondary antibodies were visualized using fluorescence detection on a Typhoon 9400 Molecular Imager (GE Healthcare) or chemiluminescence using an X-OMAT 2000A Processor (Kodak), respectively.

**Flow cytometry**

Gel-filtered platelets (10 µL; [0.5-1 x 10⁹/mL]) were incubated with the indicated concentrations of inhibitor for 20 minutes. Samples were then exposed to 5 µM SFLLRN and analyzed by flow cytometry using a Becton Dickinson FACSCalibur flow cytometer as previously described.³ PE-conjugated antihuman P-selectin (BD Biosciences) was used to detect P-selectin exposure. For evaluation of mitochondrial membrane potential, platelets were incubated with 1 µM JC-1
(Invitrogen) for 10 minutes and either buffer alone, mdivi-1, or actimycin A (Sigma) for 20 minutes. Platelets were then exposed to either buffer or SFLLRN for 20 minutes and evaluated by flow cytometry. Fluorescent channels were set at logarithmic gain and $1 \times 10^4$ particles were acquired for each sample. A 530/30 band pass filter was used to measure FL1 fluorescence and a 585/42 band pass filter was used to measure FL-2 fluorescence. Data were analyzed using CellQuest software (BD Biosciences) on a MacIntosh PowerPC (Apple). Data using JC-1 are represented using the ratio of FL2/FL1 as previously reported.\textsuperscript{4}

**Detection of adenine nucleotide release**

A luciferin-luciferase detection system was used to quantify ADP/ATP release to monitor bulk dense granule secretion.\textsuperscript{5} For these experiments, 9 µL human platelets (0.5-1 $\times 10^8$/mL) were incubated in the presence or absence of mdivi-1 and then stimulated with SFLLRN. Samples were then incubated with 1 µL luciferin-luciferase (final concentration of 3 mg/mL) at the indicated time following addition of agonists. Chemoluminescence was quantified using a luminometer (TD 20/20; Turner Design).

**Confocal Microscopy**

For colocalization measurements, platelet mitochondria were labeled by incubation of the PRP with 100 nM Mitotracker Red (Life Technologies) for 45 min at 37 °C. PRP with a platelet count of $10^7$ platelets/mL was then transferred to poly-L-lysine containing coverslips. Adhered platelets were fixed and permeabilized with 4% formaldehyde (in Tyrode’s buffer) and 0.2% Triton X-100, respectively. After incubating with IF blocking buffer (10% FCS, 1% BSA and 0.05% sodium azide in PBS) overnight, platelets were labeled with mouse anti-human Drp-1 antibody (Abcam). FITC-conjugated goat anti-mouse IgG antibody (Abcam) used as a secondary antibody for imaging the Drp-1.\textsuperscript{6} An Olympus FluoView F1000 upright confocal microscope was used for capturing images. Sequential scanning with 488 and 543 nm lasers enabled selective imaging
of the two fluorescent labels (mitotracker red and FITC) without spectral crosstalk. ImageJ, with
the Mander’s coefficients plugin, was used for colocalization analysis.
Supplementary Figures

Figure I. Localization of Drp1 in resting platelets. (A) Immunogold staining of resting platelets processed with anti-Drp1 antibody demonstrates Drp1 associated with plasma membrane (black arrows), granule membranes (white arrows), and cytosol (black arrowheads). Scale bar, 250 nm. (B) Immunogold staining using anti-Drp1 antibody as described in A. Scale bar, 400 nm. (C) Immunogold staining processed using a non-immune antibody instead of anti-Drp1 antibody. Staining is nearly absent. Scale bar, 300 nm.
Fig. II. Effect of compound E on rabbit platelet pore formation as measured by single cell amperometry. Compound E (10 μM), an mdivi-1 analog that lacks activity against Drp1, did not significantly affect quantal release (A), number of granule released per cell (B), time required for transition from fusion pore to full fusion (C), total time for release (D), % kiss and run events (E), or % foot processes (F). Data represent the mean ± S.E.M of 107 control tracings and 31 tracings from platelets exposed to mdivi-1.
Figure III. Inhibition of platelet accumulation, but not fibrin generation, during thrombus formation following laser-induced injury of murine cremaster arterioles. The distribution of fluorescence intensities representing (A) platelet accumulation and (B) fibrin generation during thrombus formation is shown. Each point represents the area under the curve of platelet or fibrin fluorescence measured over the 3 minute interval following laser injury. A 59% decrease in platelet accumulation is observed in the presence of mdivi-1 (p = 0.001). In contrast, mdivi-1 has no effect on the fibrin generation.
**Figure IV. Colocalization of Drp1 with platelet mitochondria.** Double staining confocal immunofluorescence microscopy of resting human platelets demonstrates colocalization of anti-Drp1 antibody with the mitochondrial stain, Mitotracker Red. This representative image shows staining of (A) Drp1, (B) mitochondria, and (C) the merged images. Scale bar, 5 μm. Analysis of colocalization demonstrate a Pearson’s coefficient of 0.675±0.025, an M1 value (Drp1 associated with mitochondria) of 0.428±0.038, and an M2 value (mitochondria associated with Drp1) of 0.731±0.067. Staining with non-immune antibody showed no fluorescence, and no crossover was observed between fluorescence channels.
Figure V. Platelet mitochondrial membrane potential is not affected by mdivi-1. (A,B) Human platelets were incubated for 20 minutes with buffer (*No addition*) and either (A) 3 μM antimycin A or (B) 30 μM mdivi-1. Samples were then incubated in the presence or absence or 5 μM SFLLRN for 10 minutes and mitochondrial membrane potential was subsequently measured using JC-1. The ratio of red (FL2) to green (FL1) fluorescence was used to monitor mitochondrial membrane potential as previously described. Data represent the ratio of FL-2/FL-1 ± S.E.M. of four independent samples. P-values are indicated above the graph. (C,D) Platelets were incubated for 20 minutes with buffer (*No addition*) and either (C) 3 μM antimycin A or (D) 30 μM mdivi-1. Samples were then incubated in the presence or absence or 5 μM SFLLRN for 10 minutes. P-selectin surface expression was subsequently evaluated by flow cytometry. Data represent the mean ± S.E.M. of 3 samples. P-values are indicated above the graph.
**Supplementary video 1.** Thrombus formation following laser injury was monitored in the mouse cremaster arteriole following infusion of DMSO. The accumulation of platelets (*red*) and fibrin (*green*) is visualized during thrombus formation following laser-induced injury of a cremaster arteriole.

**Supplementary video 2.** Thrombus formation following laser injury was monitored in the mouse cremaster arteriole following infusion of 50 mg/kg mdivi-1. The accumulation of platelets (*red*) and fibrin (*green*) is visualized during thrombus formation following laser-induced injury of a cremaster arteriole. Following infusion of mdivi-1, fibrin formation occurs normally, but platelet accumulation is decreased.

**Supplementary References**